The identification, cloning and characterisation of centaurin-alpha2

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THE IDENTIFICATION, CLONING AND CHARACTERISATION OF CENTAURIN-α2

Submitted by Alison M Gibbard
for the degree of PhD
of the University of Bath
2001

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Alison Gibbard
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ABSTRACT

The principal aim of this thesis was to investigate whether centaurin-α related proteins were present in insulin-sensitive tissues. The screening of an adipocyte library using a centaurin-α probe had identified a partial clone that corresponded to the 3′ terminus of centaurin-α sharing 58% identity. Rapid Amplification of cDNA Ends (RACE) was employed to amplify the 5′ sequence and subsequently the full-length clone. Analysis of the deduced amino acid sequence revealed the domain structure of this novel clone to consist of an N-terminal zinc-finger domain (with homology to the ADP ribosylation factor-GTPase activating protein (ARF-GAP) domain) and two Pleckstrin Homology (PH) domains, PH-N and PH-C. This clone shared 51% and 57-59% identity with centaurin-α and centaurin-α1, respectively. Considering the similarity with the centaurin-α family, the novel clone identified herein was named centaurin-α2. Interestingly the northern blot analysis indicated centaurin-α2 to be ubiquitously expressed, contrasting with the brain specific centaurin-αs. Anti-centaurin-α2 antiserum was raised, purified and used to investigate the protein expression at a cellular and subcellular level. Centaurin-α2’s distribution appeared to be quite distinct from the other family members, and highly abundant in insulin-sensitive tissues. In contrast to centaurin-α and centaurin-α1, centaurin-α2 was not detected in the cytosolic fraction. The constitutive membrane association might be explained by the high-affinity for phosphatidylinositol 4,5-bisphosphate (PtdIns 4,5-P2) as well as phosphatidylinositol 3,4,5-trisphosphate (PtdIns 3,4,5-P3). Preliminary binding analysis using centaurin-α2 proteins with point mutations of single arginine residues in the PH domains (R151C, R275C and R151C/R275C) indicated that PtdIns 3,4,5-P3 and PtdIns 4,5-P2 bind to the PH-N domain, whereas phosphatidylinositol 3,4-bisphosphate (PtdIns 3,4-P2) binds to PH-C. In conclusion, centaurin-α2 appears to be an interesting new addition to the centaurin-α family illustrating that these proteins are not restricted to brain tissue, however the cellular importance of this family remains unclear.
ACKNOWLEDGEMENTS

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I would especially like to thank Dr Sue Oldfield, who originally identified the centaurin-α2 partial clone, which was pivotal to the development of this research. I am also indebted to Dr Paul Whitely for his expert advice on the cloning of centaurin-α2 and for his continual support and encouragement. I would like to express my gratitude to the members of the Department of Biology and Biochemistry at Bath University for providing the resources for this research. Special thanks go to Dr Barbara Reaves for the gift of the liver Marathon-Ready cDNA library and β-tubulin antibody and to Paul Jones for performing the DNA sequencing. Thanks go to Professor Glenn Prestwich for the gift of the phosphoinositide photoaffinity labels.

Special thanks go to the members of the Holman laboratory, both past and present. On a technical note I would like to say a big thank you to Al and Jing for isolating cardiomyocytes for the confocal experiments. On a personal level I would like to say thank you to Al and Jazz who have been a constant source of fun and friendship during my time in Bath. Thanks to all the friends who have supported me through the writing of this thesis, especially Debs, with her sound advice and empathy that were gratefully received in moments of despair. I would also like to show my appreciation to the Dr John’s (Argent, Clapham and Rockett) each of whom have been a source of inspiration and encouragement.

Finally I would like to express my sincere appreciation to my family for their continuous love and support throughout my studies. Especially my parents who welcomed me home after many years of having an empty nest. I know I can’t have been fun to live with!
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ACAP</td>
<td>ARF-GAP with coiled coil, ankyrin repeat and PH domains</td>
</tr>
<tr>
<td>AEBSF</td>
<td>4-(2-aminoethyl) benzenesulfonyl fluoride</td>
</tr>
<tr>
<td>AP1</td>
<td>adaptor primer 1</td>
</tr>
<tr>
<td>AP-1</td>
<td>adaptor protein-1</td>
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<tr>
<td>AP-2</td>
<td>adaptor protein-2</td>
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<tr>
<td>AP-3</td>
<td>adaptor protein-3</td>
</tr>
<tr>
<td>ARF</td>
<td>ADP-ribosylation factor</td>
</tr>
<tr>
<td>ARL</td>
<td>ARF-like protein</td>
</tr>
<tr>
<td>ARNO</td>
<td>ARF nucleotide-binding site opener</td>
</tr>
<tr>
<td>ASAP</td>
<td>ARF-GAP containing a SH3 domain, ANK repeats and a PH domain</td>
</tr>
<tr>
<td>BFA</td>
<td>brefeldin A</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>Btk</td>
<td>Bruton's tyrosine kinase</td>
</tr>
<tr>
<td>C2 domains</td>
<td>cysteine-rich regions</td>
</tr>
<tr>
<td>CKI$\alpha$</td>
<td>casein kinase-I$\alpha$</td>
</tr>
<tr>
<td>COP</td>
<td>coat protein complex</td>
</tr>
<tr>
<td>CYT</td>
<td>cytosol</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>double-distilled water</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DIG</td>
<td>digoxigenin</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
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<tr>
<td>EEA1</td>
<td>early-endosomal autoantigen 1</td>
</tr>
<tr>
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<td>exchange factor for ARF6</td>
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<td>epidermal growth factor</td>
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</tr>
<tr>
<td>FAK</td>
<td>focal adhesion tyrosine kinase</td>
</tr>
<tr>
<td>FAPP1</td>
<td>phosphatidylinositol-four-phosphate adaptor protein-1</td>
</tr>
<tr>
<td>G3PDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PtdIns</td>
<td>phosphatidylinositol</td>
</tr>
<tr>
<td>PtdIns 3-P</td>
<td>phosphatidylinositol 3-phosphate</td>
</tr>
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<td>phosphatidylinositol 3,5-bisphosphate</td>
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<td>PtdIns 4,5-P₂</td>
<td>phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PtdIns 3,4,5-P₃</td>
<td>phosphatidylinositol 3,4,5-trisphosphate</td>
</tr>
<tr>
<td>RACE</td>
<td>rapid amplification of cDNA ends</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription-polymerase chain reaction</td>
</tr>
<tr>
<td>SH2 domain</td>
<td>Src homology 2 domain</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-buffered saline with Tween 20</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N,N'-tetramethylethylenediamine</td>
</tr>
<tr>
<td>TGN</td>
<td>trans-Golgi network</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
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1.0 INTRODUCTION

1.1 Forward

As summarised in the title, this thesis describes the identification and investigation of a new member of the centaurin-α family. The physiological function of this family of phosphoinositide-binding proteins remains unclear and this topic will be discussed in detail towards the end of this introduction. Firstly, I aim to introduce phosphoinositides and some of their varied functions within the cell. I will then discuss the importance of these phospholipids and their interacting proteins within signalling and vesicle trafficking before focusing on the centaurin-α protein family. Finally, I will introduce the area of research of the Holman Laboratory, the insulin-regulated glucose transporter, Glut4. This subject area provides a good illustration for the potential role for phosphoinositides in vesicle trafficking.

1.2 Lipid Signalling

According to the fluid mosaic model, the basic structure of biological membranes is provided by the lipid bilayer and the membrane proteins perform most of the specific functions such as nutrient uptake, ion transport and signal transduction. However, if the sole function of membrane lipids is to form a permeability barrier around the cell and its organelles, you might expect them to have quite uniform structures. In fact, they are highly diverse. Animal cell plasma membranes contain cholesterol, glycolipids and phospholipids with the latter group in greatest abundance. Phospholipids have an amphipathic structure consisting of a polar head group and two hydrophobic hydrocarbon tails. Four major phospholipids predominate in the plasma membrane of many mammalian cells: phosphatidylcholine, sphingomyelin, phosphotidylserine and phosphatidylethanolamine. The lipid composition of membranes differs between cells, organelles and the two halves of the lipid bilayer. Inositol containing phospholipids are minor constituents of eukaryotic cell membranes (2 to 8% by weight), and are predominantly localised to the cytoplasmic face of cellular membranes (Majerus et al. 1986). Phosphatidylinositol (PtdIns) represents the highest proportion (more than 80%)
of these phosphoinositides. PtdIns can undergo reversible phosphorylation at multiple sites on the inositol ring (positions D-3, D-4 and D-5) generating a variety of phosphoinositides. Seven phosphoinositides are currently known (Figure 1.1). The inositol head group is linked via a phosphodiester phosphate group at the D-1 position to the 3-carbon of the glycerol backbone. PtdIns usually contains a high proportion of stearic acid (saturated fatty acid, $C_{18}$) at the 1-carbon and a high proportion of arachidonic acid (unsaturated fatty acid, $C_{20}$) at the 2-carbon (Figure 1.1).

For many years the principle function of phospholipids was considered to be as precursors for second messengers in the signalling pathways conveying information into the cell from receptors located on the plasma membrane (PM). The classic example being phospholipase C (PLC)-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns 4,5-P$_2$). This yields the water-soluble head group moiety, inositol 1,4,5-trisphosphate (Ins 1,4,5-P$_3$) that triggers mobilisation of intracellular Ca$^{2+}$, and diacylglycerol (DAG), the lipid portion that activates protein kinase C (PKC) (Berridge and Irvine 1989). In addition to being precursors of inositol phosphates, more recently, phosphoinositides have been identified as key signalling molecules in their own right, acting as site-specific signals on membranes that recruit and regulate protein complexes. Moreover, membrane phosphoinositides are reported to function in signalling transduction, cytoskeletal assembly and membrane budding, docking and fusion processes (Section 1.4).

1.3 Generation of Phosphoinositides

PtdIns, the precursor of all phosphoinositides, is synthesised in the endoplasmic reticulum (ER) and transported to the cellular membranes. The synthesis and turnover of phosphoinositides can be spatially and temporally regulated by a series of kinases, phosphatases and phospholipases (Figure 1.2). The localisation of these enzymes to discrete membrane sites restricts signalling to a specific membrane or domain within the membrane. Novel enzymes continue to be identified and characterised, thus adding to the complexity of what is already an intricate network of reactions.

The classical research into phosphatidylinositol 4-phosphate (PtdIns 4-P) and PtdIns 4,5-P$_2$ by Grado and Ballou provided the first view of the interconvertible diversity of
Figure 1.1. Phosphatidylinositol (PtdIns) and phosphorylated phosphoinositide derivatives. (A) The inositol head group of PtdIns can be phosphorylated in the D-3, D-4 and D-5 positions (OH). Stearic acid and arachidonic acid are located at the 1-carbon and 2-carbon positions on the glycerol backbone, respectively. (B) The inositol ring of the seven known phosphoinositides are depicted, with the phosphates shown in red (P). The phosphate at position 1 links the inositol head group to the glycerol moiety. Also shown is the inositol phosphate, Ins 1,3,4,5-P₄, which is the inositol head group analogue of PtdIns 3,4,5-P₃.
Figure 1.2. A scheme showing synthetic pathways for phosphoinositides. Solid lines represent kinase reactions, dotted arrows indicate phosphatase reactions. Several PtdIns 3-kinase isoforms have been identified with different substrate specificity. PtdIns 4-P 5-kinase type I also acts on PtdIns 3-P as well as PtdIns 4-P and PtdIns 3,4-P$_2$. PtdIns 4-P 5-kinase type II is really a PtdIns 4-kinase. Fab1p and PIKfyve are yeast and mammalian PtdIns 3-P 5-kinase orthologues. The phosphatase reactions are shown but are not discussed further.
these phospholipids (Grado and Ballou 1960). For many years the accepted synthesis route for PtdIns 4,5-P2 has been via the phosphorylation of PtdIns by PtdIns 4-kinases, generating PtdIns 4-P which in turn was phosphorylated by PtdIns 4-P 5-kinases. More recently an alternative pathway has been identified. The type II PtdIns 4-P 5-kinase was shown to phosphorylate the D-4 position of phosphatidylinositol 5-phosphate (PtdIns 5-P), a lipid not previously known to exist (Rameh et al. 1997a). Consequently it has been proposed that these enzymes are renamed phosphatidylinositol phosphate 4-kinases (PIP 4-kinases) (Toker 1998).

Some phosphoinositides, such as PtdIns 4,5-P2 and phosphatidylinositol 3-phosphate (PtdIns 3-P), are ubiquitously expressed and remain at relatively constant levels, whereas the D-3 phosphoinositide products phosphatidylinositol 3,4-bisphosphate (PtdIns 3,4-P2) and phosphatidylinositol 3,4,5-trisphosphate (PtdIns 3,4,5-P3) dramatically increase upon activation of PtdIns 3-kinase. The D-3 phosphoinositides are generated by the multiple isoforms of PtdIns 3-kinase which can be sub-divided into three classes on the basis of homology, structure and substrate specificity (Vanhaesebroeck et al. 1997; Vanhaesebroeck and Waterfield 1999). Class I enzymes are heterodimers consisting of an adaptor subunit and a 110 kDa catalytic subunit (p110) and utilise PtdIns, PtdIns 4-P and PtdIns 4,5-P2 as substrates in vitro. The latter phospholipid is thought to be the preferred substrate in vivo, thus generating PtdIns 3,4,5-P3. This class is further divided into class IA, which are activated by the recruitment to tyrosine-phosphorylated proteins and interact with a 85 kDa adaptor subunit (p85), and class IB that are activated by heterotrimeric G proteins. Class II enzymes contain a characteristic C-terminal C2 domain and have been shown to utilise PtdIns > PtdIns 4-P >>> PtdIns 4,5-P2. Class III enzymes that only phosphorylate PtdIns, consist of heterodimers of a catalytic subunit (Vps34p in yeast) associated with a serine/threonine protein kinase (Vps15p in yeast).

Basal levels of PtdIns 3,4,5-P3 are generally low and increase dramatically in response to a variety of extracellular signals (Conricode 1995; Cross et al. 1997; Hawkins et al. 1992; Stephens et al. 1991; van der Kaay et al. 1997). Agonist binding stimulates receptor autophosphorylation generating phosphotyrosine residues that recruit and activate downstream signalling proteins resulting in the activation of class IA PtdIns 3-kinase (insulin-stimulated PtdIns 3-kinase activity is discussed further in Section 1.7). The cellular levels of PtdIns 3,4-P2 also increase upon agonist stimulation, however the
accumulation is slightly delayed and more sustained than that of PtdIns 3,4,5-P₃ (Stephens et al. 1993). The slow time-course can be explained by the proposal that growth factor stimulated PtdIns 3,4-P₂ production results from 5'-phosphatase action on PtdIns 3,4,5-P₃ and not the phosphorylation of PtdIns 4-P by PtdIns 3-kinase (Hawkins et al. 1992). Several 5'-phosphatases are able to act on PtdIns 3,4,5-P₃, and one isoform, SHIP₂, is reported to be regulated by growth factors and insulin (Guilherme et al. 1996; Habib et al. 1998). More recently PtdIns 3,4-P₂ production has been postulated to occur without the preceding PtdIns 3,4,5-P₃ accumulation. In platelets a novel integrin-activated pathway leads to the transient PtdIns 3-P formation that is then a substrate for PtdIns 3-P 4-kinase (Banfic et al. 1998). Similarly, in mouse fibroblasts oxidative stress induced relatively selective and sustained accumulation of PtdIns 3,4-P₂ independently of PtdIns 3,4,5-P₃ (van der Kaay et al. 1999). Furthermore the type II PtdIns 4-P 5-kinase (PIP 4-kinase) shown to phosphorylate PtdIns 5-P at the D-4 position can also phosphorylate PtdIns 3-P (Rameh et al. 1997a).

As described above there appears to be several synthesis pathways for PtdIns 4,5-P₂ and PtdIns 3,4-P₂. Similarly, further to the classical agonist stimulated PtdIns 3,4,5-P₃ synthesis by PtdIns 3-kinase, the type I and II PtdIns 5-kinases are reported to act concurrently, generating PtdIns 3,4,5-P₃ from PtdIns 3-P (Zhang et al. 1997). Type II phosphorylates PtdIns 3-P at the D-4 position generating PtdIns 3,4-P₂, which in turn is phosphorylated at the D-5 position by type I thus producing PtdIns 3,4,5-P₃.

Recently a fourth D-3 phosphatidylinositol, phosphatidylinositol 3,5-bisphosphate (PtdIns 3,5-P₂), was discovered in yeast and mammalian cells (Dove et al. 1997; Whiteford et al. 1997). In yeast the PtdIns 3-P 5-kinase, Fab1p, phosphorylates the Vps34p product PtdIns 3-P (Cooke et al. 1998; Gary et al. 1998) and a similar reaction is performed by the mammalian orthologue PIKfyve in vitro (Sbrissa et al. 1999). In 1997, Rameh and colleagues demonstrated two synthesis pathways for PtdIns 3,5-P₂. First, type I PtdIns 4-P 5-kinase can also utilise PtdIns 3-P as a substrate, and secondly class IA PtdIns 3-kinase is able to phosphorylate the novel lipid PtdIns 5-P in vitro.

In addition to the kinase reactions, interconversion of phosphoinositides by the many phosphatases adds to the complexity of phosphoinositide generation (Majerus et al. 1999). Most of the phosphatases are substrate specific and remove the phosphate at one position on the inositol ring. For example PtdIns 3,4,5-P₃ can be dephosphorylated at
the D-5 position by the SHIP enzymes (Krystal et al. 1999; Majerus et al. 1999) or at the D-3 position by a phosphatase encoded by the human tumour suppressor gene PTEN (Vazquez and Sellers 2000). Finally, further to kinases and phosphatases, there are phospholipase enzymes that hydrolyse phosphoinositides generating inositol phosphates. However, PtdIns 3,4,5-P$_3$ is not a substrate for any known phospholipases and thus is not the precursor of inositol 1,3,4,5-tetrakisphosphate (Ins 1,3,4,5-P$_4$) (Figure 1.1, B) which is generated by D-3 phosphorylation of Ins 1,4,5-P$_3$.

1.4 The Role of Phosphoinositides in Cellular Functions

Phosphoinositides have been implicated as key regulators in a diverse range of cellular functions. These include vesicle trafficking, insulin-stimulated translocation of the glucose transporter Glut4, chemotaxis, membrane ruffling, cytoskeletal assembly and apoptosis (reviewed in Corvera et al. 1999; Czech 1995; Martin 1998; Rameh and Cantley 1999; Toker 1998; Toker and Cantley 1997).

1.4.1 The Role of Phosphoinositides in Membrane Trafficking

It is now a quarter of a century since George Palade proposed that the homeostasis of eukaryotic membranous organelles is maintained through the exchange of membrane vesicles (Palade 1975). Transport vesicles containing specific ‘cargo’ of proteins and lipids, bud from the donor membrane and are driven along microtubules and actin filaments by motor proteins, and then dock and fuse with the acceptor membrane (reviewed by Rogers and Gelfand 2000). A simplified model of this complex transport system is shown in Figure 1.3. In all cells a constitutive biosynthetic pathway transports components from the ER via the Golgi to the plasma membrane (constitutive exocytosis). In specialised cells vesicles can remain in intracellular pools and only fuse with the plasma membrane in response to stimulation (regulated exocytosis). Examples include the release of histamine from mast cells, neurotransmitters from synapses and the translocation of the glucose transporter Glut4 to the plasma membrane in response to insulin. Endocytosis is the converse reaction in which vesicles bud from the plasma membrane and fuse with the early endosome where the cargo is sorted and can either be recycled back to the plasma membrane or transported to the lysosome via the late
Figure 1.3. Simplified model of membrane trafficking. Vesicles bud from a donor membrane and fuse with an acceptor membrane transporting cargo proteins and lipids. The constitutive biosynthetic pathway transports cargo from the endoplasmic reticulum (ER) to through to the Golgi, trans-Golgi network (TGN) and finally to the plasma membrane. Exocytosis can also occur via the regulated pathway in which vesicles fuse with the plasma membrane in response to stimulation. Vesicles can also transport cargo into the cell by endocytosis. Vesicles bud off from the plasma membrane and fuse with the early endosome where the cargo is sorted and either recycled back to the plasma membrane or transported to the lysosome via the late endosome.
endosome. The fidelity of the vectorial transport is governed by a network of complex interactions between regulatory molecules and mechanical components during the budding and docking/fusion stages.

Formation of transport vesicles is initiated by the assembly of coat proteins at specific sites on the cytoplasmic face of the membrane. As well as inducing curvature into the membrane, coat proteins are involved in the selection and concentration of the cargo (Rothman and Wieland 1996). Several coat proteins have been identified and appear to be localised to specific trafficking pathways. Coat protein complex-I (COPI)-coated vesicles carry proteins within the early secretory pathway (ER and Golgi), COP-II vesicles allow exit from the ER, and clathrin-coated vesicles mediate transport from the trans-Golgi network (TGN) and endocytic transport from the plasma membrane (Rothman and Wieland 1996; Wieland and Harter 1999). GTPases are known to regulate both the formation and docking/fusion stages of membrane transport. ADP-ribosylation factors (ARFs) form part of the primer in the vesicle budding of many vesicles and Rabs are implicated to function in targeting and fusion with acceptor membranes (Chavrier and Goud 1999). Docking and fusion stages are controlled by the protein-protein interactions of the components of the fusion apparatus, such as the SNARE proteins. The SNARE hypothesis for membrane fusion postulates that a v-SNARE on the vesicle must interact with a t-SNARE on the acceptor membrane to form the 'core complex' and mediate fusion (Sollner et al. 1993). In the last few years a series of experiments analysing the interaction of phosphoinositides with the mechanical and regulatory components of vesicle trafficking has generated considerable evidence supporting the importance of phosphoinositides as site-specific signals that recruit and possibly activate proteins essential in the budding, docking and fusion processes.

The first direct evidence implicating a critical role for phosphoinositides in membrane trafficking was the discovery that the yeast gene VPS34, which is important in trafficking to the vacuole, encoded a PtdIns 3-kinase (Schu et al. 1993). The protein product, Vps34p, phosphorylates PtdIns generating PtdIns 3-P, and temperature-sensitive Vps34p mutant cells exhibit a defect in protein sorting to the vacuole at non-permissive temperatures (Stack et al. 1995). The serine/threonine protein kinase Vps15p mediates membrane association and activation of Vps34p. Together they are localised on Golgi or endosome-enriched membrane fractions. These studies suggest a functional role for the Vps34p/Vps15p complex in the trafficking of hydrolytic enzymes.
to the vacuole. The equivalent trafficking pathway in mammalian cells, from the Golgi to lysosomes, was blocked by wortmannin, a potent inhibitor of PtdIns 3-kinase (Brown et al. 1995; Davidson 1995). Following many years of research it is now generally accepted that PtdIns 3-kinases and PtdIns 3,4,5-P₃ play a pivotal role in the insulin-stimulated translocation of the glucose transporter, Glut4 from internal storage pools to the plasma membrane. However, the precise signalling pathway remains uncertain (Section 1.7).

In 1997 Patki et al. undertook experiments to identify downstream effectors of PtdIns 3-kinase by searching for peripheral membrane proteins that bind to membranes in a wortmannin-sensitive manner. These studies lead to the identification of mammalian early-endosomal autoantigen-1 (EEA1) (Patki et al. 1997), which directly binds PtdIns 3-P through a 70-residue motif, referred to as the FYVE domain (Section 1.5.1). EEA1 also binds to Rab5 GTPase and stimulates homotypic endosome fusion (Simonsen et al. 1998). Similarly, Vac1p, the yeast orthologue of EEA1, interacts with the yeast Rab5 GTPase (Vps21p) and the endosomal t-SNARE Pep12p, thus co-ordinating the endosome fusion process (Peterson et al. 1999; Tall et al. 1999 and reviewed in Odorizzi et al. 2000; Wurmser et al. 1999).

Recent studies to determine the localisation of PtdIns 3-P in both mammalian and yeast cells confirmed that this phospholipid is highly enriched on early endosomes and in the internal vesicles of multivesicular endosomes (Gillooly et al. 2000). These vesicles, together with PtdIns 3-P are degraded by the hydrolases in the vacuole/lysosome. Although the bulk of PtdIns 3-P is metabolised via this route, Fab1p also uses it as a substrate to generate PtdIns 3,5-P₂ (Gary et al. 1998). Fab1p also contains a FYVE domain that binds PtdIns 3-P and may function to recruit this kinase to membranes containing its substrate. Golgi to vacuole trafficking is unaffected in FAB1 deletion mutants, however the vacuole in these cells is dramatically enlarged suggesting a distinct role for PtdIns 3,5-P₂ in regulating vacuole morphology. A model proposed by Gary and colleagues suggests that whereas Vps34p and PtdIns 3-P are essential for anterograde trafficking of membranes and cargoes to the vacuole, Fab1p and PtdIns 3,5-P₂ may play an important compensatory role in the efflux of vacuolar membranes (Gary et al. 1998).
Vesicle formation begins with the recruitment of a GTPase, ARFs (Section 1.6), to the donor membrane (Springer et al. 1999). ARF effector proteins that regulate its activation state, guanine nucleotide exchange factors (GEFs; Section 1.6.4) and GTPase activating proteins (GAPs; Section 1.6.5), contain a pleckstrin homology (PH) domain (Section 1.5.2) that bind phosphoinositides, namely PtdIns 3,4,5-P3 and PtdIns 4,5-P2. These findings agree with the proposed role of phosphoinositides as local modulators that recruit and may activate effector proteins involved in membrane trafficking. Additional evidence to support this theory came from experiments indicating the interaction between phosphoinositides and proteins involved in coat assembly. Adaptor protein-2 (AP-2), which is involved in clathrin coat formation and selection of cargo for internalisation at the plasma membrane, specifically binds PtdIns 3,4,5-P3 resulting in the inhibition of clathrin coat assembly (Gaidarov et al. 1996). Similarly adaptor protein-3 (AP-3), which is thought to function in synaptic vesicles biogenesis, and α-COP, one of the seven subunits that make up the complex of COPI, also display specific interactions with PtdIns 3,4,5-P3 (Chaudhary et al. 1998; Hao et al. 1997). β-arrestin and β-arrestin-2 are also thought to function as adaptors by linking agonist-activated G-protein coupled receptors with the clathrin-coated pit machinery and thus mediating their internalisation (Gaidarov et al. 1999; Lin et al. 1997). β-arrestin proteins contain a PH domain that could bind PtdIns 3,4,5-P3 or PtdIns 4,5-P2 in vitro, however β-arrestin recruitment to coated pits was shown to be wortmannin-insensitive and therefore PtdIns 4,5-P2 is the more likely binding candidate (Gaidarov et al. 1999).

There is now considerable evidence implicating PtdIns 4,5-P2 in vesicle trafficking. Firstly, the binding of PtdIns 4,5-P2 to ARF1 is proposed to promote its interaction with ARF-GAP (Randazzo 1997a). Secondly, in an assay measuring the formation of AP-2 mediated clathrin coated vesicle formation, the introduction of PH domains that specifically bind PtdIns 4,5-P2 inhibits the formation (Jost et al. 1998). Furthermore, the PtdIns 4,5-P2-sequestering PH domain also inhibits dynamin mediated coated pit formation and the dynamin mutant unable to bind PtdIns 4,5-P2 leads to inhibition of endocytosis (Achiriloaie et al. 1999; Lee et al. 1999; Vallis et al. 1999).

As well as functioning in endocytosis, evidence indicates a role for PtdIns 4,5-P2 in regulated exocytosis. Synaptotagmin, a synaptic vesicle membrane protein that interacts with SNARE proteins contains a C2B domain (Section 1.5) which binds both PtdIns 3,4,5-P3 and PtdIns 4,5-P2. However, in the presence of calcium, at levels
indicative of those required for neurotransmitter release, the specificity of binding switches to PtdIns 4,5-P$_2$ (Schiavo et al. 1996). Another synaptic vesicle protein rabphilin-3A, an effector of Rab3A, contains two C2 domains that bind PtdIns 3,4,5-P$_3$ and PtdIns 4,5-P$_2$. In vitro liposome-binding assays revealed calcium-dependent PtdIns 4,5-P$_2$ binding (Chung et al. 1998; Schiavo et al. 1996). It is proposed that these proteins may function concertedly in PtdIns 4,5-P$_2$-dependent docking of synaptic vesicles at the plasma membrane following calcium activation (Corvera et al. 1999).

1.5 Lipid Binding Motifs

There are several lipid-binding motifs that interact with phosphoinositides and inositol phosphates including: C2 domains (cysteine-rich regions), Src homology 2 (SH2) domains, FYVE domains (Section 1.5.1), PH domains (Section 1.5.2) and phosphotyrosine binding (PTB) domains. As described above for synaptotagmin and rabphilin-3A, C2 domains can bind to phosphoinositides in a calcium-dependent manner and are found in a number of other proteins including PKCs and phospholipase A (Hurley and Misra 2000). SH2 domains bind primarily to tyrosine-phosphorylated proteins. However, the SH2 domains of phospholipase C$_\gamma$, Src tyrosine kinase, and the p85 regulatory subunit of PtdIns 3-kinase have also been shown to bind specifically to PtdIns 3,4,5-P$_3$ (Rameh et al. 1995). The in vivo relevance of this interaction is supported by the observation that inhibition of PtdIns 3,4,5-P$_3$ production resulted in enhanced association of p85 with phosphotyrosine residues on upstream effectors such as the insulin receptor and the insulin receptor substrates (IRSs). Thus suggesting that PtdIns 3,4,5-P$_3$ binds directly to the SH2 domains of p85 and causes dissociation of PtdIns 3-kinase from tyrosine-phosphorylated proteins, thereby providing a negative feedback control. Similarly PtdIns 3,4,5-P$_3$ was shown to bind to the SH2 domains of PLC$_\gamma$ and enhanced its phospholipase activity toward PtdIns 4,5-P$_2$ (Bae et al. 1998). However more recently, structural and biochemical evidence argues against the theory of phosphoinositides binding to SH2 domains (Surdo et al. 1999).

PTB domains are related to PH domains and as their name suggests they bind phosphotyrosine proteins (reviewed by Lemmon and Ferguson 2000). The PTB domain of Shc was found to bind PtdIns 3,4,5-P$_3$ and PtdIns 4,5-P$_2$ (Rameh et al. 1997b).
Interestingly the binding of the latter phosphoinositide was shown to be displaced with phosphopeptides, analogous to the results observed with SH2 domains.

Since both FYVE domains and PH domains retain their lipid recognition properties when expressed independent from the complete protein, these domains can be utilised as experimental tools for studying the spatial and temporal characteristics of phosphoinositides (Balla et al. 2000). Using confocal microscopy, green fluorescent protein (GFP)-coupled domains, with specific binding, can be used to identify sites of synthesis of the phosphoinositides and examine changes in location in response to stimuli. One such experiment employed the PtdIns 3,4,5-P3 specific PH domains of the ARF effector proteins ARNO (ARF nucleotide-binding site opener) and GRP1 (general receptor for phosphoinositides-1) (Section 1.6.4). Localisation changes of these PH domains were studies in response to insulin and platelet-derived growth factor (PDGF) stimulation (Oatey et al. 1999).

1.5.1 FYVE Domains

FYVE domains acquired their name from the first letters of the founding members of this family (Fab1, YGO23, YPS27 and EEA1) (Stenmark et al. 1996). These domains, of 60-80 residues, contain eight conserved cysteines that form two zinc co-ordination centres, with a characteristic motif surrounding the third cysteine that is involved in binding the inositol head group of PtdIns 3-P. Structural investigations support the biochemical binding studies that reveal selectivity for PtdIns 3-P (Fruman et al. 1999). The importance of this lipid in recruiting proteins to the early endosome in vesicle trafficking is now well accepted and there may be many more cellular functions in which PtdIns 3-P and FYVE domain containing proteins are involved.

1.5.2 PH Domains

PH domains are protein modules of around 120 amino acids located in a large number of proteins involved in cellular signalling, cytoskeletal organisation, regulation of membrane trafficking, and modification of membrane phospholipids (Lemmon and Ferguson 1998). These domains were first identified as a protein region present at the N- and C-termini of pleckstrin, the major substrate of PKC in platelets (Haslam et al.
Nuclear magnetic resonance (NMR) or crystal structures are now known for several PH domains. In each case, the structure of the domain is remarkably similar, despite quite divergent sequence homology. The conserved structure contains two orthogonal antiparallel β-sheets (four β-strands, β1-β4 and three β-strands β5-β7, respectively), and a C-terminal amphipathic α-helix (reviewed by Lemmon and Ferguson 2000). Despite the lack of conserved sequence homology there is an invariant tryptophan residue situated in the C-terminal α-helix. The strands of each β-sheet are arranged as a β-meander, with each strand re-entering the sheet at the same side from which it left, with the strands occurring in the same order along the sheet as they do in the sequence. The connecting loops between β1/β2, β3/β4 and β6/β7 were found to be most variable in length. Sequence alignments suggest (by analogy to immunoglobulins) that these regions serve as variable loops and may constitute a ligand-binding site.

It was in 1994 that Harlan and colleagues demonstrated phosphoinositides binding to PH domains and proposed that this interaction may be important for membrane localisation of proteins containing these domains (Harlan et al. 1994). Subsequent crystallography analysis of PH domains bound to phosphoinositides or inositol phosphates provided valuable information of this interaction. In the crystal structure of the PLCγ PH domain bound to the head group of PtdIns 4,5-P2, the inositol moiety was located in a pocket flanked by the β1/β2 and β3/β4 loops (Ferguson et al. 1995). Specific basic residues in the β1 and β2 strands were identified as sites of interaction with the phosphate groups. Similarly the crystal structure of the Bruton's tyrosine kinase (Btk) PH domain in complex with Ins 1,3,4,5-P4 has been used to identify critical residues that interact with the phosphates on the inositol ring. An arginine residue at position 28 in the Btk PH domain (Arg-28), that interacts with the 3-phosphate of Ins 1,3,4,5-P4 was found to be mutated to a cysteine in X-linked immunodeficiency (Fukuda et al. 1996).

In 1997, Rameh et al. compared the ability of six PH domains to bind five different phosphoinositides. Four distinct PH domain subgroups were created on the basis of substrate specificity (Rameh et al. 1997b) (Figure 1.4, B). Group I PH domains display high affinity for PtdIns 3,4,5-P3, Btk and GRP1 are examples of such PH domains. Group II PH domains display low affinity for this lipid and have little or no preference
Group I: Highly specific for PtdIns 3,4,5-P_3 (or Ins 1,3,4,5-P_4)
- GRP1
- Btk
- PDK1

Group II: PtdIns 4,5-P_2
- PLC_δ
- β-spectrin pleckstrin (PH-N)

Group III: PtdIns 3,4-P_2 and PtdIns 3,4,5-P_3
- PKB (Akt)
- DAPPI/PHISH

Group IV: Low affinity for phosphoinositides
- Dynamin

(C) Lys-Xaa-Sma-Xaa_{6-11}-Arg/Lys-Xaa-Arg-Hyd-Hyd

(D) [LVIMF]-X-K-X-[GASP]-X_{m-}[WFA]-[K/R]-X-R-X-[FL]-[LM]-X_{n-}[LIF]-X-Y

Figure 1.4. Pleckstrin homology domains. (A) Ribbon diagram of the structure of the PH domain from human pleckstrin protein. (http://www-cryst.bioc.cam.ac.uk/~robert/cpgs/introduction/PHstructure.html.) The C-terminal amphipathic α-helix, common to all PH domains, is shown in blue; β-strands are shown in green and labelled 1-7; The N and C termini are also indicated. (B) Subdivision of PH domains into four groups according to the binding specificity, adapted from Rameh et al., (1997). (C) PPBM motif, showing the six conserved residues where ‘Arg’ is arginine, ‘Lys’ is lysine, ‘Xaa’ is any amino acid, ‘Sma’ is a small uncharged amino acid and ‘Hyd’ is a hydrophobic amino acid (Dowler et al., 2000). (D) “Signature motif” for PH domains known and/or predicted to bind 3-phosphoinositide with high affinity. A list of residues enclosed in square brackets represents the observed range of sequence variability at a given position; X denotes any residue; m= 5-10; n= 6-13; (Lietzke et al., 2000).
over PtdIns 4,5-P_2. The PH domains of protein kinase B (PKB, also known as Akt) and DAPP1 make up group III, which bind both PtdIns 3,4-P_2 and PtdIns 3,4,5-P_3. A final group of PH domains displayed low affinity for phosphoinositides, group IV. This group includes the dynamin PH domain that is unable to bind phosphoinositides unless it is dimerised. Oligomerisation is proposed to promote binding to membranes containing phosphoinositides which is critical for stimulation of its GTPase activity and its ability to perform scission of endocytic vesicles from the plasma membrane (Klein et al. 1998).

An in vitro assay called TOPIS (Targets of PtdIns 3-kinase Identification System) was devised by Skolnik and colleagues to identify proteins that bind to the plasma membrane in a PtdIns 3-kinase-dependent manner (Isakoff et al. 1998). A temperature sensitive yeast strain was transformed with a construct that expresses a constitutively active form of the GTPase Ras, fused with a PH domain of interest. This strain can only grow at restrictive temperatures if the Ras is targeted to the plasma membrane. This was achieved using the PLCγ PH domain, which drives membrane localisation by binding to PtdIns 4,5-P_2, thus rescuing the temperature-sensitive phenotype. Co-expression of a constitutively active form of PtdIns 3-kinase results in accumulation of PtdIns 3,4,5-P_3 and PtdIns 3,4-P_2 at the plasma membrane enabling analysis of PtdIns 3-kinase-dependent localisation of the different PH domains. From this study, a consensus sequence for high affinity binding to PtdIns 3,4-P_2 and/or PtdIns 3,4,5-P_3 was derived (Isakoff et al. 1998). More recently Dowler and colleagues noted that all of the specific PtdIns 3,4,5-P_3-binding proteins possessed a common sequence at the N-terminal regions of the PH domains, the putative PtdIns 3,4,5-P_3 binding motif (PPBM) (Figure 1.4, C). The expressed sequence tag (EST) database was searched for proteins possessing a PH domain containing this consensus sequence of six basic residues that appears to correlate with high-affinity PtdIns 3,4,5-P_3 binding (Dowler et al. 2000). Surprisingly this search to identify novel proteins that interact with PtdIns 3,4,5-P_3 identified several proteins that do not bind this phosphoinositide, but instead posses novel and unexpected in vitro phosphoinositide-binding specificity. Until the discovery of PEPP1 (phosphatidylinositol-three-phosphate-binding PH-domain protein-1), no PH domains were known to bind PtdIns 3-P, the exclusive ligand of FYVE domains. Likewise centaurin-β2 (Section 1.6.5) was the first PH domain protein found to exhibit selective binding for PtdIns 3,5-P_2 and FAPP1 (phosphatidylinositol-four-phosphate adaptor protein-1) for PtdIns 4-P (Dowler et al. 2000). Sequence analysis of the PPBM
region of these novel proteins revealed that one residue of the consensus sequence was altered. However, not all PH domains containing the six residues of the consensus sequence were able to bind PtdIns 3,4,5-P$_3$, thus emphasising the importance of residues outside the PPBM.

Lietzke and colleagues observed that some of the invariant basic residues are also conserved in the PLC$_8$ PH domain which binds PtdIns 4,5-P$_2$ rather than 3-phosphoinositides, but that a tyrosine residue located in the β3 strand was strictly conserved to PH domain that bind 3-phosphoinositides with high affinity. The “signature motif” for 3-phosphoinositide binding was redefined (Lietzke et al. 2000) (Figure 1.4, D).

The structural basis of 3-phosphoinositides recognition by PH domains, and the discrimination between phospholipids, has been most clearly revealed by X-ray crystallography studies. Structural analysis of the PH domains of GRP1 (Section 1.6.4), (which is specific for PtdIns 3,4,5-P$_3$) and DAPP1 (that binds both PtdIns 3,4,5-P$_3$ and PtdIns 3,4-P$_2$) identified key differences in the residues that interact with the phosphates (Ferguson et al. 2000). Although the two PH domains share the common core structure of seven β-strands and an α-helix, there is a 20 amino acid insertion in the β6/β7 loop of GRP1 (Lietzke et al. 2000). This insertion forms two additional β-strands, β6’ and β6’’, and appears to deepen the inositol phosphate-binding site. Hydrogen bonding, common to both PH domains, only occurs with the 1-, 3-, and 4-phosphates of Ins 1,3,4,5-P$_4$. In addition to these shared bonds, DAPP1-PH and GRP1-PH make three unique side-chain hydrogen bonds. All of the interactions unique to DAPP1 are made with the 4-phosphate, whereas GRP1 connects with both the 4- and 5-phosphates. This variation explains how only Ins 1,3,4,5-P$_4$ or PtdIns 3,4,5-P$_3$ satisfy the phosphate group recognition requirements for GRP1-PH, while PtdIns 3,4,5-P$_3$, PtdIns 3,4-P$_2$ and their inositol phosphate equivalents are able to satisfy the requirements for DAPP1-PH.

Since cellular levels of PtdIns 4,5-P$_2$ are considerably higher than PtdIns 3,4,5-P$_3$ it is estimated that in order to achieve PtdIns 3-kinase dependent membrane recruitment, a PH domain must display substantially higher (at least 25-fold) affinity for PtdIns 3,4,5-P$_3$ over PtdIns 4,5-P$_2$ (Lemmon and Ferguson 2000). All PH domains of groups I and III satisfy these conditions, including those of GRP1, PKB and DAPP1. Whereas all three of these PH domains are recruited to the plasma membrane following stimulation.
of PtdIns 3-kinase, PtdIns 3,4-P<sub>2</sub> accumulation in response to oxidative stress stimulates the translocation of PKB but not GRP1 (Gray <i>et al.</i> 1999; Isakoff <i>et al.</i> 1998; Venkateswarlu <i>et al.</i> 1998a). Thus, it can be concluded that a PH domain which recognises only PtdIns 3,4,5-P<sub>3</sub> (such as GRP1) will be recruited transiently to the plasma membrane, whereas one that binds both PtdIns 3,4,5-P<sub>3</sub> and PtdIns 3,4-P<sub>2</sub> (PKB and DAPP1) may have more sustained binding.

1.6 The Role of ARF Proteins in Vesicle Trafficking

ARFs are 20 kDa guanine nucleotide-binding proteins, members of the Ras GTPase superfamily, that were initially isolated because of their ability to stimulate the ADP-ribosyltransferase activity of the cholera toxin A subunit (Kahn and Gilman 1986). ARFs function as molecular switches, cycling between inactive GDP- and active GTP-bound states. The cycle is controlled by two sets of regulatory proteins, GEFs that facilitate the exchange of GDP for GTP, thereby activating ARF, and GAPs that stimulate GTP hydrolysis. Since ARFs possess a low intrinsic GTPase activity, ARF-GAP proteins are essential for ARF function.

Mammalian ARFs, of which there are six, can be divided into three classes: Class I, (ARF1-ARF3), Class II (ARF4 and ARF5) and Class III (ARF6) (Moss and Vaughan 1995). It is well established that mammalian Class I ARFs regulate coat assembly in vesicle formation from the ER, Golgi and TGN (Section 1.6.1). The function of Class II ARFs remains unknown. ARF6, the least conserved ARF protein, localises to the plasma membrane and endosomal compartments and is proposed to regulate membrane transport and cytoskeletal organisation (D'Souza-Schorey <i>et al.</i> 1998; Radhakrishna <i>et al.</i> 1999) (Section 1.6.2). In the yeast <i>Saccharomyces cerevisiae</i>, there are three ARF proteins. ARF1 and ARF2 are functionally interchangeable, and yeast cells require at least one of these proteins for viability. Yeast ARF3 is not essential for growth and probably corresponds to the mammalian ARF6.

In its inactive GDP-bound form ARF is predominantly cytosolic, whereas the GTP-bound conformation is membrane associated. ARF proteins possess a 17-residue myristoylated amphipathic α-helix at their N-terminus and tight binding to the membrane requires that this myristoyl group insert into the lipid bilayer. Early models of ARF function
proposed that, in the GDP-bound form, the N-terminal helix would be packed against
the protein core and that the exchange for GTP would induce a conformational change
allowing the helix to insert into the lipid bilayer (Amor et al. 1994). Therefore the site
of ARF activity would be determined by the location of the GEF protein responsible for
the release of GDP, thus dictating the location of vesicle formation. However,
subsequent studies have established that the myristate group is exposed and interacts
with the phospholipids when ARF is in the GDP-bound conformation (Franco et al.
1995; Goldberg 1998). However, the conformational change induced by GDP/GTP
exchange was found to expose several hydrophobic residues in the N-terminal helix,
thus facilitating the insertion into the lipid bilayer and stabilising ARFs membrane
association (Antonny et al. 1997). These authors went on to demonstrate a requirement
for membrane association of ARF1-GDP for the stable interaction with a GEF and the
subsequent GTP exchange (Beraud-Dufour et al. 1999). Moreover, the conformational
switch of amino-terminal helix (from a hydrophobic pocket in ARF-GDP to tight
association with lipids) was found to occur early in the exchange reaction, before the
release of GDP. Many GEFs are cytosolic proteins that associate with specific
membrane phosphoinositides via their PH domains (Section 1.6.4) and an important
conclusion of this study was that ARF-GDP and the GEFs must each contain
membrane-targeting information. Therefore localisation of the GEFs controls the site of
ARF activation thus governing the locality of vesicle formation.

1.6.1 ARF1

ARF1, the best characterised ARF protein, was originally found to be localised to the
Golgi complex where it mediates the interaction of coatamer into COPI vesicles
(Palmer et al. 1993). Interestingly, in addition to the involvement in the biosynthetic
pathway, there is growing evidence implicating ARF1 and endosomal COPs in
endosomal membrane transport (Gu and Gruenberg 2000). In addition there is evidence
to suggest a role for ARF1 in the formation of clathrin-coated vesicles. ARF1 was
shown to be required for the binding of adaptor protein-1 (AP-1) of clathrin-coated
vesicles to the Golgi membranes (Stamnes and Rothman 1993) and TGN (Zhu et al.
1998), and for the membrane recruitment of AP-3 (Ooi et al. 1998).
Using a cell free system Schekman and colleagues demonstrated coatomer, ARF1-GTPyS (non-hydrolysable GTP analogue) and liposomes to be sufficient components to reproduce Golgi vesicle budding (Spang et al. 1998). Confirmation of ARF’s regulatory role in vesicle trafficking \textit{in vivo} was achieved using ARF1 mutants that were defective in either the binding or hydrolysis of GTP. The mutant defective in GTP-binding prevented coat proteins from binding to the Golgi membrane, resulting in the retrograde transport of these membranes back to the ER. Intriguingly, the mutant defective in GTP-hydrolysis caused irreversible binding of COPI to the membranes, thus preventing uncoating and fusion with acceptor membranes (reviewed in Rothman and Wieland 1996; Schekman and Orci 1996).

### 1.6.2 ARF6

Initial studies by D’Souza-Schorey \textit{et al.} demonstrated that ARF6 was localised to the plasma membrane and intracellular endosomal compartments (D’Souza-Schorey \textit{et al.} 1995). Subsequent electron microscopy analysis revealed that at low levels of protein expression, wild-type ARF6 localised predominantly to intracellular compartments around the pericentriolar region of the cell (D’Souza-Schorey \textit{et al.} 1998). Whereas other ARF proteins are cytosolic in the GDP-bound form, ARF6-GDP accumulates in the pericentriolar region, characteristic of the recycling endosomal compartment. ARF6 mutants that are constitutively GDP-bound cause an intracellular accumulation of transferrin receptors. Conversely GTP-bound ARF6 mutants are localised at the plasma membrane where they reduce transferrin receptor uptake into the cell and stimulate the redistribution of receptors back to the cell surface (D’Souza-Schorey \textit{et al.} 1995). As well as functioning in membrane transport, ARF6 is able to stimulate cortical actin rearrangement (Radhakrishna and Donaldson 1997). ARF6 co-localises with Rac1, a Rho family GTPase implicated in cortical actin rearrangements, and is thought to control the ability of lamellipodia formation that is induced by Rac1 (Radhakrishna \textit{et al.} 1999). ARF6 has also been implicated in the regulation of cell surface levels of Glut4, as insulin-stimulated glucose transport and Glut4 translocation were partially inhibited (~50%) using the myristolated N-terminal peptide of ARF6 in adipocyte cells (Millar \textit{et al.} 1999).
1.6.3 Coat Assembly and Vesicle Formation

As described above, formation of the vesicle begins upon activation of membrane associated ARF, which then recruits the coat protein complex coatomer. Using a yeast two hybrid system this interaction with ARF-GTP was recently shown to occur with subunits β- and ε-COP (Eugster et al. 2000). Coatomer polymerisation induces membrane deformation, in the process cargo proteins and targeting machinery are incorporated and budding occurs. Finally, a vesicle associated ARF-GAP stimulates hydrolysis of bound GTP to release ARF, triggering uncoating of the vesicle which is required prior to docking and fusion with the acceptor membrane. This model describes in brief the stages that occur in vesicle formation (reviewed by Chavrier and Goud 1999; Moss and Vaughan 1998; Roth 1999; Springer et al. 1999), however there may be many more proteins involved in the regulation of this process.

For vesicles to mediate selective transport, coats must distinguish between cargo and resident proteins. Sorting information may be encoded in cytoplasmic sorting signals that interact directly with the coat. For example, ER-resident proteins with the dilysine trafficking motif (KKXX) are recognised by coatomer for retrograde Golgi-to-ER transport in COPI-coated vesicles (Bremser et al. 1999). Similarly, in COPII vesicle transport from ER to Golgi, the coat subunit Sec23/24p complex interacts with the GTPase Sar1p-GTP and Bet1p (v-SNARE) (Springer and Schekman 1998). Springer et al. (1999) proposed a model of vesicle formation that involved the capture of cargo protein into a priming complex with a small GTPase together with one or several other subunits of the vesicular coat, including a GAP protein. The KDEL receptor is a further example of sorting signal for COPI mediated transport for retrieval from the Golgi to the ER. The ligand bound KDEL receptor has been shown to recruit ARF-GAP into the priming complex. Using the yeast two-hybrid system described above for the identification of COPI subunits interacting with ARF-GTP, it was also found that γ- and β'-COP interact with the ARF-GAP called Glo3p (Eugster et al. 2000). In the same study the authors demonstrated that recombinant hexa-His-tagged Glo3p interacts with coatomer from cytosol in vitro. Therefore, cargo proteins and targeting machinery may play an active role in coat assembly and/or control uncoating through regulation of ARF-GAP activity.
Figure 1.5. A hypothetical model for the involvement of ARF in vesicle formation. In the inactive GDP-bound form ARF has low affinity for membrane phosphoinositides and shuttles between the membrane and cytosol. ARF-GEF proteins promote nucleotide exchange, which induces a conformational change resulting in stable association of ARF-GTP. This interaction is dependent on the myristate group, which inserts into the lipid bilayer. Coatomer is recruited and assembled through a bivalent interaction with ARF-GTP and the cytosolic sorting signal on membrane cargo proteins. An ARF-GAP protein is recruited during this process by its interaction with the membrane cargo protein. Coatomer polymerisation induces membrane deformation and budding occurs. Adapted from Chavrier et al. (1999).
As described above ARF-GAPs appear to be incorporated into the priming complex during vesicle formation, however the point at which they stimulate GTPase activity remains unknown. Although GTP hydrolysis is required for the release of ARF from the membrane and the subsequent uncoating of the vesicles, it may occur prior to vesicle scission. In 1999, Goldberg presented the crystal structure of ARF1 complexed to ARF-GAP. An ARF1 mutant in which the myristolated N-terminal α-helix was truncated (termed ARF1Δ or Δ17-ARF1) was crystallised in complex with a 130-amino acid catalytic fragment of GAP1 (Goldberg 1999). Unlike Ras GTPases, the GAP-binding domain of ARF1 does not overlap with the effector-binding site. This allows ARF1-GTP to simultaneously interact with coatmer (the effector) and ARF-GAP, described as the tripartite model (Goldberg 1999). Biochemical studies have shown that coatmer stimulated GTP hydrolysis by 1000-fold in an ARF-GAP-dependent manner, further strengthening the tripartite model. More recently, Goldberg suggested that coatmer recognition of sorting signals on cargo proteins might be a selection mechanism conferring differentiation through GTP hydrolysis (Goldberg 2000). However, it is important to note that the assays using truncated ARF1 and the catalytic domain of GAP1 were performed in a membrane-free environment. When these experiments were repeated using wild-type myristolated ARF1-GTP in the presence of phospholipid vesicles, GAP1 stimulated GTP hydrolysis in the absence of coatmer therefore challenging the tripartite model (Szafer et al. 2000). Furthermore, coatmer was unable to enhance the GTPase activity. Disruption of the vesicle phospholipids with detergent resulted in reduced GAP activity, which was coatmer-sensitive, a pattern similar to the activity of Δ17-ARF1. Taken together these findings indicate that coatmer may function to recruit the GAP protein and that the binding of both ARF and its GAP to membrane phospholipids may be sufficient for the stimulation of ARF1 GTPase activity (Eugster et al. 2000; Szafer et al. 2000).

1.6.4 ARF-GEF Protein Family

ARF-GEFs are a family of proteins that share a common domain of ~200 amino acids, termed the Sec7 domain. Chardin and colleagues established that the guanine exchange activity resides in the Sec7 domain and that this domain alone is sufficient for exchange activity (Chardin et al. 1996). This highly divergent family of proteins has been subdivided into four distinct subfamilies on the basis of size and sequence homology.
At present there are two families of large GEFs (> 100 kDa) and two families of small GEFs (Jackson and Casanova 2000). In addition to the distinct domain structure, these proteins also differ in their sensitivity to the fungal metabolite brefeldin A (BFA). BFA inhibits protein secretion and induces disassembly of the Golgi complex and redistribution of resident Golgi markers to the ER (Chardin and McCormick 1999). By stabilising the ARF-GDP-GEF complex, BFA inhibits GEF-catalysed ARF activation.

The large (>100 kDa) ARF-GEFs of the Gea and Sec7/BIG subfamilies function in the ER-Golgi system, and the majority are BFA-sensitive. In contrast the cytohesin (ARNO/cytohesin/GRP1) and EFA6 subfamilies function primarily in the endosomal-PM system and are resistant to the adverse effects of BFA (reviewed by Donaldson and Jackson 2000).

ARNO, cytohesin-1 and GRP1 are closely related in size and sequence (77% identity), and share a common domain structure. This consists of an N-terminal coiled coil region, a central Sec7 domain, a PH domain and an adjacent carboxyl-terminal polybasic domain. GRP1 was first identified from a screen for phosphoinositide-binding proteins and its PH domain specifically binds to PtdIns 3,4,5-P3 (Klarlund et al. 1997). Similarly, ARNO and cytohesin-1 display high affinity for PtdIns 3,4,5-P3 although they do not exhibit the same degree of selectivity for PtdIns 3,4,5-P3 over PtdIns 4,5-P2 as that exhibited by GRP1 (Klarlund et al. 2000). The remarkable PtdIns 3,4,5-P3 binding selectivity of GRP1 was found to be created by the unique diglycine motif in the β1/β2 loop of the PH domain, as opposed to the triglycine motif in ARNO and cytohesin-1 (Cullen and Chardin 2000; Klarlund et al. 2000). The addition of an extra glycine in GRP1 greatly increased the binding affinity of PtdIns 4,5-P2, while deletion of a single glycine in the ARNO PH domain markedly reduced its binding affinity for PtdIns 4,5-P2. However, in both situations the PtdIns 3,4,5-P3 binding was not altered (Klarlund et al. 2000).

As described above (Section 1.5) PH domains that selectively bind PtdIns 3,4,5-P3 have been utilised for the investigation of cellular distribution of this phosphoinositide. Moreover, recruitment of GFP-tagged ARNO to the plasma membrane was demonstrated in insulin-stimulated 3T3-L1 adipocytes (Venkateswarlu et al. 1998b). Similar studies have shown membrane recruitment in response to PtdIns 3-kinase
Figure 1.6 The Sec7 family of ARF-GEFs. All proteins share homology in the Sec7 domain and have been subdivided into the four subgroups according to their size and sequence homology. The small (<100 kDa) ARF-GEFs of the cytohesin (ARNO/cytohesin/GRP1) and EFA6 subfamilies are (at the top of the figure) are relatively insensitive to BFA. In contrast the majority of the large (>100 kDa) ARF-GEFs of the Gea and Sec7/BIG subfamilies are BFA-sensitive. This diagram has been adapted from Jackson and Casanova 2000, with proportional representation of the domain size and arrangement.
stimulation for GRP1 (Langille et al. 1999) and cytohesin-1 (Nagel et al. 1998; Venkateswarlu et al. 1999a). It was clear, both from using the PtdIns 3-kinase inhibitors wortmannin and LY294002, and by co-expression with a dominant-negative p85 mutant, that the translocation is a consequence of agonist-stimulated PtdIns 3-kinase activity. Interestingly, overexpression of ARNO and ARNO3 (the human orthologue of GRP1) results in fragmentation of the Golgi complex, implying a possible role in Golgi to ER transport (Franco et al. 1998; Monier et al. 1998). More recently the N-terminal coiled coil region of B2-1 (identical to human cytohesin-1) was shown to be responsible for Golgi targeting (Lee and Pohajdak 2000). In agreement with this study both endogenous and transfected B2-1 were found to be localised in a perinuclear compartment that co-localises with Golgi complex markers (Lee et al. 2000). As observed with ARNO and ARNO3, overexpression of B2-1 results in disruption of the Golgi complex.

The physiological substrate for the cytohesin family of GEFs has been the focus of some debate. GRP1 was found to catalyse guanine nucleotide exchange of ARF1 and ARF5, but not ARF6 (Klarlund et al. 1998), cytohesin-1 was active towards ARF1 and ARF3, but not ARF5 (Meacci et al. 1997) and both ARF1 and ARF6 were substrates for ARNO (Frank et al. 1998a). In contrast to these individual studies, Franco and colleagues analysed the substrate specificity of all three proteins in the cytohesin family and found that they all act as ARF1 GEFs in vitro, whereas they had little effect on ARF6 (Franco et al. 1998). The relevance of the in vitro nucleotide exchange assays therefore remains disputable. Nevertheless in vivo data is providing some useful clues as to the function of these small GEF proteins.

The observation of plasma membrane localisation of the cytohesin family of ARF-GEFs has led to the proposal that ARF6 may be their physiological substrate. In support of this hypothesis Frank and colleagues discovered that overexpression of ARNO in HeLa cells induced disassembly of actin stress fibres, changes similar to those resulting from ARF6 activation (Frank et al. 1998b). Subsequent treatment of ARNO transfectants with a PKC agonist stimulated the dramatic redistribution of ARNO, ARF6 and actin into membrane protrusions that resembled lamellipodia. Moreover, GRP1 was found to co-localise with ARF6 in plasma membrane ruffles in response to insulin and epidermal growth factor (EGF) stimulation (Dowler et al. 1999). Venkateswarlu and Cullen have proposed a link between agonist stimulation of PtdIns 3-kinase and ARF6 activation
and redistribution to the plasma membrane. Membrane recruitment of ARNO (through PtdIns 3,4,5-P$_3$ binding) resulted in an increase in ARF6 exchange activity. GDP/GTP exchange is thought to occur when the recycling endosomal compartment comes into juxtaposition with the plasma membrane bound ARF-GEF (Venkateswarlu and Cullen 2000). This experiment demonstrates that agonist stimulation of PtdIns 3-kinase increases the catalytic activity of the cytohesin family ARF-GEFs by inducing their recruitment to the plasma membrane.

A specific ARF6 GEF has been identified and entitled exchange factor for ARF6 (EFA6). This is structurally distinct from the cytohesin family of GEFs (Franco et al. 1999). Studies suggest that EFA may regulate trafficking from the recycling endosome to the plasma membrane and the redistribution of the actin cytoskeleton. The latter may facilitate endosomal trafficking to the cell surface.

### 1.6.5 ARF-GAP Protein Family

ARF-GAP1, the first ARF-GAP protein to be identified, was cloned from rat liver and found to contain an N-terminal zinc-finger motif that was critical for GAP activity (Cukierman et al. 1995). In the last few years a number of new ARF-GAP proteins have been identified and they all share a common GAP domain of ~70 amino acids which contains the zinc-finger motif of CX$_2$CX$_{16-17}$CX$_2$C (where C is cysteine and X is any amino acid). In addition to the GAP domain, some ARF-GAP proteins contain ankyrin repeats and/or PH domains. The size and arrangement of the domains are quite distinct and allows the multidomain proteins to be divided into sub-groups (Figure 1.7).

The molecular action of ARF-GAPs appears to differ to that of the GAP proteins of other GTPases, such as Ras and Rho. The crystal structure of ARF1-GDP complexed to ARF-GAP revealed that ARF-GAP binds to an unexpected region away from the catalytic site (Goldberg 1999). In addition ARF-GAP does not appear to provide any catalytic residues, a principle feature of Ras-GAP (Scheffzek et al. 1997).

Randazzo and colleagues designed an assay to determine ARF-GAP activity, which measured a single round of hydrolysis (Randazzo and Kahn 1994). In brief, the ARF proteins (purified or recombinantly expressed in myristolated and non-myristolated
Figure 1.7 The ARF-GAP family. All of these proteins possess the ARF GAP domain that contains a zinc-finger binding motif $\text{CX}_2\text{CX}_{16-17}\text{CX}_2\text{C}$ (where C is cysteine and X is any amino acid). Closely related proteins are grouped together. There are four subgroups of centaurin proteins: α, β, δ and γ. Gcs1 and Glo3 are from S. cerevisiae. This diagram has been adapted from Donaldson and Jackson 2000, with proportional representation of the domain size and arrangement.
forms) were pre-loaded with $[\alpha-^{32}P]GTP$ and added to a GTPase reaction with the GAP protein. Other additions, such as phospholipids, were also tested in the assay. Protein-bound nucleotide was trapped on nitrocellulose filters, extracted and fractionated by thin-layer chromatography, which was then visualised and quantified for $^{32}P$. This assay has been used to determine the specificity of several of the ARF-GAP proteins.

ARF-GAP1 regulates GTP hydrolysis on ARF1 in the Golgi (Cukierman et al. 1995). Two ARF1 GAP proteins from rat liver have been resolved and found to have different phospholipid dependencies (Randazzo 1997b). ARF-GAP1 was activated by dioleoglycerols, whereas PtdIns 4,5-P$_2$ and phosphatidic acid activate ARF-GAP2 (Randazzo 1997b). The yeast ARF-GAPs, Gcs1 and Glo3, which act on yeast ARF1 and ARF2, mediate retrograde vesicular transport from the Golgi to the ER (Poon et al. 1996; Poon et al. 1999).

ASAP1 (so called because it is an ARF-GAP containing a SH3 domain, ANK repeats and a PH domain, also known as centaurin-β4) was the first peripheral ARF-GAP to be characterised. Identified in a screen for proteins interacting with the SH3 domain of Src (Brown et al. 1998), ASAP1 has been shown to bind to Src and focal adhesion tyrosine kinase (FAK) and is localised to focal adhesions (Randazzo et al. 2000). Overexpression of ASAP1 altered the morphology of focal adhesions and inhibited both cell spreading and formation of PDGF-induced dorsal membrane ruffles. ASAP1, in which a mutation was introduced that disrupts the GAP activity, had a reduced effect on spreading and increased the number of cells forming ruffles in response to PDGF (Randazzo et al. 2000). As ARF6 has been implicated in the cortical actin rearrangement, it would appear that ASAP might function as an ARF6 GAP. Interestingly, an ASAP1 construct (consisting of the PH domain, zinc-finger motif and ankyrin repeat regions) was found to have PtdIns 4,5-P$_2$-dependent GAP activity for ARF1 and ARF5, and less activity for ARF6 (Andreev et al. 1999; Brown et al. 1998).

Closely related to ASAP1 is Pyk2-associated protein (PAP; also known as centaurin-β3) which also displays strong GAP activity towards ARF1 and ARF5, and weak activity for ARF6 (Andreev et al. 1999; Brown et al. 1998). There appears to be two splice variants of the PAP, the larger human homologue designated PAPα and also known as PAG3 (Kondo et al. 2000), and the shorter murine PAPβ (Andreev et al. 1999). All ARF-GAPs contain a conserved arginine residue within the GAP domain (five residues...
after the fourth cysteine residue) and in ASAP1 and PAPβ mutation of this arginine residue reduced GTPase activity by 100,000-fold (Mandiyan et al. 1999; Randazzo et al. 2000). Crystal structure analysis of the GAP domain and ankyrin repeats of PAPβ revealed that this conserved arginine was situated on the surface of the protein and not at the site of ARF interaction (Goldberg 1999).

ACAP1 and ACAP2 (for ARF-GAP with coiled coil, ankyrin repeat and PH domains; also known as centaurin-β1 and centaurin-β2 respectively) have been grouped with ASAP1 and PAP as they share the general domain structure but lack the SH3-domain (Jackson et al. 2000a). The GAP activity of the ACAP proteins was found to be dependent on PtdIns 4,5-P₂ and phosphatidic acid. However in contrast to ASAP1 and PAP, ACAP proteins possess GAP activity for ARF6 in preference to ARF1 and ARF5 (Jackson et al. 2000a). As for ASAP1, studies have demonstrated the recruitment of ACAP proteins to focal adhesions, and overexpression produced similar results altering the morphology of the focal adhesions (Jackson et al. 2000a). Another ARF-GAP protein to be identified through its interaction with focal adhesion proteins was Pkl (paxillin-kinase-linker), which as its name suggests, interacts with paxillin (Turner et al. 1999).

A distinct family of ARF-GAPs has been discovered, the GITs are G-protein-coupled receptor kinase interacting proteins whose GAP activity is stimulated by PtdIns 3,4,5-P₃. GIT1 was identified in a yeast two-hybrid screen with G-protein coupled receptor kinase-2 (GRK-2). Overexpression reduced β₂-adrenergic receptor signalling and increased receptor phosphorylation, these effects result from reduced receptor internalisation and resensitisation (Premont et al. 1998). Later studies have revealed GIT1 to be specific for receptors internalised via clathrin-coated vesicles, but was not limited to G protein-coupled receptors as the EGF receptor was similarly affected by overexpression (Claing et al. 2000). Interestingly GIT1 and its shorter splice variant GIT2 stimulate GTP hydrolysis on all five ARFs tested (ARF1-3 and ARF5-6) (Vitale et al. 2000). The findings of these studies suggest the involvement of this ARF-GAP subfamily in the regulation of agonist-stimulated receptor internalisation including PtdIns 3-kinase activating receptors such as EGF.

The PH domain of ASAP1 corresponds to that of group I and would therefore be expected to bind PtdIns 3,4,5-P₃ with high affinity. Studies have shown that although
PtdIns 3,4,5-P₃ was sufficient for membrane recruitment it did not induce GAP activity (Kam et al. 2000). Activation of ASAP1 was mediated by PtdIns 4,5-P₂ which induced a specific conformational change in ASAP1, and possibly ARF as well (Kam et al. 2000). Surprisingly ASAP1 mutants, which do not bind phosphoinositides, or which completely lack the PH domain, are recruited to membrane ruffles. This suggests that although the PH domain may contribute to targeting, it is not required. Therefore, phosphoinositides appear to play different roles in ARF-GAP and ARF-GEF function.

Finally, there is a group of zinc-finger containing proteins, the centaurin-αs, for which ARF-GAP activity has not been detected (Figure 1.7 & 1.8; Section 1.6.5.1). These proteins have been grouped together on the basis of sequence homology and size. Rat centaurin-α, the first to be cloned and characterised, is the least conserved of the group and contains ankyrin repeats and one PH domain (Hammonds-Odie et al. 1996). The remaining proteins, which are more closely related, possess two PH domains. Although ARF-GAP activity has not been demonstrated for any of the centaurin-α proteins to date (Jackson et al. 2000b; Tanaka et al. 1999; Venkateswarlu and Cullen 1999), human centaurin-α1 was shown to functionally complement the yeast ARF-GAP, Gcs1 (Venkateswarlu et al. 1999b).

1.6.5.1 The Centaurin-α Protein Family
In 1996 Hammonds-Odie et al. described the identification and cloning of centaurin-α. Affinity chromatography, using aminopropyl-Ins 1,3,4,5-P₄ linked to Affi-Gel 10 matrix, was employed to purify potential PtdIns 3,4,5-P₃ receptors from rat brain homogenates. Initial sequence analysis performed by Theibert and colleagues revealed a 419 amino acid protein with an N-terminal zinc-finger binding motif and ten divergent ankyrin-like repeats. The chimeric nature of centaurin-α and its homology to GAP proteins appeared reminiscent of the chimaerins, and consequently the protein was named after the centaur (half-man, half-horse) of Greek mythology (Hammonds-Odie et al. 1996). The high affinity binding of PtdIns 3,4,5-P₃ and homology to ARF-GAPs lead to the suggestion that PtdIns 3-kinase receptors might act via centaurin-α to regulate ARF-dependent membrane trafficking events.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Protein isolated from:</th>
<th>cDNA cloned from:</th>
<th>Protein size (amino acids)</th>
<th>Calculated molecular weight</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centaurin-α</td>
<td>Rat whole brain</td>
<td>Rat brain cDNA</td>
<td>419</td>
<td>46 kDa</td>
<td>Hammonds-Odie et al., 1996</td>
</tr>
<tr>
<td>PIP₃-BP</td>
<td>Bovine brain</td>
<td>Brain cDNA</td>
<td>373</td>
<td>43.2 kDa</td>
<td>Tanaka et al., 1997</td>
</tr>
<tr>
<td>p42ᵧp₄</td>
<td>Porcine cerebellum membranes</td>
<td>Porcine cerebellum cDNA</td>
<td>374</td>
<td>43.4 kDa</td>
<td>Stricker et al., 1997</td>
</tr>
<tr>
<td>p42ᵧp₄</td>
<td>–</td>
<td>Rat brain</td>
<td>374</td>
<td>43.4 kDa</td>
<td>Aggensteiner et al., 1998</td>
</tr>
<tr>
<td>Centaurin-α₁</td>
<td>–</td>
<td>Human blood cDNA</td>
<td>374</td>
<td>43.4 kDa</td>
<td>Venkateswarlu et al. 1999.</td>
</tr>
</tbody>
</table>

**Figure 1.8. Summary of the centaurin-α protein family.** Rat centaurin-α (accession number U51013); bovine PIP3-BP (accession number D89940); porcine p42ᵧp₄ (accession number U88368); rat p42ᵧp₄ (accession number AJ007422); human centaurin-α₁ (accession number AJ006422).
In 1997 two further members of the centaurin-α family were identified from cow and pig and were named PIP3-BP (PtdIns 3,4,5-P_3-binding protein) and p42^{IP4}, respectively (Stricker et al. 1997; Tanaka et al. 1997). Like rat centaurin-α, PIP3-BP was also purified from brain by affinity chromatography, but using a PtdIns 3,4,5-P_3 analogue. In 1999, the cloning and characterisation of another orthologue, human centaurin-α1 was reported (Venkateswarlu and Cullen 1999). PIP3-BP, p42^{IP4} and centaurin-α1 share high homology (> 90% identity) and as such are considered to be orthologues, the same protein from different species, and are collectively referred to as centaurin-α1. Although centaurin-α shares relatively high homology to centaurin-α1 (~75-79% identity) there are two regions of distinction. Firstly, centaurin-α contains ankyrin repeats in the PH-N region of centaurin-α1. Secondly, centaurin-α has an extended sequence that terminates approximately 46 amino acids later than centaurin-α1. Thus the centaurin-α sequence encodes a protein with a predicted molecular mass of 48 kDa protein in comparison to centaurin-α1 42-43.5 kDa (Figure 1.8). Doubt was cast on the authenticity of the rat centaurin-α sequence by the findings of Reiser and colleagues. Reverse transcription-polymerase chain reaction (RT-PCR) and western blotting approaches were employed to search for the presence of centaurin-α in rat brain (Aggensteiner et al. 1998). Several clones were identified that were analogous to p42^{IP4} in both the PH-N domain and termination position. Furthermore, none of the clones contained the sequence characteristics of rat centaurin-α. The anti-peptide antiserum designed to both centaurin-α and p42^{IP4} detected a single 42 kDa protein band that comigrated with pig p42^{IP4} (Aggensteiner et al. 1998). Thus, no molecular or biochemical evidence to support the presence of centaurin-α was found in this study.

Centaurin-α and centaurin-α1 transcripts are highly abundant in brain, and much lower levels of expression have been detected in other tissues, such as lung, spleen, kidney and peripheral blood leukocytes (Hammonds-Odie et al. 1996; Tanaka et al. 1997; Venkateswarlu and Cullen 1999). For the centaurin-α1s whose tissue-distribution has not been investigated, they were either purified from brain tissue and/or cloned from brain cDNA (Aggensteiner et al. 1998; Stricker et al. 1997).

Western blot analysis and phospholipid binding assays suggest that the centaurin-α1 proteins are both membrane-associated and cytosolic. However, the distribution varies quite considerably between studies, for example, rat centaurin-α and pig p42^{IP4} are
reported to have membrane:cytosol distribution ratios of 1:2 and 3:1, respectively (Hammonds-Odie et al. 1996; Stricker et al. 1997). Immunostaining with a monoclonal PIP3-BP antibody indicated PIP3-BP to be localised in nuclei of neuronal cells and similar results were observed with GFP-tagged constructs of the N-terminal 14 amino acids and full-length PIP3-BP (Tanaka et al. 1999). Similarly GFP-tagged centaurin-α1 was found to be localised in the nucleus and cytoplasm of PC12 cells (Venkateswarlu et al. 1999b). These results were reproduced with the nuclear localisation of both HA-tagged and GFP-tagged human centaurin-α1 in CHO.T cells (Venkateswarlu and Cullen 1999). Interestingly EGF-stimulation caused translocation of human centaurin-α1 to the plasma membrane in PC12 cells (Venkateswarlu et al. 1999b). Membrane recruitment of centaurin-α1 was also observed in CHO.T cells co-transfected with the constitutively active, membrane targeted p110 catalytic subunit of PtdIns 3-kinase (Venkateswarlu and Cullen 1999). The transient nature of this membrane recruitment is consistent with the generation of PtdIns 3,4,5-P₃ and appears to be dependent on PtdIns 3-kinase (Venkateswarlu et al. 1999b).

Although all centaurin-α proteins have been shown to bind PtdIns 3,4,5-P₃, the specificity and selectivity characterisation of these proteins remains incomplete. Comparison of data is complex due to the differences in assay conditions used by the various groups. For example, Tanaka and colleagues investigated the ability of phospholipids to displace glutathione S-transferase (GST)-tagged PIP3-BP from the PtdIns 3,4,5-P₃ analogue resin used for its purification (Tanaka et al. 1997). In this technique the two PH domains behave individually, and therefore it may be possible for a phospholipid to bind to one PH domain while PIP3-BP remains bound to the resin via the second PH domain. Hammonds-Odie et al. assessed the relative binding affinities of native centaurin-α using [³H]-p-benzoyledihydrocinnamoyl- ([³H]BZDC-) Ins 1,3,4,5-P₄, a photoprobe that covalently links to the protein to which it was bound (Hammonds-Odie et al. 1996). Despite the differing investigative approaches, the general consensus of the binding data shows that the centaurin-α proteins possess high affinity for PtdIns 3,4,5-P₃ and 1,3,4,5-P₄ (Hammonds-Odie et al. 1996; Stricker et al. 1997; Tanaka et al. 1997; Venkateswarlu and Cullen 1999).

As stated above no ARF-GAP activity has been shown for any of the centaurin-α proteins to date (Jackson et al. 2000b; Tanaka et al. 1999; Venkateswarlu and Cullen 1999).
However, Cullen and colleagues have shown human centaurin-α1 to functionally complement the yeast ARF-GAP Gcs1 (Venkateswarlu et al. 1999b). This study utilised a Gcs1 deleted yeast strain Δgcs1, which was unable to grow on fluoride containing media. Transformation of the Δgcs1 yeast strain with centaurin-α1 or Gcs1 rescued growth indicating that centaurin-α1 may function as an ARF-GAP despite the lack of \textit{in vitro} activity. More recently centaurin-α1 was found to copurify with casein kinase-Iα (CKIα) (Dubois et al. 2001). Upon investigation it was discovered that centaurin-α and centaurin-α1 associate specifically and directly with CKIα within the kinase domain, which is well conserved in all seven CKI isoforms. However, the data suggest that CKIα does not phosphorylate centaurin-α, and centaurin-α had no affect of the activity of this serine/threonine protein kinase. The CKI isoforms are implicated in a diverse range of cellular function including membrane trafficking and CKIα has recently been shown to associate with the clathrin adaptor AP-3 in synaptic vesicle coating (Faundez and Kelly 2000). CKIα represents the first protein partner identified for the centaurin-α proteins, however the physiological relevance of this association remains to be demonstrated.

1.7 The Role of PtdIns 3-kinase and PtdIns 3,4,5-P$_3$ in Insulin-Stimulated Glut4 Translocation

Glut4 is unique among the glucose transporter isoforms in that its subcellular localisation is regulated by insulin. In insulin-sensitive tissues, such as muscle and adipose tissue, Glut4 is sequestered in perinuclear tubulovesicular structures and vesicles known as Glut4 storage vesicles when in the basal, non-stimulated state (Rea and James 1997). However, upon stimulation with insulin, Glut4 is released from its storage sites and translocates to the plasma membrane. Cell surface levels of Glut4 increase by approximately 20-fold, resulting in increased transport of glucose into the muscle and adipose cells with concurrent reduction in blood glucose levels (Holman \textit{et al}. 1990).

Insulin binds to and activates the insulin receptor, which then initiates the signalling cascade (Figure 1.9). There is considerable evidence supporting the role of PtdIns 3-kinase in Glut4 translocation and the concomitant increase in glucose transport
Figure 1.9. A simplified model for insulin-stimulated Glut4 translocation. Insulin stimulates glucose uptake into skeletal muscle, heart and adipose tissue. This effect begins with the interaction of insulin with its receptor, which triggers autophosphorylation of the receptor and cellular substrates including the insulin receptor substrate (IRS) proteins. Tyrosine phosphorylated IRS interacts with the SH2 domain of the p85 regulatory subunit of PtdIns 3-kinase and serves both to activate the p110 catalytic subunit and to appropriately target the PtdIns 3-kinase to its subcellular site of action. PtdIns 3-kinase phosphorylates phosphoinositides at the D-3 position on the inositol ring. The synthesis of PtdIns 3,4,5-P₃, the major product of PtdIns 3-kinase is illustrated. Multiple lines of evidence, including the use of the PtdIns 3-kinase inhibitor wortmannin, demonstrate the requirement for PtdIns 3-kinase activity in the insulin stimulation of glucose transport through the translocation of Glut 4.
Ptdlns 3-kinase inhibitors, wortmannin and LY294002, block insulin-stimulated glucose transport and the translocation of Glut4 to the plasma membrane (Kotani et al. 1995; Okada et al. 1994). Membrane permeant analogues of PtdIns 3,4,5-P_3 were able to restore glucose uptake in insulin-stimulated cells inhibited with wortmannin, however this phosphoinositide had no effect in the absence of insulin (Jiang et al. 1998). An alternative approach using the dominant negative form of the p85 subunit of PtdIns 3-kinase which was shown to be unable to stimulate glucose uptake (Hara et al. 1994). Overexpression of wild-type or constitutively active form of p110 catalytic subunit of PtdIns 3-kinase was sufficient to stimulate the translocation of Glut4 to the plasma membrane of cultured 3T3-L1 adipocytes (Katagiri et al. 1996; Martin et al. 1996) and isolated rat adipocytes (Tanti et al. 1996). Therefore it is generally accepted that the generation of the PtdIns 3-kinase product PtdIns 3,4,5-P_3 mediates Glut4 translocation, although the precise mechanisms are unclear.

One important characteristic is that other growth factors activate PtdIns 3-kinase, but are unable to stimulate Glut4 translocation or glucose uptake. It has been proposed that the specificity of insulin activation may be mediated by differential compartmentalisation of PtdIns 3-kinase. In support of this hypothesis, subcellular fractionation analysis in 3T3-L1 adipocytes has revealed that insulin- and PDGF-stimulated PtdIns 3-kinase activities are localised in the high speed pellet and plasma membrane, respectively (Clark et al. 1998). This study also suggested the potential involvement of the actin cytoskeleton in targeting of PtdIns 3-kinase to Glut4 vesicles. This was supported by Klip and colleagues who used cytochalasin D, a fungal metabolite, to prevent polymerisation of actin filaments and inhibit glucose transport (Wang et al. 1999). Other studies have indicated that microtubules may also function in the trafficking of Glut4 vesicles. For example the microtubule protein α-tubulin and the intermediate filament protein vimentin were identified as components of purified Glut4 storage vesicles (Guilherme et al. 2000). Furthermore, disruption of the microtubule network with the depolymerising agent nocodazole inhibited insulin-mediated Glut4 translocation by more than 80% in 3T3-L1 cultured adipocytes (Olson et al. 2001). By using in vitro microtubule binding, the group demonstrated that Glut4 vesicles bind specifically to microtubules, implicating microtubules in both insulin signalling and Glut4 translocation. Moreover Emoto et al. have reported Glut4 translocation to be dependent on both microtubule and actin-based cytoskeletal structures in 3T3-L1.
adipocytes suggesting the co-operative action of F-actin and microtubules in Glut4 translocation (Emoto et al. 2001).

The missing link between PtdIns 3,4,5-P$_3$ production and Glut4 translocation is still to be established. The discovery of new PtdIns 3,4,5-P$_3$ binding proteins, such as the ARF-GEFs and ARF-GAPs, may provide some exciting new advancement in this area.

1.8 Experimental Aims and Approaches of the Research Described Herein

The discovery of the PtdIns 3,4,5-P$_3$-binding centaurin-α proteins together with the known importance of PtdIns 3-kinase in insulin-stimulated Glut4 translocation prompted us to investigate whether centaurin-α related proteins were present in insulin-sensitive tissues.

Initial experiments were focused on the cloning of a centaurin-α related clone from an adipocyte cDNA library using RACE protocols. Following the identification and cloning, sequence analysis was performed to confirm the sequence identity of the novel homologue and compare it with the published centaurin-α sequences. Northern blot analysis was used to examine the expression and distribution of the transcript and determine whether this was specific to insulin-sensitive tissues. Characterisation of the protein expression of the centaurin-α homologue was undertaken using immunochemical techniques. These were used to investigate the tissue distribution and subcellular distribution of the native protein. Recombinant protein was generated for use in phosphoinositide binding assays to analyse the specificity of the PH domains. Point mutations were introduced into the PH domains, by site-directed mutagenesis, to further characterise the binding properties of this novel centaurin-α family protein.
2.0 MATERIALS AND METHODS

2.1 Materials

2.1.1 General Materials

All general laboratory chemicals were purchased from either Sigma-Aldrich Chemical Company, Fisons Scientific UK Ltd. or BDH Laboratory Supplies unless otherwise specified. Other reagents were acquired from:

Amersham Pharmacia Biotech Ltd.
- ECL Western blotting detection reagents
- Hybond N
- Hyperfilm ECL
- Hyperfilm MP
- MicroSpin Columns
- Multiprime DNA Labelling System
- NovaBlot Electrode paper
- Redivue [α-³²P]dCTP

Bio-Rad
- Ammonium persulphate
- N,N,N,N'-tetramethylethylenediamine (TEMED)

Clontech
- Advantage cDNA PCR Kit
- DpnI restriction enzyme
- Marathon-Ready cDNA
- PfuTurbo DNA Polymerase
- TALON Metal Affinity Resin

Novagen
- Introductory pT7Blue Perfectly Blunt Cloning Kit
- pET15b vector
Oligonucleotides were purchased from either Perkin Elmer or Eurogentec. PolyATtract mRNA Isolation System IV was obtained from Promega. ProtoGel was purchased from National Diagnostics, BioTrace NT nitrocellulose membrane from Gelman Sciences Ltd., and BioDesign GelWrap from BioDesign Inc.

### 2.1.2 Antibodies

Rabbit anti-centaurin-α2 antisera were raised at AstraZeneca using a fourteen amino acid peptide coupled to ovalbumin. The peptide was designed to a region of the rat centaurin-α2 sequence located at the C-terminus of the PH-C domain. Glut4 antisera was raised in the laboratory of Dr. S. Cushman (National Institutes of Health, Bethesda, USA) using C-terminal peptides produced in our laboratory (Holman et al. 1990).

The source of antibodies and their appropriate dilutions for western blotting (Section 2.4.6) and confocal microscopy (Section 2.4.7) are shown in Table 2.1.
Table 2.1. Antibodies utilised in this study. The primary (1°) and secondary (2°) antibodies used in western blot analysis and confocal microscopy are indicated along with their appropriate dilutions and the source of the antibody.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Primary (1°) or Secondary (2°)</th>
<th>Application</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit anti-Centaurin-a2</td>
<td>1°</td>
<td>Western blotting and confocal</td>
<td>1:60 - 1:10,000</td>
<td>AstraZeneca</td>
</tr>
<tr>
<td>Monoclonal anti-pentaHis</td>
<td>1°</td>
<td>Western blotting</td>
<td>1:2,000</td>
<td>Qiagen</td>
</tr>
<tr>
<td>Rabbit anti-Glut4</td>
<td>1°</td>
<td>Western blotting</td>
<td>1:4,000</td>
<td>Gift from Dr S Cushman</td>
</tr>
<tr>
<td>Monoclonal anti-β-tubulin</td>
<td>1°</td>
<td>Western blotting and confocal</td>
<td>1:220 - 1,000</td>
<td>Sigma</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG peroxidase conjugate</td>
<td>2°</td>
<td>Western blotting</td>
<td>1:4,000</td>
<td>Sigma</td>
</tr>
<tr>
<td>Goat anti-mouse IgG peroxidase conjugate</td>
<td>2°</td>
<td>Western blotting</td>
<td>1:1,000</td>
<td>Sigma</td>
</tr>
<tr>
<td>Fluorescein labelled anti-rabbit IgG</td>
<td>2°</td>
<td>Confocal</td>
<td>1:100</td>
<td>Jackson Laboratories</td>
</tr>
<tr>
<td>Rhodamine labelled anti-mouse IgG</td>
<td>2°</td>
<td>Confocal</td>
<td>1:60</td>
<td>Jackson Laboratories</td>
</tr>
</tbody>
</table>
2.1.3 Phosphoinositides and Inositol Phosphates

D-[\textsuperscript{3}H]Ins \textsubscript{1,3,4,5-P\textsubscript{4}} (777 GBq/mmol) was purchased from NEN Life Science Products and DL-\textit{myo}-Ins \textsubscript{1,3,4,5-P\textsubscript{4}} tetrapotassium salt and D-\textit{myo}-Ins \textsubscript{1,4,5-P\textsubscript{3}} hexasodium salt were purchased from Alexis Biochemicals. The photoaffinity labels, \([\textsuperscript{3}H]BZDC\)-phosphatidylinositol triesters of PtdIns \textsubscript{3,4-P\textsubscript{2}} and PtdIns \textsubscript{3,4,5-P\textsubscript{3}} (Figure 2.1) were a gift from Professor G. Prestwich (University of Utah, USA) (Thum et al. 1996; Chen et al. 1996). The D-\textit{myo}-dipalmitoyl phosphatidylinositides: diC\textsubscript{16}PtdIns \textsubscript{3,4-P\textsubscript{2}}, diC\textsubscript{16}PtdIns \textsubscript{3,5-P\textsubscript{2}}, diC\textsubscript{16}PtdIns \textsubscript{4,5-P\textsubscript{2}} and diC\textsubscript{16}PtdIns \textsubscript{3,4,5-P\textsubscript{3}} were purchased from Echelon.

2.2 General Molecular Biology Techniques

2.2.1 Small Scale Plasmid Preparation from \textit{E. coli} using the QIAprep Miniprep Kit

Single colonies of \textit{E. coli} were used to inoculate 10 ml Luria-Bertani broth (LB broth: 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl) containing ampicillin (100 \(\mu\)g/ml) and shaken overnight at 37°C. Cells were pelleted and the DNA isolated using the manufacturer’s protocol. The purified DNA was eluted from the spin columns using 50 \(\mu\)l autoclaved double-distilled H\textsubscript{2}O (ddH\textsubscript{2}O).

2.2.2 Agarose Gel Electrophoresis

A 1% (w/v) agarose gel was prepared using 1 g of agarose which was heated in 100 ml of 1X TAE buffer (40 mM Tris-acetate, pH 7.6, 1 mM EDTA). Once dissolved, the solution was allowed to cool slightly, ethidium bromide (0.5 \(\mu\)g/ml) was added and the gel poured into a cast. DNA samples were prepared by adding 1 \(\mu\)l loading dye (30% (v/v) glycerol, 0.25% (w/v) bromophenol blue) to 5 \(\mu\)l DNA and loaded into the wells. Electrophoresis was performed at 100 volts in 1X TAE until the dye front had migrated sufficient distance for the samples to be resolved when visualised on an ultraviolet (UV)
$[^3]H$BZDC-PtdIns 3,4,5-$P_3$

$[^3]H$BZDC-PtdIns 3,4-$P_2$

Figure 2.1. Structures of the phosphoinositide benzophenone photoaffinity probes. $[^3]H$BZDC-phosphoinositide triesters of PtdIns 3,4,5-$P_3$ and PtdIns 3,4-$P_2$. The phosphoinositide group is attached via a flexible linker to the photophore benzophenone group, which is activated at 350-360 nm. Photoaffinity labelling produces a covalent attachment and the labelled proteins can be denatured and resolved by SDS-PAGE.
transilluminator. RNA integrity was also examined by agarose gel electrophoresis using a gel tank pre-treated with 3% (v/v) H$_2$O$_2$ to remove any contaminating ribonuclease (RNase) activity.

### 2.2.3 Extraction of DNA from Agarose Gels

DNA fragments were extracted from agarose gel using the QIAquick Gel Extraction Kit using the manufacturer’s protocol. In brief, DNA bands were excised from agarose gels and placed in pre-weighed microcentrifuge tubes. The weight of the gel slice was determined and converted into an equivalent volume, with 100 mg equating to 100 μl. The gel slice was solubilised in 3 volumes of QG buffer (components not disclosed) and heated at 50°C for 10 min with occasional vortexing. One volume of isopropanol was added to the gel solution, mixed and applied to the QIAquick spin column. All wash steps were performed as outlined in the protocol and the DNA eluted in 30 μl autoclaved ddH$_2$O.

### 2.2.4 Sequencing

Purified DNA from plasmid miniprep isolation or polymerase chain reaction (PCR) was sequenced using a PE Biosystems ABI 377 DNA Sequencer (Perkin Elmer). Sequencing reactions containing 7 pmol of primer and template DNA (50-100 ng of single-stranded DNA, 200-500 ng double-stranded DNA) were prepared in a 6 μl final volume in 0.5 ml microcentrifuge tubes.

### 2.2.5 Rapid Amplification of cDNA Ends

Rapid amplification of cDNA ends (RACE) was performed in both the 5’ and 3’ direction using Marathon-Ready cDNA (Clontech). Firstly 5’RACE reactions were prepared using the components from the kit: 1.75 mM MgCl$_2$, 400 μM dNTP, 200 nM adaptor primer-1 (API), 200 nM gene-specific primer-1 (GSP1), reaction buffer (the composition was not disclosed by the manufacturer) and Expand Long Template PCR enzyme mix. All components of the reaction were combined and the sample mix was overlaid with 30 μl mineral oil and heated to 94°C before the addition of the enzyme.
and the initiation of the thermocycle programme (Section 3.2; Figure 3.2). Similarly
3'RACE reactions were performed using AP1 and GSP2 primers and the enzyme from
the Advantage cDNA PCR Kit (Section 3.3; Figure 3.5).

2.2.6 Site-Directed Mutagenesis

Single amino acid changes were generated using the QuikChange Site-Directed
Mutagenesis method (Stratagene). A complementary pair of oligonucleotides, whose
sequence contained the desired mutation, were used in the PCR reaction. Reactions
were prepared in a thin walled PCR tube with the following reagents: 10 mM KCl, 10
mM (NH₄)₂SO₄, 20 mM Tris-HCl, pH 8.8, 2 mM MgSO₄, 0.1% (v/v) Triton X-100, 100
µg/ml bovine serum albumin (BSA), 2.5 ng/µl of each of the sense and antisense
primers, 200 µM dNTP mix, 1 µl template (5-50 ng). PfuTurbo (2.5 U) was then added,
and the 50 µl reaction was overlaid with 30 µl mineral oil. DNA amplification was
performed using the following cycle parameters: an initial step of 95°C for 30 sec, 16
cycles of 95°C for 30 sec, 55°C for 1 min and 68°C for 14 min. Following temperature
cycling the reaction was cooled to approximately 37°C by incubation on ice for 2 min
before the digestion of the amplified DNA with DpnI (10 U) at 37°C for 1 h.

2.2.7 Preparation of Competent Cells

Competent cells were streaked onto a plate of LB agar (LB broth and 1.5% (w/v) agar)
and incubated overnight at 37°C. An overnight culture was prepared by inoculating 10
ml LB broth with a single colony from the LB agar plate and incubating at 37°C in a
shaking incubator. An aliquot of 1 ml of the overnight culture was used to inoculate
100 ml LB broth that was incubated as above for approximately 1½ h until the optical
density at 600 nm (OD₆₀₀) reached an absorbance of 0.5. The cells were harvested by
centrifugation and resuspended in 10 ml ice-cold TSB buffer (1% (w/v) tryptone, 0.5%
(w/v) yeast extract, 10% (w/v) polyethylene glycol (PEG)₃₃₅₀, 5% (v/v) DMSO, 100
mM NaCl, 10 mM MgSO₄, 10 mM MgCl₂, pH 6.1). Following a 10-min incubation on
ice, aliquots were stored at -70°C.
2.2.8 Transformation of Competent Cells

Competent cells were thawed on ice and 100 μl aliquots transferred into 1.5 ml microcentrifuge tubes. Plasmid DNA (1-10 ng) was added to the cells and the mixture was incubated on ice for 15 min. A negative control, using ddH₂O in place of the DNA was also prepared. The samples were heat shocked at 42°C for 30 sec. For cells transformed with ligation reactions (rather than intact plasmid DNA), an additional incubation of shaking at 37°C for 30 min was performed. An appropriate volume of cells was plated onto LB agar plates containing the required antibiotics and incubated overnight at 37°C.

2.3 Northern Blotting

Two detection methods have been utilised in this research: ³²P-labelled DNA probes, following the method outlined in Maniatis et al. 1982, and the digoxigenin (DIG) system used in accordance to the manufacturer’s guidelines (Roche Molecular Biochemicals). Some methods described below, such as RNA isolation and capillary transfer, are similar in both systems, whereas hybridisation and detection techniques are quite distinct and hence are explained separately.

2.3.1 Preparation of RNase-free ddH₂O, Buffers and Apparatus

Buffers used for RNA analysis were generally treated with diethyl pyrocarbonate (DEPC) to inactivate ribonuclease (RNase) enzymes before being autoclaved. RNase-free ddH₂O was prepared by adding DEPC to 1% (v/v) in ddH₂O, mixing vigorously and incubating overnight at room temperature before autoclaving. Pipette tips, microcentrifuge tubes and glass Corex tubes were all autoclaved to reduce the risk of RNase contamination. Apparatus including the gel electrophoresis tank, hybridisation bottles and Tupperware boxes were treated with 3% (v/v) H₂O₂ for 20 to 30 min and rinsed with DEPC-treated ddH₂O prior to use.
2.3.2 Isolation of Total RNA

Total RNA was isolated from rat tissues using the TriPure Isolation Reagent, a monophasic solution of phenol and guanidine thiocyanate. Tissues were removed from male Wistar rats and snap frozen in liquid nitrogen. Using a pestle and mortar, approximately 1 g of tissue was ground to a fine powder under liquid nitrogen. TriPure reagent was added (1 ml per 100 mg tissue) and the samples were quickly homogenised using a Teflon plunger. The homogenates were transferred to autoclaved glass Corex tubes and incubated for 5 min at room temperature. Chloroform was added (0.2 ml per 1 ml TriPure reagent) and the samples were vortexed for 15 sec, and then incubated for 2 to 3 min at room temperature. Centrifugation at 12,000 x g for 15 min at 4°C separated the sample into three phases: a colourless upper aqueous phase containing RNA, a cloudy white interface containing DNA, and a pink lower organic phase containing protein. The colourless upper aqueous phase was transferred to a clean Corex tube and the RNA precipitated by the addition of isopropanol (0.5 ml per 1 ml TriPure reagent). The samples were mixed by inversion, incubated for 10 min at room temperature and centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was discarded and the pellet washed with 75% ethanol (1 ml per 1 ml TriPure reagent) and centrifuged at 7,500 x g for 5 min at 4°C. Excess ethanol was removed and the RNA pellets were briefly air-dried before resuspension in DEPC-treated ddH2O. The concentration and purity of the RNA were determined by spectrophotometry. To calculate the RNA concentration it was assumed that 40 μg/ml RNA solution would have an absorbance of 1.0 at 260 nm. RNA purity is indicated by the $A_{260}/A_{280}$ absorbance ratio, with pure RNA having a ratio of 2.0. The manufacturers predict a ratio of 1.6-2.0 for RNA isolated using this method and this level of purity is suitable for applications such as northern blot analysis. A small sample of RNA was also analysed by gel electrophoresis.

2.3.3 Purification of PolyA⁺ RNA from Total RNA

The PolyATtract mRNA Isolation System was used to purify PolyA⁺ RNA from total RNA using the method outlined by the manufacturer. In brief, 1 mg total RNA, in a final volume of 500 μl, was heated for 10 min at 65°C. The biotinylated oligo dT probe (3 μl) and 13 μl 20X SSC (where 1X SSC is 150 mM NaCl, 15 mM sodium citrate)
were added to the RNA and left at room temperature until the samples had cooled. During this time the streptavidin magnetic particles were washed three times in 0.5X SSC, collecting the beads between each wash using a magnetic stand. The cooled RNA samples were incubated with the magnetic beads for 10 min at room temperature, with mixing by inversion every few minutes. The beads were collected as described above, the supernatant was discarded, and the beads washed a further four times with 0.1X SSC. PolyA⁺ RNA was eluted from the beads with 250 µl RNase-free water (included in the PolyATtract mRNA Isolation System). The RNA was then precipitated by the addition of 25 µl 3M NaOAc (DEPC-treated), 250 µl isopropanol and incubated overnight at -70°C. The RNA was pelleted by centrifugation at 12,000 x g for 10 min, washed with 70% ethanol and resuspended in RNase-free water and stored at -70°C until required.

2.3.4 Formaldehyde Denaturing Gel Electrophoresis

The technique employed for RNA electrophoresis in formaldehyde containing gels followed that outlined by Maniatis et al. (1982). A denaturing formaldehyde gel (1% (w/v) agarose, 6.6% (v/v) formaldehyde, 20 mM 3-(N-morpholino)propanesulfonic acid (MOPS), 8 mM NaOAc, 1 mM EDTA) was prepared by melting the agarose in DEPC-treated ddH₂O, cooling to 60°C, and adding the appropriate volumes of formaldehyde gel-running buffer (0.1 M MOPS; pH 7.0, 40 mM NaOAc, 5 mM EDTA; pH 8.0) and 37% (v/v) formaldehyde. The formaldehyde gel used in the DIG system was prepared using virtually the same method although the component proportions were modified to produce a gel with the following composition: 1% (w/v) agarose, 2% (v/v) formaldehyde, 1X MOPS buffer (20 mM MOPS, 5 mM NaOAc, 1 mM EDTA, pH 7.0).

Total RNA samples (20 µg) were made up to a final volume of 4.5 µl with DEPC-treated ddH₂O. In the case of PolyA⁺ RNA, the precipitated RNA was resuspended in 4.5 µl RNase-free water. The RNA molecular weight marker II (10 µl) sample was prepared in the same way as the total RNA samples. A master mix of denaturing solution was prepared (1 part formaldehyde gel-running buffer, 1.75 parts formaldehyde and 5 parts formamide) and 15.5 µl was then added to each RNA sample before heating for 15 min at 65°C. Following a short incubation on ice and brief centrifugation, 2 µl formaldehyde gel-loading buffer (50% (v/v) glycerol, 1mM EDTA, pH 8.0, 0.25%
(w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF) was added to the samples. The gel tank was filled with formaldehyde gel-running buffer (diluted 1:5 in DEPC-treated ddH₂O) and the gel was pre-run for 5 min at 5 V/cm. The samples were carefully loaded with the markers in the outside lanes and the gel electrophoresis performed at 3-4 V/cm (70-92 V) for between 4-4½ h, until the furthest dye front had migrated about ¾ the length of the tank. Following electrophoresis, the gel was transferred to a Tupperware box and washed in DEPC-treated ddH₂O for 30 min.

PolyA⁺ RNA samples were prepared using the sample preparation protocol that accompanied the DIG-labelled RNA Molecular Weight Marker II. One volume of polyA⁺ RNA (5 µl) was mixed with 4 volumes of freshly prepared sample buffer (62.5 % (v/v) formamide, 7.7% (v/v) formaldehyde, 25 mM MOPS, 6.25 mM NaOAc, 1.25 mM EDTA, pH 7.0, 0.01% (w/v) bromophenol blue). The gel tank was carefully filled with 1X MOPS buffer until the buffer reached a height equivalent with the gel top, but did not submerge it. Heat-denatured samples were loaded into the wells and electrophoresis was performed at 100 mA for 5 min. Once the samples had entered the gel (i.e. migrated from the wells), additional MOPS buffer was added and the electrophoresis continued at 3-4 V/cm (70-92 V) for approximately 4 h.

2.3.5 Capillary Transfer of Denatured RNA onto a Membrane

The method employed for the capillary transfer of RNA from the gel onto a membrane was common to both detection systems utilised herein, albeit using different membranes. A nitrocellulose membrane was used for the radioactively-labelled DNA probes, whereas the DIG detection system required a positively charged nylon membrane. Before constructing the transfer assembly, a sheet of 3MM paper measuring 160 x 300 mm was cut in order to create a wick. Two further pieces of 3MM paper and a piece of nitrocellulose were cut to the size of gel, 110 x 165 mm. The three pieces of 3MM paper were briefly soaked in 20X SSC and the largest piece was placed on a glass platform with opposite ends submerged in a tray containing a large volume of 20X SSC to create a wick. The gel was positioned on top of the wick, base side up, and air bubbles were removed by rolling with a sterile plastic pipette. The nitrocellulose membrane was carefully placed on the gel and the air bubbles removed again. The edges of the membrane were covered with thin strips of Saran wrap. The two pre-
wetted pieces of 3MM paper were then placed on top followed by a stack of tissues cut to the same size as the gel. A glass plate and a 500 g weight were placed on the top of the stack which was left overnight with in a sufficient volume of 20X SSC.

The northern transfer assembly was carefully dismantled and the position of the wells marked on the membrane using a pencil. The RNA was fixed to the nitrocellulose membrane by baking for one h at 80°C, whereas the nylon membrane was UV crosslinked at 120,000 μJ/cm². The outer marker lanes were cut off and stained with a solution of 0.5 M NaOAc (pH 5.2) and 0.04% (w/v) methylene blue for 5 min at room temperature. Once rinsed under the cold water tap for about 5 min, the ribosomal bands and markers were visible and the positions were marked with pencil.

2.3.6 Random Labelling of DNA Probes with $^{32}$P

DNA probes labelled with $^{32}$P were synthesised using the Multiprime DNA Labelling System. Double-stranded template DNA was diluted to a concentration of 2-25 μg/ml in 10 μl ddH₂O and denatured by heating at 95°C for 5 min, then chilled on ice. Random sequence hexanucleotides were used to prime DNA synthesis on the denatured template DNA at numerous sites along its length and [$\alpha$-32P]dCTP incorporated by the Klenow fragment of DNA polymerase I. The reaction was prepared by the addition of 18 μl ddH₂O, 10 μl 10X reaction buffer (Tris-HCl, pH 7.8, MgCl₂, and 2-mercaptoethanol, concentrations not disclosed), 5 μl primer solution, 5 μl [$\alpha$-32P]dCTP (~3000 Ci/mmol) and 2 μl enzyme. The sample was incubated for 1 h at 37°C and the unincorporated radioactivity was removed by gel filtration using MircoSpin columns. The columns were pre-centrifuged for 1 min at 750 x g and the samples were applied followed by 50 μl TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and centrifuged as above. The eluate was heat denatured at 95°C for 5 min before use in the hybridisation reaction.
2.3.7 Hybridisation and Detection of Radioactively-Labelled DNA Probes

Prehybridisation of the nitrocellulose membrane was performed for 90 min at 65°C in 15 ml 5X SSC, Denhardt’s Solution (0.02% (v/v) Ficoll, 0.02% (v/v) polyvinylpyrrolidone, 0.02% (w/v) BSA), 100 µg/ml sheared-herring sperm DNA and 0.5% (w/v) SDS. The denatured [$\alpha$-$^{32}$P]dCTP-labelled DNA probe (Section 2.3.6) was added directly into the prehybridisation buffer and hybridised overnight at 65°C. Two 30-min post-hybridisation washes were performed at 65°C. Firstly, the membrane was washed in 100 ml 2X SSC, 0.1% SDS, followed by 100 ml 0.5X SSC, 0.1% (w/v) SDS. Some probes required more stringent washing conditions with 0.2X SSC and 0.1% (w/v) SDS for the second wash. Autoradiography was performed at -70°C with Hyperfilm MP for varying time intervals from 8-48 h.

Immediately after exposure to film the probe was stripped from the membrane by the addition of a boiling solution of 0.5% (w/v) SDS, the membrane was then washed in this solution on a rocking platform until cool. The membrane was rinsed in 2X SSC and incubated in prehybridisation buffer prior to further hybridisation reactions using radioactively-labelled probes.

2.3.8 Generation of DIG-Labelled Riboprobes

A DIG RNA Labelling Kit was used to generate DIG-labelled, single-stranded RNA probes as described in the manufacturer's protocol. DNA encoding the mRNA of interest was subcloned into the multiple cloning site of the pSPT18 transcription vector (supplied in the kit), adjacent to the SP6 RNA polymerase promoter site. The DNA template was linearised with Smal and the DNA fragment was separated on an agarose gel and purified before quantification by spectrometry (A$_{260}$). The DNA was precipitated by the addition of 25 µl NaOAc and 625 µl ethanol and by incubation at -20°C overnight. After centrifugation the DNA was washed with 70% ethanol and resuspended in DEPC-treated ddH$_2$O. RNA transcripts of uniform length were synthesised by in vitro transcription, incorporating a DIG-labelled UTP residue every 20-25 nucleotides. A standard labelling reaction comprised 1 µg purified linearised DNA template, NTP labelling mix (1 mM ATP, 1mM CTP, 1 mM GTP, 0.65 mM UTP,
0.35 mM DIG-11-UTP, pH 7.5), transcription buffer (components not disclosed), 20 U RNase inhibitor, and 40 U SP6 RNA polymerase. The transcription reaction was incubated for 2 h at 37°C and stopped by the addition of 2 μl 0.2 M EDTA. The yield of DIG-labelled RNA was determined in a spot assay (Section 2.3.9) and the riboprobes were stored at -70°C.

2.3.9 Estimation of DIG-Labelled RNA Yield

An estimation of the yield of DIG-labelled RNA was assessed using a spot assay in comparison to the DIG-labelled control RNA provided in the kit. In a standard reaction 10 μg DIG-labelled RNA was transcribed from 1 μg DNA template. A dilution series of the DIG-labelled sample and the RNA control were prepared and spotted on a piece of positively charge nylon membrane. The RNA was fixed to the membrane by UV crosslinking at 120,000 μJ/cm² and then was directly detected using DIG Luminescent Detection Kit (Section 2.3.10). Concentration of the experimental probe was estimated by comparing the intensities of the spot for the control and experimental dilutions.

2.3.10 Hybridisation and Detection of DIG-Labelled Riboprobes

Prehybridisation of the nylon membrane was performed with at least 20 ml of hybridisation buffer (50% (v/v) formamide, 5X SSC, 2% (w/v) blocking reagent, 0.1% (w/v) N-lauroyl-sarcosine, 0.02% (w/v) SDS) per 100 cm² of membrane in a 68°C hybridisation oven for 60-90 min. The prehybridisation buffer was replaced with fresh hybridisation buffer (2.5 ml per 100 cm²) containing 50-200 ng/ml DIG-labelled RNA and incubated at 68°C overnight. After hybridisation the membrane was transferred to a Tupperware box and washed twice with at least 50 ml of 2X SSC, 0.1% (w/v) SDS per 100 cm², for 5 min on a rocking platform at room temperature. The membrane was placed in a clean hybridisation bottle and washed twice in 0.1X SSC, 0.1% (w/v) SDS at 68°C for 15 min. The DIG Luminescent Detection Kit was used for the subsequent blocking, washing and detection stages, which were all performed on a rocking platform at room temperature. Following the post-hybridisation washes, the membrane was rinsed for 2 min with washing buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5, 0.3% (v/v) Tween-20) and blocked by incubating in 1% (w/v) blocking buffer for 30 min.
Anti-DIG-alkaline phosphatase conjugate was diluted 1:10,000 in Blocking Reagent (supplied in DIG Luminescent Detection Kit, components not disclosed) and the membrane was incubated in this antibody solution for 30 min. Following two 15-min washes in washing buffer the membrane was equilibrated in detection buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl) for 2 min. CSPD (disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1.3,7]decan}-4-yl)phenyl phosphate) substrate was diluted 1:100 in detection buffer and added to the membrane for 5 min. The membrane was sealed in a bag, incubated for 10 min at 37°C and exposed to Hyperfilm-ECL for periods ranging from 10 sec to 1 h for visualisation of the chemiluminescent signal.

Immediately after exposure to film, the membrane was stripped of hybridisation probe in preparation for hybridisation with a new probe. Firstly the membrane was rinsed in DEPC-treated ddH₂O and the probes were stripped from the membrane by washing in boiling 0.1% SDS (w/v) and by leaving to wash on a rocking platform until cool. The membrane was washed in washing buffer for 5 min and twice in 2X SSC for 5 min. The stripped membrane was sealed in a clean bag and stored at 4°C until use in further hybridisation reactions.

2.4 General Protein Biochemistry Techniques

2.4.1 BCA Protein Assay

Protein concentrations were determined using the Pierce Bicinchoninic Acid (BCA) protein assay. A standard curve (0.1-1 μg/μl BSA) was constructed by diluting a 1 mg/ml stock solution of BSA in 0.1 M NaOH. Protein samples were either measured undiluted or diluted in 0.1 M NaOH. Triplicate 10 μl aliquots of both the standards and protein samples (neat or diluted in 0.1 M NaOH) were placed in a microtitre plate. Reagent A (1% (w/v) BCA-NO₂, 2% (w/v) Na₂CO₃.H₂O, 0.16% (w/v) Na₂tartrate, 0.4% (w/v) NaOH, 0.95% (w/v) NaHCO₃) and Reagent B (4% (w/v) CuSO₄.5H₂O) were mixed in the ratio 50:1 and 200 μl of this solution was added to each well. The plate was incubated at 37°C for 30 min or until sufficient colour development had occurred. The absorption was measured at 562 nm in a microplate spectrophotometer.
2.4.2 SDS-PAGE

Generally protein samples were prepared for SDS-PAGE by the addition of 0.5 volumes of 3X electrophoresis sample buffer (where 1X is 62.5 mM Tris-HCl, pH 6.8, 1% (w/v) SDS, 10% (v/v) glycerol and 0.01% (w/v) bromophenol blue). A 5X concentrate of electrophoresis sample buffer was used for larger volumes of protein samples. Dithiothreitol (DTT, final concentration 100 mM) was added to the protein samples prior to heating at 95°C for 5 min.

Electrophoresis was performed following the discontinuous buffer system of Laemmli 1970, using the Protean II Xl system for large gels and either the Mini-Protean II electrophoresis cell (Bio-Rad) or the Atto Dual Mini Vertical Page cell system for the smaller gels. The gels were prepared according to the manufacturer's instructions. The compositions of reagents for the Protean II Xl system are shown in Table 2.2. Volumes were scaled down appropriately for the mini gels. Resolving gels consisted of 0.375 M Tris-HCl, 0.1% (w/v) SDS and 10% (w/v) acrylamide, and the stacking gels were made to 0.125 M Tris-HCl and 0.1% (w/v) SDS with 6.375% (w/v) acrylamide. Polymerisation was initiated by the addition of the ammonium persulphate and TEMED.

The gel tank was filled with electrophoresis buffer (0.025 M Tris-HCl, 0.1% (w/v) SDS, 0.192 M glycine, pH 8.3) and the samples and molecular weight markers were loaded into the wells. Large gels were run overnight at 12-15 mA for 1.5 mm thickness gels (24-30 mA for 3 mm gels) until the bromophenol blue tracking dye had run off the bottom of the gel. Small gels were run at a constant voltage of 150-200 Volts for approximately 90 min or until the dye front had run off. Following electrophoresis, the protein was either visualised by Coomassie staining (Section 2.4.3) or transferred to nitrocellulose membrane (Section 2.4.4).

2.4.3 Coomassie Staining

Protein bands were visualised by staining with Coomassie brilliant blue reagent (0.2% (w/v) Coomassie brilliant blue R-250 in destaining solution (10% (v/v) glacial acetic acid, 30% (v/v) methanol, 60% (v/v) ddH2O)) for 20-30 min, followed by incubation in
Table 2.2. Composition and preparation of resolving and stacking gels for SDS-PAGE. The component volumes indicated are those required for the preparation of a 1.5 mm gel for the Protean II XI system using 16x18 cm plates. The volumes were doubled when preparing a 3 mm gel and scaled down for the smaller gels.

<table>
<thead>
<tr>
<th>Component</th>
<th>Composition of Stock</th>
<th>10% Resolving Gel</th>
<th>6.375% Stacking Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>30% (w/v) acrylamide/methylene bisacrylamide</td>
<td>25 ml</td>
<td>3.4 ml</td>
</tr>
<tr>
<td>ddH₂O</td>
<td></td>
<td>25 ml</td>
<td>7.5 ml</td>
</tr>
<tr>
<td>Resolving gel buffer</td>
<td>1.5 M Tris-HCl, pH 8.8, 0.4% (w/v) SDS</td>
<td>25 ml</td>
<td>-</td>
</tr>
<tr>
<td>Stacking gel buffer</td>
<td>0.5 M Tris-HCl, pH 6.8, 0.4% (w/v) SDS</td>
<td>-</td>
<td>5 ml</td>
</tr>
<tr>
<td>Ammonium persulfate</td>
<td>10% (w/v)</td>
<td>500 μl</td>
<td>80 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylenediamine</td>
<td>40 μl</td>
<td>20 μl</td>
</tr>
</tbody>
</table>
destaining solution. Some analytical gels were dried so as to save the results. This was performed at room temperature by placing the gels between two layers of BioDesign GelWrap which were pre-soaked in destaining solution containing 8.7% (v/v) glycerol.

2.4.4 Electrotransfer of Protein from SDS-PAGE Gels to Nitrocellulose

Following SDS-PAGE, the gel was soaked in SDS transfer buffer (0.0375% (w/v) SDS, 48 mM Tris-HCl, 39 mM glycine, pH 8.8) for 2 min together with a piece of nitrocellulose membrane and 18 sheets of electrode paper, all cut to the size of the gel. The transfer assembly was constructed by placing nine sheets of electrode paper onto the electrophore, followed by the nitrocellulose. The gel and finally another nine sheets of electrode paper were added, ensuring that any air bubbles were expelled. The second electrode plate of the Multiphor II NovaBlot electrophoretic transfer unit (Pharmacia) was placed on the top and completed the assembly. A constant current, proportional to the surface area of the blot (0.8 mA/cm²), was applied for 110 min. After electrotransfer, the nitrocellulose was rinsed in ddH₂O before staining with 0.1% (w/v) Ponceau S in 3% (w/v) trichloroacetic acid. In the marker lanes, the positions of known molecular weight proteins were marked onto the nitrocellulose with a pencil. Western blotting could be performed directly after this or the membrane might be air-dried and stored at room temperature until required.

2.4.5 Affinity Purification of Antiserum

The centaurin-α2 antiserum was affinity purified using a column composed of the immunising peptide coupled to Reacti-Gel matrix (6%, crosslinked agarose, Pierce). Coupling of the peptide to the matrix was performed according to the manufacturer’s instructions. In brief, the Reacti-Gel was prepared by filtering 4 ml 50% slurry of gel through filter paper in a Buchner funnel connected to a vacuum and then by washing three times with 15 ml ice-cold ddH₂O and once with 15 ml ice-cold borate buffer (0.1 M sodium borate, pH 8.5). Coupling was performed by rotating the gel matrix with 4 mg peptide in borate buffer for 48 h at 4°C. The gel matrix was pelleted and washed once with 10 ml ice-cold borate buffer. Free imidazoyl-carbamate groups were then
blocked by rotating in 2 ml 1 M ethanolamine in borate buffer at room temperature for 3 h. A column was packed and washed successively with 1 M NaCl, ddH2O and phosphate-buffered saline (PBS; 12.5 mM Na2HPO4·12H2O, pH 7.2, 154 mM NaCl). The column was stored between use in PBS, 0.02% (w/v) sodium azide at 4°C.

Before use the column was washed extensively with PBS. A small volume of antiserum (500 μl) was diluted in PBS to generate sufficient volume for recirculation through the column. This was then placed at 4°C and the antiserum solution recirculated overnight. The column was extensively washed with PBS and 2 M NaCl in 10 mM Na2HPO4, pH 7.2. The bound antibodies were eluted with 15 ml of 3.5 M NaSCN, 10 mM Na2HPO4, pH 6.6 and immediately transferred into dialysis tubing. Dialysis was performed for at least 18 h with several changes of PBS. The antibody solution was concentrated using PEG20,000 whilst in the dialysis tubing and was then extracted and stored at 4°C.

2.4.6 Western Blotting

The standard western blotting protocol described here employs the use of Tris-buffered saline (TBS; 10 mM Tris-HCl, pH 7.4, 154 mM NaCl) containing Tween-20 at a final concentration of 0.1% (v/v) (TBS-T). However, for some western blotting experiments the Tween-20 was omitted from the TBS buffer at all stages including washing (Section 4.3.2). Following electrophoretic transfer the nitrocellulose was rinsed briefly in TBS-T. Dry membranes that had been stored were re-hydrated in SDS-transfer buffer before rinsing with TBS-T. Blocking was performed in 5% (w/v) Marvel dried skimmed milk in TBS-T for 30 min. All incubations were performed on a rocking platform at room temperature. Following a brief rinse with TBS-T, the nitrocellulose was incubated with the primary antibody at the required dilution, in TBS-T with 1% (w/v) BSA for 60 min. After six 5-min washes with TBS-T, the nitrocellulose was incubated with the secondary antibody at the required dilution in blocking solution (5% (w/v) Marvel in TBS-T) for 30 min. The nitrocellulose was then washed as above and the bound antibodies were visualised using enhanced chemiluminescence (ECL) which was used as described by the manufacturers. The nitrocellulose was placed between two transparency sheets before exposure to autoradiography film (Hyperfilm ECL) for varying lengths of time and until a sufficient signal was detected upon development.
2.4.7 Confocal Microscopy

Rat cardiomyocytes were isolated by Dr A Gillingham and Dr J Yang using a modified method of that originally described by Fischer et al. (1991). The isolated cardiomyocytes were allowed to recover at 37°C for 20-30 min before fixation in 4% (w/v) paraformaldehyde in PBS pH 7.2 for 20 min. Unless otherwise stated, all incubations were performed with gentle agitation on a rocking platform at room temperature. The cells were washed twice in PBS, permeabilised by incubation in methanol at -20°C for 5 min and then washed three times with wash buffer (1% (v/v) BSA in PBS, pH 7.2). Before incubation with the primary antibodies, the cells were transferred to microcentrifuge tubes and were incubated in blocking buffer (3% (v/v) goat serum, 1% (v/v) BSA in PBS pH 7.2) for 30 min. Primary antibodies, in blocking buffer, were incubated with the cells for 1 h. The cells were washed three times in wash buffer before incubating with the rhodamine-labelled anti-mouse IgG antibody and/or fluorescein-labelled anti-rabbit IgG antibody (1:60 and 1:100 dilutions respectively; Table 2.1) in blocking buffer for 30 min. Washing (in wash buffer) was performed overnight and the cells mounted onto a glass coverslip using Vector Shield mounting medium (Vector Laboratories). Cardiomyocytes were viewed using a Zeiss LSM 510 confocal scanning microscope with a 458/488 nm laser (for fluorescein-labelled samples) or a 543 nm laser (for rhodamine-labelled samples).

2.4.8 Expression and Purification of 6xHis-tagged Proteins

To assist the purification of recombinant centaurin-α2, the protein was expressed with an N-terminal six-histidine (6xHis) tag using the bacterial expression system pET15b. LB broth containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol was inoculated with BL21(DE3)pLysS cells that had been transformed with pET15b-centaurin-α2. This culture was shaken overnight at 37°C until the optical density at 600 nm (OD_{600}) reached an absorbance of 0.5. Once the cells were at the appropriate density, expression was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h. The cells were harvested by centrifugation at 1,000 x g for 10 min at 4°C and resuspended in 2 ml lysis buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl) per 25 ml of cell culture. The bacterial cell walls were disrupted by flash freezing the cell suspension in a dry ice-ethanol bath. This was followed by
thawing in cool water. The cells were sonicated using eight 15-sec pulses with 15-sec intervals and centrifuged at 12,000 x g for 10 min at 4°C.

The 6xHis proteins were purified using a batch/gravity-flow column method with Talon Metal Affinity Resin as described by the manufacturers. The resin was prepared by washing in five volumes of lysis buffer and pelleted by centrifugation at 700 x g for 5 min. The washed resin was incubated with the cleared lysate for 20 min at room temperature with gentle agitation. The resin was centrifuged as above and washed twice in 10 volumes of lysis buffer for 10 min and transferred to a gravity column and washed with five volumes of lysis buffer. Weakly binding proteins were washed from the column with five volumes 10 mM imidazole in lysis buffer and the 6xHis proteins were eluted with 100 mM imidazole in lysis buffer. Fractions of 1 ml were collected, 20 μl of each fraction was analysed by SDS-PAGE and the remaining volume was placed into Slide-A-Lyser dialysis cassettes and dialysed against lysis buffer. The concentration of the purified protein was calculated from the A280 and examined by SDS-PAGE. Molecular weight markers of known concentration were resolved alongside the protein samples to allow comparison of both the protein size and quantity.

2.5 Protein Isolation and Subcellular Fractionation

2.5.1 Isolation of Rat Adipocytes

Adipocytes were isolated from whole epididymal fat pads removed from male Wistar rats (180-200 g) as described by Simpson et al. (1983). The epididymal fat pads were quickly removed and washed in 1% (w/v) BSA/Krebs-Ringers-HEPES (KRH) buffer (140 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.25 mM MgSO₄, 2.5 mM NaH₂PO₄, 10 mM HEPES, pH 7.6) at 37°C. The fat pads were placed in KRH buffer containing 3.5% (w/v) BSA, 5 mM glucose and 0.7 mg/ml collagenase (4 fat pads/5 ml) and were finely chopped with scissors. Digestion was performed with vigorous shaking in a 37°C water bath for 40-50 min until a fine cell suspension was achieved. The cell suspension was filtered through 250 μm nylon gauze and the cells returned to 37°C and allowed to float. The infranatant buffer was removed using a 13 gauge x 10 cm needle, and the cells were gently resuspended in 1% (w/v) BSA/KRH buffer, and then returned to 37°C
and allowed to float. This wash step was repeated three times and the cell suspension was adjusted to 40% cytocrit.

For some preparations the cell suspension was divided and half of the suspension was treated with 20 nM insulin 1% (w/v) BSA/KRH buffer and incubated at 37°C for 30 min (basal cells incubated in 1% (w/v) BSA/KRH buffer). For all preparations, the cells were washed in HES buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, 255 mM sucrose containing protease inhibitors: 100 μM (4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF) and 1 μg/ml antipain, aprotinin, leupeptin and pepstatin A) at 18°C to remove the BSA.

### 2.5.2 Subfractionation of Rat Adipocytes

Isolated adipocytes were washed in HES buffer (18°C) and resuspended to 40% cytocrit. The basal cells were homogenised with 10 strokes of a 55 ml Potter-Elvehjem homogeniser (Thomas Scientific). The procedure was then repeated for the insulin stimulated cells. Initially the homogenate was centrifuged at 1000 x g for 2 min at 4°C to pellet any unhomogenised tissue and the tubes were then placed on ice for a few minutes to allow the fat to solidify at the neck of the tubes. The infranatants were removed with a syringe and needle before being subjected to centrifugation as described by Weber et al. (1988) (*Figure 2.2*). All centrifugation steps were performed in a Beckman TL-100 bench-top ultracentrifuge with a TLA-100.3 fixed rotor (or a TLS-55 swing-out rotor, where specified). The first spin at 17,500 x g for 20 min produced a crude plasma membrane pellet and a microsomal/cytosol fraction. The supernatant was then transferred to a new tube and centrifuged at 49,000 x g for 9 min to pellet the high-density microsomes (HDM). The post-HDM supernatant was centrifuged at 541,000 x g for 17 min to separate the low-density microsomes (LDM) from the cytosolic fraction. The HDM and LDM pellets were resuspended in HES buffer.

The crude plasma membrane pellet was resuspended in 300 μl HES, loaded on top of a 600 μl sucrose cushion (1.12 M sucrose in HES buffer) and centrifuged at 105,000 x g for 20 min in the swing-out rotor. The supernatant was collected, resuspended in HES buffer to 3 ml and centrifuged at 74,000 x g for 9 min. The plasma membrane pellet was resuspended in 3 ml HES buffer and pelleted as before. The final plasma
Figure 2.2. Subcellular fractionation protocol for isolated rat adipocytes. The initial centrifugation step (1000 x g for 2 min) was performed to separate the fat, which forms a cake at the surface and any unhomogenised tissue. The homogenate was then separated by sequential centrifugation using a Beckman TL-100 bench-top ultracentrifuge with a TLA-100.3 fixed rotor. The crude plasma membrane pellet was resuspended in HES buffer and overlaid on a sucrose cushion (1.12 M sucrose in HES buffer) and centrifugation using a TLS-55 swing-out rotor. HDM, high-density microsomes; and LDM, low-density microsomes.
membrane fraction was resuspended in HES buffer using a syringe and 25G needle and was assayed for protein content along with the other subcellular fractions (Section 2.4.1). The fractions were divided into aliquots, which were stored at -20°C.

### 2.5.3 Separation of Total Particulate and Cytosolic Fractions

Tissues were removed from male Wistar rats (180-280 g) and placed in ice-cold HES buffer. The tissues were homogenised in HES buffer (5 ml per gram) and were centrifuged at 1,000 x g for 2 min at 4°C. The supernatant was centrifuged at 541,000 x g for 30 min. The cytosolic fraction was collected and the particulate fraction resuspended in HES buffer using a syringe and 25G needle. The protein content was assayed (Section 2.4.1) and the fractions stored at -20°C.

### 2.6 Analysis of PH Domain Binding Characteristics

#### 2.6.1 Ins 1,3,4,5-P₄ Binding Studies

Binding of Ins 1,3,4,5-P₄ to recombinant centaurin-α2 was performed using a modification to methods described by Stricker et al. (1996) and Cullen et al. (1995a). In brief, each 500 μl assay contained 500 ng recombinant centaurin-α2, binding buffer (25 mM sodium acetate/25 mM potassium phosphate, pH 5.0, 0.05% (w/v) Brij58, 1 mM EDTA), 1 nM [³H]Ins 1,3,4,5-P₄ and varying concentrations of unlabelled Ins 1,3,4,5-P₄ or other phosphoinositides as specified. The reactions were maintained on ice for 15 min, after which the receptor-ligand complex was precipitated by addition of 100 μl 5 mg/ml γ-globulin and 1 ml 25% (w/v) PEG3350. The samples were then incubated on ice for a further 5 min and centrifuged at 13,000 x g for 10 min. The supernatants were removed and the pellets quickly washed with 500 μl 25% PEG3350. The resulting pellets were resuspended in 100 μl 0.1 M NaOH and 7 ml of scintillation fluid (Optiphase Safe) was added the ³H counts determined using a Packard Tri-Carb 1600 Scintillation counter.
2.6.2 $[^3]H$BZDC-PtdInsP$_n$ Photoaffinity Labelling

Recombinant centaurin-α2 (3 μg) was incubated with either 0.1 μCi $[^3]H$BZDC-PtdIns 3,4-P$_2$ or $[^3]H$BZDC-PtdIns 3,4,5-P$_3$ probes in lysis buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl) using a modification of protocols described by Profit et al. (1998) and Chaudhary et al. (1998). Samples were equilibrated on ice for 10 min in a 96-well microtitre plate and UV-irradiated at 350 nM for four 5-min periods with 5-min intervals. Photolabelled protein samples were then subjected to electrophoresis on large 3 mm thick 10% SDS-PAGE gels (Section 2.4.2). After Coomassie staining and destaining (Section 2.4.3) the gels were cut into slices and dried in open scintillation vials in an 80°C oven for 2 h. The dried gel slices were solubilised in 500 μl alkaline hydrogen peroxide (2% (v/v) NH$_4$OH in 30% (v/v) H$_2$O$_2$) in sealed vials at 80°C for a further 2 h. The samples were cooled before the addition of 7 ml scintillation fluid (Optiphase Safe). The $^3$H counts measured using a Packard Tri-Carb 1600 Scintillation counter.
3.0 IDENTIFICATION AND CLONING OF CENTAURIN-α2

3.1 Introduction

In adipose tissue and muscle, insulin initiates a signalling cascade resulting in the translocation of Glut4 from the internal storage compartment to the plasma membrane, where it transports glucose into the cell. It is now well established that PtdIns 3-kinase plays a pivotal role in this response. PtdIns 3,4,5-P₃, the major product of this kinase, was shown to be specifically bound by centaurin-α and centaurin-α1. In 1997, when this research project was initiated, very little was known about the function of the centaurin-α proteins. Their abundant expression in brain tissue and high affinity for PtdIns 3,4,5-P₃ lead to the suggestion that they might function in signal transduction and vesicle transport (Hammonds-Odie et al. 1996; Kreutz et al. 1997; Strieker et al. 1997; Tanaka et al. 1997). The discovery of this PtdIns 3,4,5-P₃ binding ARF-GAP-related protein family inspired us to investigate the presence of related proteins in insulin-sensitive tissues.

3.1.1 The Discovery of a Novel Partial Clone with Homology to the Centaurin-α Family

At the outset of my research, a former member of our laboratory, Dr Sue Oldfield, had discovered a partial clone, relating to the C-terminal portion of the later identified centaurin-α2. This initial finding was achieved using a centaurin-α DNA probe (encoding the majority of the coding region of rat centaurin-α) to screen a rat adipocyte λgt 11 library (a gift from C. Londos, NIH, Bethesda) at low stringency. The rat centaurin-α 1218 bp DNA fragment was assembled into pBluescript from two PCR products amplified from rat brain cDNA. The centaurin-α fragment was excised and labelled with [α-³²P]dCTP by random priming (Section 2.3.6). Almost all of the high intensity positive plaques were found to contain identical inserts of approximately one kilobase. The sequence contained an incomplete open-reading frame coding for 193
amino acids that had 58% identity to amino acids 184-376 of centaurin-α, plus 405 nucleotides of 3' untranslated region (Figure 3.1, A).

3.1.2 Identification of the Human Centaurin-α2 Clone

A search of the EMBL databases for nucleotide sequences showing similarity to rat centaurin-α identified several human ESTs. These sequences appeared to fall into two groups. The first set shared greater than 80% identity with the centaurin-α family and approximately 65% identity with the rat centaurin-α2 partial clone, whereas the second group had the converse relationship. From this latter group, one EST (accession no. AA256750) of 451 bp was 63% identical to the 5' region of centaurin-α and did not overlap with the centaurin-α2 partial clone. This clone was obtained from the UK HGMP Resource Centre, Hinxton, Cambridge. The complete sequencing of the clone revealed a 1.8 kb cDNA encoding for a protein with 81% identity to the centaurin-α2 partial clone (Figure 3.1B).

3.2 5'RACE Using Marathon-Ready cDNA

A sample of a rat liver Marathon-Ready cDNA library (a gift from Dr B Reaves, University of Bath, UK) was used as a template for the amplification of the 5' terminus of rat centaurin-α2 as described in the manufacturer's protocol (Section 2.2.5). A gene-specific reverse primer (Figure 3.2, A) was designed to the 5' sequence of the rat centaurin-α2 partial clone using the guidelines described in the Marathon-Ready cDNA User Manual. A 24 base oligonucleotide was designed with a G/C content of 62.5% and a melting temperature (T_m) of 74.5°C, as determined by nearest neighbour analysis. A high-stringency thermal cycling program was selected, which incorporated a high annealing/elongation temperature that was compatible with both the gene-specific primer and the enzyme (Expand Long Template PCR System) (Figure 3.2, B). A positive control reaction was performed using the control 5'RACE primer for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) in place of the gene-specific primer. Following amplification, the products were examined by agarose gel electrophoresis. Visualisation of the gel with an UV transilluminator revealed that
Figure 3.1. Sequence alignment of the rat centaurin-α2 partial clone sequence with related centaurin proteins. (A) Rat centaurin-α2 partial clone sequence (top sequence) was aligned with the rat centaurin-α sequence (lower sequence; accession number U51013). (B) Rat centaurin-α2 partial clone sequence was aligned with the human centaurin-α2 sequence (accession number AJ238994) for the overlapping region (amino acids 186-376). The rat centaurin-α2 sequence is at the top, human centaurin-α2 at the bottom.
Figure 3.2. Amplification of the 5' terminus of centaurin-α2 using 5'RACE. (A) Overview of the marathon protocol. The specificity of marathon RACE reactions is enhanced by the absence of an AP1 binding site on the adaptor ligated cDNAs. This site is created on the cDNA of interest by the extension from the gene-specific primer during the first RACE cycle. GSP1, gene-specific primer-1 (CGGGCTGCA CGGA-GAGCATTGAAC). (B) Thermal cycling programmes used to amplify centaurin-α2. Protocol 1 was used in the initial reaction and Protocol 2 outlines the conditions of the modified amplification programme.
absence of DNA bands from both the sample and positive control lanes (data not shown). A 1.1 kb DNA fragment was expected for the G3PDH positive control, the absence of this band indicated that the amplification was unsuccessful.

A number of modifications were made to the 5'RACE protocol. Firstly, the PCR product from the previous 5'RACE reaction was diluted (1:100 and 1:500 in ddH2O) and used as a template. Secondly, a lower annealing temperature was employed (Figure 3.2, B). Three sample reactions were prepared with different template conditions for each reaction; the first reaction contained 5 μl Marathon-Ready cDNA, whereas diluted 5'RACE product was used in the other two reactions (1:100 and 1:500 dilutions, respectively). Analysis of the products by agarose gel electrophoresis revealed a single band of approximately 800 bp in the reactions using the diluted 5'RACE product as a template (Figure 3.3, A). No bands were visible for the reaction performed with the Marathon-Ready cDNA template.

Triplicate reactions were performed using the 1:100 diluted 5'RACE product as a template. A negative control was also performed with the gene-specific primer omitted from the reaction. These samples were run on an agarose gel (Figure 3.3, B) and gel slices containing the DNA fragment were excised and the DNA purified (Section 2.2.3).

### 3.2.1 Cloning of the 5'RACE Fragment into pT7Blue Cloning Vector

The purified DNA fragment was ligated into the pT7Blue cloning vector (Figure 3.4) using the Perfectly Blunt Cloning Kit. In brief, the ends of the DNA fragment were converted to blunt, phosphorylated ends, by adding 5 μl End Conversion Mix to 3 μl purified 5'RACE product and making the final volume to 10 μl with ddH2O. The reaction was incubated at 22°C for 15 min then terminated by heating at 75°C for 5 min and briefly chilled on ice. Blunt pT7Blue vector, dephosphorylated at the EcoRV site (50 ng) was added to the end conversion reaction and ligation was performed using T4 DNA ligase (4U) for 2 h at 22°C. The ligase was heat inactivated at 60°C for 5 min and the reaction cooled prior to transformation into NovaBlue Singles Competent Cells (Section 2.2.8). In brief, 1 μl ligation reaction was added to the competent cells and chilled on ice for 5 min prior to a heat shock step for 30 sec at 42°C. The cells were chilled on ice for a further 2 min, after which 250 μl SOC medium (2% (w/v) tryptone,
Figure 3.3. Agarose gel electrophoresis of 5'RACE reactions. (A) 5'RACE amplification using liver Marathon-Ready cDNA. Three reactions were prepared with different templates; 1:100 and 1:500 dilutions of product from the previous 5'RACE reaction and the third reaction (Library) was prepared using liver Marathon-Ready cDNA as the template. (B) Multiple 5'RACE reactions were repeated using the 1:100 reaction conditions used in (A). The negative control reaction (Control) was performed with the omission of the gene specific primer. Molecular weight markers (MW) were run alongside the samples, with appropriate band sizes indicated.
Figure 3.4. Vector map of the pT7Blue cloning vector. (A) Cloning map. (B) pT7Blue cloning/expression region. The ends of the 5'RACE DNA fragment were converted to blunt ends and ligated with the linearised vector, thus inserting the 5'RACE product into the blunt cloning site (EcoRV). These figures were reprinted with the kind permission of Novagen, Inc.
0.5% (w/v) yeast extract, 0.05% (w/v) NaCl, 2.5 mM KCl, 10 mM MgCl₂, 20 mM glucose; included in the kit) was added and the cells transferred to a shaking incubator at 37°C for 30 min. The transformation reaction (10 μl and 90 μl) was spread onto LB agar plates that were prespread with 35 μl of 20 mg/ml X-gal in dimethyl formamide and 20 μl 100 mM IPTG. The plates were inverted and incubated overnight at 37°C.

Recombinant colonies were identified using blue/white selection. The pT7Blue plasmid encodes a functional lacZ α-peptide that complements the lacZ ω-fragment expressed by the host strain (NovaBlue). The resulting active β-galactosidase can cleave the chromogenic substrate X-gal to yield a blue colony phenotype. Inserts cloned into the multiple cloning site disrupt the open reading frame of the α-peptide, resulting in the white colony phenotype. The plate that had been spread with 100 μl transformation reaction contained between 100-200 colonies with the ratio of blue and white colonies at approximately 50:50. Plasmid DNA was purified from overnight cultures inoculated with single white colonies (Section 2.2.1).

### 3.2.2 Characterisation of the DNA Inserts

The purified recombinant DNA was digested with the endonuclease enzymes HindIII and EcoRI, which cut either side of the EcoRV restriction site, within the multiple cloning site of pT7Blue (Figure 3.4). In the absence of internal restriction sites for these two enzymes within the DNA insert, two bands of 2800 and 900 bp were expected for the vector and insert respectively. The majority of clones yielded similar DNA digest patterns consisting of two DNA fragments, thus indicating the presence of an insert of similar size. However, the fragment length could not be determined due to poor visibility of the size markers (data not shown). Four of the clones were chosen for sequencing. Two clones (1 and 2) were selected because they shared the restriction pattern displayed by the majority of the clones. The additional two clones were chosen for the opposite reason, as they displayed distinct digestion patterns. All four clones were sequenced in both directions using the vector primers M13 universal primer and M13 reverse primer.

Sequence analysis revealed that the two clones exhibiting unique restriction digest patterns were artefacts, as the gene-specific primer was present at both ends of the
sequence. However, clones 1 and 2 contained the adaptor primer and gene specific primer located at the 5’ and 3’ ends respectively. The gene specific primer was situated within a 143 nucleotide overlapping region present at the 3’ end of the cDNA fragment and the 5’ sequence of the centaurin-α2 partial clone identified from the λgt 11 library. The 5’RACE DNA fragment contained an open reading frame coding for 248 amino acids. The start codon was assigned at codon 18 based on a neural network prediction of start codons (Pedersen and Nielsen 1997) and similarity to the centaurin-α protein sequences.

### 3.3 Generation of the Full-Length Rat Centaurin-α2 cDNA by 3’RACE

Following amplification of the 5’ sequence it was possible to generate the full-length clone using two different approaches. Firstly the 5’ and 3’ fragments could be combined using basic cloning techniques. This simple procedure involves the digestion of the fragments at a restriction site located in the overlapping region, followed by ligation of the compatible ends with T4 DNA ligase. However, caution should be noted when using this technique, as there is the possibility of creating a chimera by joining cDNAs derived from two different transcripts, as discussed later for the rat centaurin-α probe. Furthermore, the two cDNA fragments of centaurin-α2 were amplified from different tissues (liver and adipocyte). Therefore, 3’RACE was selected for generation of the full-length cDNA. Marathon-Ready cDNA libraries, from two tissues, adipocyte and brain (synthesised from polyA+ RNA by Dr P Whitley using the Marathon cDNA Amplification Kit, Clontech) were utilised for the 3’RACE reaction. The adaptor primer and a gene-specific primer designed to the 5’ terminus were used to amplify the full-length cDNA using a touchdown thermal cycling program (*Figure 3.5, A and B*).

A 1.8 kb band was successfully amplified from both the adipocyte and brain libraries, with an additional 1.1 kb band from the brain template (*Figure 3.6*). The three DNA fragments were cloned into pT7Blue and transformed into NovaBlue Singles (*Section 3.2.1*). Overnight cultures were prepared using colonies chosen by blue/white selection and the plasmid DNA purified. Restriction digests with *PstI* was performed to determine the presence and orientation of the insert (data not shown). Sequence analysis of the 5’RACE product and the partial clone enabled the construction of a
Figure 3.5. Amplification of centaurin-α2 using 3’RACE. (A) Overview of the marathon protocol. The full-length centaurin-α2 sequence was amplified using AP1 and GSP2 (CCGGCCATGGGCGACCGTGAACG). As with the 5’RACE protocol, the AP1 binding site is not created until extension of the cDNA of interest using GSP2. (B) Thermal cycling programmes used to amplify the complete centaurin-α2 clone.
Figure 3.6. Agarose gel electrophoresis of 3'RACE DNA fragments amplified from adipocyte and brain Marathon-Ready cDNA libraries. A 1.8 kb fragment was amplified from both the adipocyte and brain cDNA. An additional 1.1 kb band was detected in the brain sample. Molecular weight markers (MW) were run alongside and the appropriate size bands indicated.
predicted restriction map for PstI. Two internal sites were expected, with an additional site located in the multiple cloning site of the vector. The restriction pattern for both the adipocyte and brain 1.8 kb samples agree with the predicted fragment pattern. However, the 1.1 kb DNA fragment from brain was also analysed and did not contain an internal PstI site.

3.4 Sequence Analysis

The restriction digest data enabled the selection of suitable clones for sequencing. In addition to the vector primers described above, a series of internal primers were used to sequence the 1.8 kb inserts in both directions. The adipocyte and brain sequences were identical to the sequence assembled from the combination of 5'RACE sequence (from liver) and the partial clone isolated from the adipocyte λgt 11 library. The predicted position of the initiation codon of rat centaurin-α2 was further strengthened by the presence of an in frame stop codon in the human cDNA, located 5' of the proposed ATG start codon. The complete nucleotide sequence of 1798 bp and predicted protein sequence of 376 amino acids are shown in Figure 3.7.

BLAST searches of the protein sequence databases revealed that this clone encodes a novel protein that is most similar to porcine p42IP4 (59% identity) (Stricker et al. 1997) and other centaurin-α family proteins (Hammonds-Odie et al. 1996; Tanaka et al. 1997; Venkateswarlu and Cullen 1999) (Figure 3.8 and 3.9). The protein has a deduced molecular mass of 43.5 kDa and protein sequence analysis using ProfileScan (ISREC) detected the presence of an N-terminal zinc-finger motif (residues 21-82) with the consensus CX2CX16CX2C and two PH domains, PH-N (residues 131-232) and PH-C (residues 254-360). The size and domain arrangement of centaurin-α2 matches that of the centaurin-α1 proteins. Since the centaurin-α1 proteins share approximately 90% identity to one another, it appears that the protein identified here is distinct. However, the structure of the other centaurin protein families, centaurin-β, -δ, and -γ, bare little similarity to this novel protein (see Section 1.5.4, Figure 1.7). Consequently it was decided that p42IP4, PIP3-BP and human centaurin-α1 should collectively be referred to as the centaurin-α1 proteins, which are closely related to rat centaurin-α. The novel rat and human centaurin proteins described herein should form a related subfamily of the centaurin-α proteins entitled centaurin-α2.
Figure 3.7. Nucleotide and deduced amino acid sequence of the cloned cDNA of rat centaurin-α2. The numbers of the nucleotide and amino acid residues are indicated on the right of the sequence.
Figure 3.8. Alignment of rat and human centaurin-α2 amino acid sequences with those of the centaurin-α family; rat centaurin-α2 (accession number AJ238993); human centaurin-α2 (accession number AJ238994); rat centaurin-α (accession number U51013); rat p42IP (accession number AJ007422); human centaurin-α1 (accession number AJ006422); porcine p42IP (accession number U88368); bovine PIP3-BP (accession number D89940). A multiple sequence alignment was generated using MultAlin (INRA, Paris, France) and Boxshade (EMBn@t.ch). The domains are underlined:

- N-terminal zinc finger motif
- PH-N domain
- PH-C domain.
<table>
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<tr>
<th></th>
<th><strong>Centaurin-α</strong></th>
<th><strong>Centaurin-α1</strong></th>
<th><strong>Centaurin-α2</strong></th>
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<tr>
<td></td>
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<td><strong>Rat</strong></td>
<td><strong>Human</strong></td>
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<td><strong>Centaurin-α</strong></td>
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<td>51%</td>
</tr>
<tr>
<td></td>
<td><strong>Porcine</strong></td>
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<td><strong>Human</strong></td>
</tr>
<tr>
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<td>(p42IP4)</td>
<td>76%</td>
<td>79%</td>
</tr>
<tr>
<td></td>
<td><strong>Bovine</strong></td>
<td><strong>Bovine</strong></td>
<td><strong>Human</strong></td>
</tr>
<tr>
<td><strong>Centaurin-α1</strong></td>
<td>(p42IP4)</td>
<td>75%</td>
<td>59%</td>
</tr>
<tr>
<td></td>
<td><strong>Porcine</strong></td>
<td><strong>Porcine</strong></td>
<td><strong>Human</strong></td>
</tr>
<tr>
<td><strong>Centaurin-α2</strong></td>
<td>(p42IP4)</td>
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<td>58%</td>
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<td><strong>Bovine</strong></td>
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</tr>
<tr>
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<tr>
<td><strong>Centaurin-α2</strong></td>
<td></td>
<td>94%</td>
<td>59%</td>
</tr>
</tbody>
</table>

**Figure 3.9. Sequence identities of the centaurin-α family proteins.** Rat centaurin-α2 (accession number AJ238993); human centaurin-α2 (accession number AJ238994); rat centaurin-α (accession number U51013); rat p42IP4 (accession number AJ007422); human centaurin-α1 (accession number AJ006422); porcine p42IP4 (accession number U88368); bovine PIP3-BP (accession number D89940). The percentages were calculated by performing pair alignments using ALIGN (ExPASy).
3.5 Discussion

In order to screen the adipocyte λgt 11 library for centaurin-α related clones, the generation of a suitable probe was required. The cloning and sequencing of the centaurin-α cDNA probe revealed three differences from the published rat centaurin-α sequence: two single base changes at positions 396 and 402 and a two base deletion at position 1232. The latter deletion caused a frameshift with respect to the published sequence (Hammonds-Odie et al. 1996), which introduced a stop codon. Consequently, the cDNA encoded a shorter protein of 376 amino acids that terminated at the same point as the centaurin-α1 proteins. Although sharing similarity in size, our rat centaurin-α clone differed from the centaurin-α1 proteins within the PH-N region (amino acids 139-161). In this region the identity was interrupted and our centaurin-α clone was identical to the published rat centaurin-α sequence. This occurrence can be explained by the method employed in the generation of the clone, which was assembled from two PCR products. Therefore, it would appear this cDNA is a hybrid generated from transcripts encoding the 5' portion of centaurin-α and the 3' region of centaurin-α1.

Until now, centaurin-α was the only member of the centaurin-α protein family to have ankyrin repeats in place of the PH-N domain (Hammonds-Odie et al. 1996). In 1998, Reiser and colleagues investigated this anomaly using a variety of molecular biology and biochemical approaches. Firstly, RT-PCR was employed to analysis the mRNA from rat brain using PCR primers complementary to the regions spanning the start and stop codons of rat centaurin-α. Several cDNA clones with identical sequence were identified and designated rat p42IP4 as the sequence was identical to pig p42IP4 between amino acids 139-161, terminated at the same position and shared 94.5% identity (Aggensteiner et al. 1998). Secondly, antiserum raised against an 18 amino acid peptide corresponding to amino acids 353-371 of porcine p42IP4 and amino acids 355-373 of rat centaurin-α was used in western blot analysis. Protein fractions were purified using the chromatography methods employed for the purification of porcine p42IP4 from pig cerebellum (Donié and Reiser 1991). A single 42 kDa protein band that comigrated with pig p42IP4 was observed (Aggensteiner et al. 1998). Although this study by Reiser and colleagues cast some doubt on the existence of rat centaurin-α, our results
strengthen the authenticity of the ankyrin repeat region of the rat centaurin-α sequence. However, the position of the stop codon of rat centaurin-α remains questionable. Attempts to isolate the full-length centaurin-α2 clone from the adipocyte λgt 11 library were unsuccessful. The initial effort to amplify the 5' region of the partial clone using 5'RACE was also unsuccessful. The absence of G3PDH (positive control) signal indicated that the amplification conditions were unsuitable. Modification of the thermal cycling programme alone was not sufficient to yield amplified cDNA from the liver Marathon-Ready cDNA template. However, the 5' fragment was successfully amplified in reactions where the template was changed to the diluted PCR product from the previous 5'RACE reaction (Figure 3.3, A). The reasons for these results remain ambiguous. No cDNA bands appeared to have been amplified in the first reaction, however dilution of the reaction product may simply have diluted the template cDNA to levels appropriate for amplification. Alternatively components that were inhibiting or interfering with the amplification reaction may have been diluted.

The rat and human centaurin-α2 sequences are on the EMBL Nucleotide Database, accession numbers AJ238993/4 respectively. Shortly before the release of these sequences into the public domain, an identical human centaurin-α2 sequence was released by another group, who also entitled the protein centaurin-α2 (accession number AJ272195). Jenne and co-workers identified the centaurin-α2 gene to be one of a common set of 11 functional genes which were lost in the majority of neurofibromatosis type-1 patients with gross deletions (Jenne et al. 2000). Neurofibromatosis type-1 (NF1), also known as von Recklinghausen disease, is an autosomal dominant disorder with an estimated prevalence of 1 in 3000. It is caused by mutations of the NF1 gene (Rasmussen and Friedman 2000; von Deimling et al. 1995). Neurofibromin, the protein encoded by the NF1 gene, is a GAP protein for the ras oncogene, and has been suggested to act as a tumour suppressor (Xu et al. 1990). Neurofibromas, benign tumours, can arise in the skin or in fibrous tissue surrounding peripheral and cranial nerves and some of the characteristic symptoms include café-au-lait spots, optic glioma, Lisch nodules (iris hamartomas), distinctive osseous lesions and mental retardation (von Deimling et al. 1995). Jenne et al. proposed that most of the NF1 patients with the entire NF1 gene deletion also lacked the same set of 11 genes (including centaurin-α2) which are adjacent to the NF1 gene located at chromosome 17q11.2 (Jenne et al. 2000).
Centaurin-α2 contains the zinc-finger motif with the consensus CX$_2$CX$_{16-17}$CX$_2$C that is conserved throughout the family of ARF-GAP proteins. The invariant arginine residue located five residues following the fourth cysteine residue is conserved in centaurin-α2. Although centaurin-α1 has been shown to functionally compliment Gcs1 (Venkateswarlu et al. 1999b) no in vitro ARF-GAP activity has been detected for centaurin-α or the centaurin-α1 proteins (Jackson et al. 2000b). Jackson and colleagues went on to speculate that these proteins may require another cofactor, or may act on a different ARF or ARF-like (ARL) partner, or they may not be GAP proteins and have an alternative function (Jackson et al. 2000b). Interestingly the GAP domain of the Ras-GAP neurofibromin mentioned above has been shown to interact with microtubules as well as activating the GTPase activity of Ras (Gregory et al. 1993).
4.0 INVESTIGATION OF CENTAURIN-α2 mRNA AND PROTEIN EXPRESSION

4.1 Introduction

The transcripts for centaurin-α and several of the centaurin-α1s have been found to be highly abundant in brain, the tissue from which the majority of these proteins were identified (Hammonds-Odie et al. 1996; Stricker et al. 1997; Tanaka et al. 1997; Venkateswarlu and Cullen 1999). Although centaurin-α was detected in lung, kidney and spleen and centaurin-α1 found in spleen, kidney and peripheral blood leukocytes, the level of expression was considerably lower than that observed for brain. Simply from the cloning of rat centaurin-α2, we expected a wider distribution of expression, as the 5'RACE product was amplified from a liver cDNA library and the full-length sequence from adipocyte and brain.

In order to determine the distribution of centaurin-α2 expression, studies were undertaken to examine the relative abundance of both the transcript and the protein, in a number of tissues, including insulin-sensitive tissues of adipocytes, heart and skeletal muscle. Firstly the mRNA expression was examined using northern blot analysis. Secondly, centaurin-α2 antiserum was employed to determine the tissue distribution and subcellular localisation of the native protein using western blot analysis. Finally, further analysis of the subcellular localisation was performed using confocal laser microscopy.

4.2 Northern Blot Analysis

Before the identification of the full-length centaurin-α2 sequence, the expression of the partial clone was analysed using total RNA northern blots and radioactively-labelled DNA probes. Later experiments were performed using DIG-labelled RNA probes encoding the 5'RACE product of centaurin-α2 with polyA⁺ RNA blots. In order to ascertain the relative abundance of centaurin-α2, normalisation of the signal, to
compensate for differences in RNA loading, was required. Ubiquitous housekeeping
genes, such as β-actin, an essential cytoskeletal protein, and the glycolytic enzyme
G3PDH are among the most common genes traditionally employed for RNA
normalisation.

4.2.1 Northern Blot Analysis using Radioactively-Labelled DNA
Probes

4.2.1.1 Purification of Total RNA
Total RNA was isolated from nine rat tissues: adipose, brain, heart, kidney, liver, lung, skeletal muscle, spleen and testis. Following the determination of RNA concentration
by spectrophotometry, 10 µg of the RNA was examined by agarose gel electrophoresis.
The 18S and 28S ribosomal RNA bands were clearly visible in all the total RNA
samples, with the exception of spleen where the bands were weak (Figure 4.1). The
smear observed in the spleen sample might have resulted from poor sample loading.
Alternatively the RNA may have been degraded, although the A$_{260}$/A$_{280}$ ratio was 1.78,
well within the 1.6-2.0 range recommended for northern blot analysis (TriPure Isolation
Reagent, Roche Molecular Biochemicals).

4.2.1.2 Preparation of 32P-Labelled DNA Probes
The cDNA encoding the partial centaurin-α2 sequence was excised from the cloning
vector pBluescript using the restriction endonuclease EcoRI. Electrophoresis of the
digested DNA showed a band at approximately 800 bp as expected (Figure 4.2). This
band was cut from the gel and the DNA purified. Similarly, the centaurin-α cDNA
(previously used to screen the rat adipocyte λgt 11 library) was excised from
pBluescript using HindIII and XbaI, yielding a 1200 bp DNA fragment. Radioactively-
labelled probes were generated by the incorporation of [α-32P]dCTP during DNA
synthesis primed with random sequence hexanucleotides (Section 2.3.6). Rat G3PDH
cDNA was also labelled using this method.

4.2.1.3 Northern Blot Analysis using 32P-Labelled DNA Probes
Total RNA isolated from the rat tissues and RNA Molecular Weight Markers were
separated on a denaturing formaldehyde agarose gel. In additional sample of 20 µg
Figure 4.1. Agarose gel electrophoresis of total RNA. Total RNA was isolated from rat tissues using TriPure Isolation Reagent and 10 μg was examined by electrophoresis on a 1% agarose gel containing 0.5 μg/ml ethidium bromide. The position of the 28S and 18S ribosomal RNA bands are indicated by the arrows. Ad, adipose tissue; Br, brain; He, heart; Ki, kidney; Li, liver; Lu, lung; Mu, skeletal muscle; Sp, spleen; and Te, testis.

Figure 4.2. Restriction digestion of the centaurin-α2 partial clone and centaurin-α. Centaurin-α2 cDNA was excised from pBluescript using EcoRI yielding a fragment of approximately 800 bp. Similarly the centaurin-α cDNA fragment (1200 bp) was cut out of pBluescript with HindIII and Xbal. Molecular weight markers (MW) were separated alongside the samples and the sizes of the appropriate markers have been indicated.
liver total RNA was run alongside the markers. Following transfer and baking of the nitrocellulose, the section of membrane containing the marker lanes was cut off and stained with methylene blue (Figure 4.3). The position of the wells, the 18S and 28S ribosomal bands of the total RNA, and the molecular weight marker bands were marked onto the nitrocellulose with a pencil. The migration distance was plotted against the transcript size (data not shown) and the mRNA transcript sizes calculated. Hybridisation reactions were performed sequentially using radioactively-labelled cDNA probes for the centaurin-α2 partial clone, centaurin-α and G3PDH.

The signal intensity was weak for all three probes and consequently the blots required long periods of exposure to the film (> 48 h). Centaurin-α2 produced a particularly weak signal, resulting in high levels of non-specific background. Although, faint bands of approximately 4.0 kb were vaguely detectable in kidney, lung and spleen when using this probe (Figure 4.4, A). Unfortunately there was an intense spot of non-specific binding, located between the adipocyte and brain lanes, that obscured these lanes in the region of the 4.0 kb centaurin-α2 band. As expected an intense band of 2.5 kb was detected in brain for centaurin-α, with weaker signals observed in kidney, lung and spleen (Figure 4.4, B). The intensity of the 1.6 kb G3PDH band varied considerably between the tissues, despite loading of equal quantities of total RNA (Figure 4.4, C).

In an attempt to intensify the signal, the amount of poly-adenylated (polyA+) messenger RNA loaded onto the gel was increased using purified polyA+ RNA northern blots. Firstly, polyA+ RNA was isolated from 100 μg total RNA for each tissue, with an expected yield of 1-2 μg. Loading total RNA (20 μg) and polyA+ RNA (1-2 μg) on the same gel enabled direct comparison of the relative signal intensities (Figure 4.5). The blots probed with G3PDH and centaurin-α clearly indicated an increase in signal intensity when using polyA+ RNA, as compared with the total RNA (Figure 4.5, B and C). As before, high background levels obscured the centaurin-α2 signal, although indistinct bands were just about detectable in the polyA+ lanes. Although it was difficult to distinguish, there appeared to be two bands in the brain PolyA+ RNA sample. The position of the lower band corresponded to that of centaurin-α. Since the centaurin-α2 probe was used after the centaurin-α probe, the lower band may have been a result of residual centaurin-α probe. Following this observation, the centaurin-α2 probe was tested before centaurin-α for subsequent blots.
Figure 4.3. Methylene blue stained nitrocellulose membrane containing RNA molecular weight markers and total RNA. RNA molecular weight markers and 20 μg total RNA from liver were separated alongside the tissue total RNA samples on a denaturing gel and transferred to nitrocellulose generating a multi-tissue northern blot. Following baking, the outer marker lanes were cut away and the membrane was stained with a solution of 0.5 M sodium acetate (pH 5.2) and 0.04% methylene blue. After rinsing with water the positions of the marker bands and the ribosomal RNA bands were marked.
Figure 4.4. Northern blot analysis of total RNA from rat tissues using $^{32}$P-labelled DNA probes. A multiple tissue northern blot was prepared using 20 μg total RNA from rat tissues. (A) Centaurin-α2 partial clone probe produced a 4.0 kb band; (B) Centaurin-α yielded an intense 2.5 kb band in brain; and (C) G3PDH 1.6 kb band was detected in almost all the tissues. Ad, adipose tissue; Br, brain; He, heart; Ki, kidney; Li, liver; Lu, lung; Mu, skeletal muscle; Sp, spleen and Te, testis. The positions of the marker bands are indicated.
Figure 4.5. Northern blot analysis using polyA⁺ RNA and total RNA. Total RNA (20 μg) and polyA⁺ RNA (1-2 μg) were separated on the same formaldehyde denaturing gel and transferred to a nitrocellulose membrane. Hybridisation with ³²P-labelled DNA probes was performed. (A) Centaurin-α² partial clone probe; (B) Centaurin-α; and (C) G3PDH. Ad, adipose tissue; Br, brain. RNA markers were not used so the positions of the rat centaurin-α bands (2.5 and 4.0 kb) are marked.
As the signal intensity was much improved with polyA⁺ RNA, a multiple tissue northern blot was generated and hybridised successively with the three ³²P-labelled DNA probes. Despite the centaurin-α2 signal remaining fairly weak with high background levels, a 4.0 kb centaurin-α2 band was discernible in liver and kidney (Figure 4.6, A). In addition there appeared to be a very faint band of approximately 5 kb in these tissues. As shown previously, a very strong centaurin-α signal was detected in brain with weaker signals visible in kidney and lung (Figure 4.6, B). The intensity of the G3PDH signal was strong and differed quite significantly between samples. Thus comparison of expression levels of the centaurin proteins in the various tissues required normalisation (Figure 4.6, C).

4.2.2 Northern Blot Analysis using the DIG Detection System

The DIG system is a non-radioactive detection system that utilises digoxigenin, a steroid hapten, coupled to dUTP, UTP or ddUTP to label DNA, RNA or oligonucleotides for hybridisation and subsequent detection. There are several methods of generating DIG-labelled probes, however DIG-labelled RNA probes are the most sensitive for northern blot analysis. Detection of the hybridised probe was achieved using an anti-digoxigenin antibody-alkaline phosphatase conjugate and the chemiluminescent alkaline phosphatase substrate, CSPD. The chemiluminescent signal was visualised upon exposure to Hyperfilm ECL. Single-stranded RNA probes were generated for centaurin-α2, centaurin-α, β-actin and G3PDH by in vitro transcription using the pSPT18 vector included in the DIG RNA Labelling Kit (Figure 4.7).

4.2.2.1 Preparation of DIG-Labelled RNA Probes

The centaurin-α2 5'RACE product was excised from pT7Blue using XbaI and SmaI, restriction endonucleases that cut either side of the EcoRV insertion site (Figure 3.4). A single DNA fragment of approximately 830 bp was cut from the pT7Blue vector (2887 bp) and extracted from the agarose gel (Figure 4.8). Likewise, the pSPT18 vector was digested with XbaI and SmaI and purified in preparation for the ligation reaction with the insert DNA. Following ligation with T4 DNA ligase, the DNA was transformed into the competent cells (NovaBlue Singles). Recombinant colonies were selected using blue/white screening and used to inoculate overnight cultures of LB
Figure 4.6. PolyA⁺ RNA northern blot analysis using ³²P-labelled DNA probes. A multiple tissue northern blot was prepared using polyA⁺ RNA. (A) Centaurin-α2 partial clone probe, (B) Centaurin-α, and (C) G3PDH. Ad, adipose tissue; Br, brain; He, heart; Ki, kidney; Li, liver; Lu, lung; Mu, skeletal muscle; Sp, spleen and Te, testis. The positions of the RNA molecular weight markers are indicated.
Figure 4.7. Multiple cloning site and vector map of the transcription vector pSPT18. The pSPT18 vector was cut with XbaI and Smal separated on an agarose gel and purified. Centaurin-α2 5’RACE product was cloned into pSPT18. The recombinant plasmid was then linearised using Smal, and RNA transcripts of uniform length were synthesised by in vitro transcription using SP6 RNA polymerase. Amp', ampicillin selective marker. This diagram was reprinted with the kind permission of Roche Molecular Biochemicals.
broth. The purified plasmid DNA was analysed by restriction digestion with \textit{Xba}I and \textit{Smal} to confirm the presence of the 781 bp insert DNA and the 3106 bp pSPT18 vector (\textit{Figure 4.9}).

The endonuclease \textit{Smal}, cuts the pSPT18 vector at the 3' end of the inserted DNA and was used to linearise the recombinant plasmid. RNA transcripts of uniform length were synthesised by \textit{in vitro} transcription using SP6 RNA polymerase. Quantification of the DIG-labelled RNA yield was determined by direct comparison with the DIG-labelled control RNA in a spot assay. The centaurin-\textalpha{}2 probe yield was calculated to be 125 ng/\textmu{}l. (\textit{Figure 4.10}). Likewise, Dr Paul Whitley generated RNA probes for centaurin-\textalpha{} (using the cDNA used to screen the rat adipocyte \textlambda{}gt 11 library, \textit{Section 3.1.1}), \textbeta{}-actin and G3PDH.

### 4.2.2.2 Northern Blot Analysis using DIG-Labelled RNA Probes

PolyA\textsuperscript{+} RNA was isolated from 1 mg total RNA, with a predicted yield of 10-20 \textmu{}g. A tenth of this RNA (1-2 \textmu{}g) was used for the first northern blot, which was probed with G3PDH and \textbeta{}-actin to determine which probe to use for the normalisation process. A single band of 1.6 kb was produced with the G3PDH probe, and \textbeta{}-actin yielded two bands, the ubiquitously expressed 2.0 kb transcript and the 1.8 kb muscle type actin in heart and skeletal muscle (\textit{Figure 4.11, A and B}). These blots, developed using the DIG-detection system, had very low levels of background signal and very little non-specific spotting. The signal intensities were inconsistent between tissue samples, for example in lung the G3PDH expression appears to be considerably lower than in other tissues. In contrast the \textbeta{}-actin signal is quite abundant in lung. No bands were detected in the spleen samples using either of the normalisation probes. The total RNA from which the spleen polyA\textsuperscript{+} RNA was isolated appeared normal on examination by electrophoresis. However, degradation may have ensued, alternatively the isolation of polyA\textsuperscript{+} RNA may have been unsuccessful.

A study comparing transcript size, tissue-specific variation and the number of hybridising bands of nine widely used housekeeping genes found \textbeta{}-actin to be the best choice for use in the normalisation process (Siebert \textit{et al.} 1999). Since there was no obvious distinction between the two probes, \textbeta{}-actin was selected for the standardisation process of the subsequent northern blots. A second northern blot was produced using
Figure 4.8. Excision of the centaurin-α2 5′RACE product cDNA from the pT7Blue cloning vector. The cloning vector pT7Blue containing the centaurin-α2 5′RACE product cDNA was digested with XbaI and SmaI. The digested recombinant plasmid was separated by agarose gel electrophoresis. RD, restriction digested sample; MW, 100 bp molecular weight markers.

Figure 4.9. Restriction digestion of the recombinant plasmid, pSPT18-5′centaurin-α2 using XbaI and SmaI. Positive recombinant clones, selected using blue/white screening were tested for the presence of the centaurin-α2 cDNA insert (781 bp) using restriction digestion with XbaI and SmaI. Molecular weight markers (MW) were separated alongside the samples and the sizes of the appropriate markers have been indicated.
Figure 4.10. Estimation of DIG-labelled centaurin-α2 RNA yield in a spot test with a DIG-labelled control RNA. The DIG-labelled centaurin-α2 RNA probe synthesised by \textit{in vitro} transcription was quantified in a spot test. A dilution series of the control DIG-labelled RNA and centaurin-α2 RNA were prepared and spotted onto nylon membrane. Detection was performed using the DIG-detection system.

(A) β-actin

(B) G3PDH

Figure 4.11. Northern blot analysis of the housekeeping genes β-actin and G3PDH. Multiple tissue northern blots were generated using polyA+ RNA and the nylon membrane was then hybridised with DIG-labelled RNA probes for (A) β-actin and (B) G3PDH. β-actin yielded two bands, the ubiquitously expressed 2.0 kb transcript and the 1.8 kb muscle type actin in heart and skeletal muscle. A single band of 1.6 kb was produced with the G3PDH probe. Ad, adipose tissue; Br, brain; He, heart; Ki, kidney; Li, liver; Lu, lung; Mu, skeletal muscle; Sp, spleen and Te, testis. RNA molecular weight markers were separated alongside the samples and the sizes of the appropriate markers have been indicated.
the same stock of polyA⁺ RNA as the previous samples. The signal intensity of the β-actin 2.0 kb band was quantified for each tissue on the duplicate blots using Molecular Analyst/PC version 1.5, (Bio-Rad Laboratories). The quantity of polyA⁺ RNA loaded onto subsequent gels was adjusted so that approximately equal β-actin signal intensities were observed. Sequential hybridisation reactions were then performed using the centaurin-α2, centaurin-α and β-actin probes (Figure 4.12). The migration distance was plotted against the logarithm of the transcript size (Figure 4.13) enabling the mRNA transcript sizes of the centaurin and β-actin bands to be determined.

The centaurin-α2 probe yielded two mRNA transcripts of 4.0 kb (major transcript) and 5.2 kb (minor transcript). These bands appeared to be ubiquitously expressed, although the abundance varied between tissues, with the highest levels observed in kidney and liver (Figure 4.12, A). Although the β-actin signal intensity appeared to be relatively uniform when examined by eye, computer analysis was used to accurately quantify the band density and the abundance of the 4.0 kb centaurin-α2 band was altered accordingly (Figure 4.14). Normalised data indicated centaurin-α2 expression to be most abundant in liver and with the levels decreasing in the following order, kidney > lung > heart > skeletal muscle > adipose tissue > brain > testis.

More than one transcript was detected using the centaurin-α probe, with the major transcript of 2.5 kb depicted by the extremely intense band in brain (Figure 4.12, B). This major band was also present, although considerably less abundant in kidney and lung as described previously. Interestingly, longer exposure times revealed the presence of this band in adipose tissue, heart and skeletal muscle. The centaurin-α probe also produced a 4.0 kb band that appeared to be ubiquitous, however the abundance pattern was different to that of the 2.5 kb transcript. Like the 2.5 kb transcript, the 4.0 kb transcript was most abundant in brain, it also appeared to be highly expressed in liver. This finding differs from that of the 2.5 kb transcript which has not been detected in liver, both in this study and those reported in the literature (Hammonds-Odie et al. 1996; Stricker et al. 1997; Venkateswarlu and Cullen 1999). A third band of approximately 3.1 kb was also produced by the centaurin-α probe. The distribution of this transcript appeared to follow that of the 2.5 kb band and was clearly visible in kidney, but in brain the band was not distinguishable as it is obscured by the broad and intense 2.5 kb band.
Figure 4.12. Northern blot analysis using DIG-labelled RNA probes. A multiple tissue northern blot was prepared using polyA⁺ RNA. (A) Centaurin-α2 (5′RACE product); (B) Centaurin-α; and (C) β-actin. Ad, adipose tissue; Br, brain; He, heart; Ki, kidney; Li, liver; Lu, lung; Mu, skeletal muscle; Sp, spleen and Te, testis. RNA molecular weight markers were separated alongside the samples and the sizes of the appropriate markers have been indicated.
Figure 4.13. *Migration curve of RNA molecular weight markers.* The logarithm of the transcript size was plotted against the migration distance (mm) for the RNA molecular weight markers. The size of mRNA transcripts was extrapolated for the samples on the northern blots.

Figure 4.14. *Graphical representation of rat centaurin-α2 tissue distribution.* Densometric analysis of the β-actin and centaurin-α2 signal intensities were used to calculate the normalised tissue distribution for the expression of the centaurin-α2 4.0 kb mRNA transcript. For each tissue the relative abundance of centaurin-α2 expression was expressed as a percentage of the maximum abundance value (liver). The values are the means ± S.E.M (n=2).
4.3 Western Blotting Analysis of the Tissue Distribution and Subcellular Localisation of the Centaurin-α2 Protein

In order to investigate the tissue distribution and subcellular localisation of the centaurin-α2 protein, anti-centaurin-α2 antibodies were raised. A fourteen amino acid peptide, corresponding to the C-terminus of the PH-C domain of rat centaurin-α2 (Figure 4.15), was coupled to ovalbumin and used to immunise two rabbits. First bleed antisera samples were taken 38 days after the initial immunisation and the final bleed samples after 94 days.

Preliminary western blot analysis examining the first bleed antisera was performed as described in Section 2.4.6, with the inclusion of 0.1% (v/v) Tween-20 in the TBS buffer. The antisera from both rabbits appeared to recognise numerous proteins ranging in size, which were absent from the pre-immune sera blots (Figure 4.16). The full-length sequence of centaurin-α2 was still unknown at this time, thus preventing prediction of the molecular weight, although this was expected to be similar to that of the centaurin-α1 proteins (42-46 kDa).

4.3.1 Affinity Purification of the Antisera

In an attempt to increase the specificity of the anti-centaurin-α2 antisera, affinity purification was performed using the immunising peptide coupled to a column matrix (Section 2.4.5). Western blots containing samples of the four subcellular fractions (20 μg protein) in duplicate were used to compare the antisera before and after purification (Figure 4.17). Affinity purification appeared to reduce the non-specific binding quite considerably, however several protein bands of varying sizes were still detected. The most prominent band detected by both antisera (rabbits A and B) at approximately 100 kDa was present in all subcellular fractions. In addition, antiserum B detected a cytosolic protein of approximately 35 kDa and two intense bands at 43 and 56 kDa in the PM and HDM fractions (Figure 4.17). These latter two bands were also detected with antiserum A, although the intensity was much weaker.
Figure 4.15. Amino acid sequence of centaurin-α2 illustrating the position of the immunising peptide. A fourteen amino acid peptide located towards the C-terminus of the PH-C domain of centaurin-α2 (CPTEKEQREWLESL) was coupled to ovalbumin and used for the immunisation of two rabbits. The ARF-GAP domain and PH domains are illustrated in different colours. The numbers of the amino acid residues are indicated on the right.

Figure 4.16. Western blot analysis of the first bleed anti-centaurin-α2 antisera. Multiple samples of adipocyte homogenate (20 μg) were resolved on a 10% SDS-PAGE gel and transferred to nitrocellulose membrane. Four identical membrane strips were blocked in 5% (w/v) Marvel in TBS-T followed by a 1 h incubation in the following primary antibody solutions: (A) first bleed antiserum from rabbit A, (A-Pi) preimmune serum from rabbit A, (B) and (B-Pi) from rabbit B. All four antiserum solutions were at a final dilution of 1:2,000 in 1% BSA-TBS-T. Detection with anti-rabbit IgG and ECL reagent was performed as described in Section 2.4.6. The positions of the molecular weight markers are shown.
Antiserum A

Final Bleed Ab Affinity Purified

PM HDM LDM CYT PM HDM LDM CYT

97 kDa
66 kDa
56 kDa
43 kDa
37 kDa
27 kDa

Antiserum B

Final Bleed Ab Affinity Purified

PM HDM LDM CYT PM HDM LDM CYT

116 kDa 97 kDa 66 kDa 56 kDa 43 kDa 37 kDa 27 kDa

Figure 4.17. Western blot comparing anti-centaurin-α2 antisera before and after affinity purification. The two anti-centaurin-α2 antisera were affinity purified using a column composed of the immunising peptide coupled to Reacti-Gel support matrix. Adipocyte subcellular fractions (20 µg) were resolved on duplicate 10% SDS-PAGE gels and transferred to nitrocellulose. PM, plasma membrane; HDM, high-density microsomes; LDM, low-density microsomes; CYT, cytosolic fraction. Western blot analysis was performed as described (Section 2.4.6) using the final bleed antisera (1:4,000 dilution) and the respective affinity purified antisera (1:1,000 dilution) in 1% BSA-TBS-T. The positions of the molecular weight markers are shown.
Identification of the full-length centaurin-α2 cDNA encoding the 396 amino acids allowed the predicted molecular mass to be calculated at 43.6 kDa, which was a very similar size to a band that was observed in the PM and HDM fractions (Figure 4.17). The absence of centaurin-α2 from the cytosolic fraction was quite unexpected as western blot analysis of centaurin-α and the centaurin-α1 proteins revealed its presence in both the soluble and membranous fractions (Hammonds-Odie et al. 1996; Stricker et al. 1997). The HES buffer used in the subcellular fractionation of adipocyte cells had a relatively low salt content (20 mM HEPES) which may have affected the centaurin-α2 distribution during the subcellular fractionation procedure. In order to investigate this hypothesis a KCl buffer (100 mM KCl, 20 mM NaCl, 10 mM HEPES/NaOH, pH 7.0, 1 mM EDTA) was used in place of the HES buffer during the fractionation of basal and insulin-stimulated adipocyte cells. The alteration to the buffer composition did not appear to affect the subcellular distribution of centaurin-α2. It was detected in the PM fractions and was absent from the both the LDM and cytosol (Figure 4.18). SDS-PAGE was performed using a large gel allowing greater resolution between protein bands and only the region approximately 10 kDa above and below the 45 kDa centaurin-α2 band was processed.

### 4.3.2 Optimisation of Western Blotting Conditions for Centaurin-α2 Antisera

A number of modifications were made to the western blotting protocol in an attempt to increase the binding specificity of the purified antisera. These included altering the blocking stage by replacing 5% (w/v) Marvel with varying dilutions of BSA, changing the dilutions of the primary and secondary antibodies and the solutions in which they were diluted. None of these alterations significantly improved the blotting results (data not shown). Finally the omission of Tween-20 from the TBS buffer at all stages of the western blot development was found to have a remarkable effect. Virtually all non-specific binding was removed, resulting in the detection of only a single protein band of approximately 45 kDa (Figure 4.19). All subsequent western blots were performed without the non-ionic detergent Tween-20 in the TBS buffer.
Figure 4.18. Western blot analysis of adipocyte subcellular fractions prepared using KCl buffer instead of HES buffer. Subcellular fractions were prepared from basal and insulin-stimulated adipocytes using KCl buffer (100 mM KCl, 20 mM NaCl, 10 mM HEPES/NaOH, pH 7.0, 1 mM EDTA) in place of HES buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, 255 mM sucrose). Fractions (20 µg) were resolved on a large 10% SDS-PAGE gel. Western blotting was performed using affinity purified antiserum B diluted 1:10,000 in 1% BSA-TBS-T. Crude PM, plasma membrane; LDM, low-density microsomes; and CYT, cytosolic fraction. The positions of the molecular weight markers are shown.

Figure 4.19. Effect of Tween-20 on western blotting with purified centaurin-α2 antisera. The adipocyte total particulate fraction was generated by centrifugation at 541,000 x g for 30 min. Duplicate samples (20 µg) were resolved on a small 10% SDS-PAGE gel. Following transfer to nitrocellulose, the blot was divided and the two halves processed in parallel. One blot was processed as normal (+Tw) and the second in the absence of Tween-20 from all stages (-Tw). Western blotting was performed using affinity purified antiserum B was diluted 1:1,000 in 1% BSA-TBS (+/-Tween-20). The positions of the molecular weight markers are shown.
4.3.3 Tissue Distribution of the Centaurin-α2 Protein

Homogenates from adipose tissue, brain, heart, kidney, liver, lung and skeletal muscle were separated into total particulate and cytosolic fractions (Section 2.5.3). These fractions (20 µg) were then separated on a large 10% SDS-PAGE gel. Centaurin-α2 was detected in the particulate fractions of all tissues tested (Figure 4.20) with highest abundance in heart, adipose tissue and skeletal muscle. Recombinantly expressed 6xHis-tagged centaurin-α2 (see Section 5.2) was resolved alongside the tissue subcellular fractions as a positive control. This protein has a hexa-histidine tag on the N-terminus making the molecular weight slightly larger than that of native centaurin-α2 at 45.7 kDa. Centaurin-α2 was not detected in any of the cytosolic fractions, even with extensive periods of exposure to film.

In adipose tissue, the total protein content isolated from the particulate and cytosolic fractions was calculated to have a ratio of approximately 1:1. However this ratio differs between tissues. Consequently the total protein was measured for the particulate and cytosolic fractions isolated from each tissue and the ratios calculated. A second western blot was performed with the protein of the particulate and cytosolic fractions loaded in the proportions representative of the cellular distribution of each tissue. Nevertheless, the centaurin-α2 band was still not detected in the any of the cytosolic fractions (data not shown).

4.3.4 Subcellular Localisation of Centaurin-α2 in Rat Adipocyte Subcellular Fractions

Subcellular fractions were previously used to examine the efficiency of antibody purification (Section 4.3.1) and although the quality of these blots was poor due to the inclusion of Tween-20, bands of approximately 43 kDa were still discernible (Figure 4.17). This band was present in the PM, HDM and LDM, but was not detected in the cytosolic fraction. However, when repeated without Tween-20 a weak centaurin-α2 band was evident in PM upon long exposure to film (Figure 4.21), and no signal was detected in the HDM, LDM and cytosol fractions. The positive control (recombinant centaurin-α2) was clearly visible and hence the detection reaction was successful.
Figure 4.20. Western blot analysis of the tissue distribution of centaurin-α2 protein expression. Total particulate and cytosolic fractions were prepared from tissue homogenates by centrifugation at 541,000 x g for 30 min. These fractions (20 μg) were resolved on a large 10% SDS-PAGE gel. Recombinant wild-type centaurin-α2 (WT; 250 ng) was resolved alongside the tissue samples providing an indication of size as well as being a positive control. Ad, adipose tissue; Br, brain; He, heart; Ki, kidney; Li, liver; Lu, lung; Mu, skeletal muscle. Western blotting was performed in the absence of Tween-20 using affinity purified antiserum B was diluted 1:500 in 1% BSA-TBS.

Figure 4.21. Western blot analysis of subcellular distribution of centaurin-α2 in rat adipocytes. Subcellular fractions were prepared as described in Section 2.5.2. The fractions (20 μg) were resolved on a small 10% SDS-PAGE gel. Western blotting was performed in the absence of Tween-20 using affinity purified antiserum B was diluted 1:500 in 1% BSA-TBS. PM, purified plasma membrane; HDM, high-density microsomes; LDM, low-density microsomes; CYT, cytosolic fraction. The positions of the molecular weight markers are shown.
As centaurin-α2 was abundant in the particulate fraction of adipocytes we expected this protein to be present in at least one of the membranous fractions generated by the subcellular fractionation. The absence of centaurin-α2 signal might be explained by protein degradation during the longer procedure required for the full subcellular fractionation. Alternatively centaurin-α2 could be displaced from the membrane and discarded during the purification of the plasma membrane fraction. To investigate these possibilities further, another adipocyte subcellular fractionation was performed, and every component was retained for analysis.

In all the previous adipocyte subcellular fractionations the pellet resulting from the sucrose cushion centrifugation (Figure 2.2, centrifugation step d) was discarded. However, this pellet was retained and resuspended in HES buffer. The supernatant was transferred to a new tube and processed as normal with the addition of more HES buffer to a final volume of 3 ml and centrifugation as described (Figure 2.2, centrifugation step e). The supernatant from this step was normally discarded and the pellet resuspended in HES buffer and centrifugation repeated. The resulting supernatant from the two centrifugation steps e and f (Figure 2.2) were combined and centrifuged for 30 min at 541,000 x g. The resulting pellet was resuspended in HES buffer. All fractions (20 μg protein) were resolved on a 10% SDS-PAGE gel for analysis by western blotting. Centaurin-α2 was detected in two fractions, the crude plasma membrane and the pellet recovered from the sucrose cushion centrifugation step, but not in the purified plasma membrane (Figure 4.22).

In order to examine the interaction of centaurin-α2 with components of the sucrose cushion pellet fraction, aliquots of this fractions were incubated in HES buffer containing a number of reagents, such as 1% Triton which is used to solubilise membranes. Incubation was performed on ice for 1 h (DNase sample incubated at 37°C) before centrifugation at 17,500 x g for 20 min. Centaurin-α1 proteins are known to bind Ins 1,3,4,5-P₄, similarly our investigations have shown Ins 1,3,4,5-P₄ to interact with the PH-N domain of centaurin-α2 (Section 5.4), thus Ins 1,3,4,5-P₄ was added to one of the samples at a final concentration of 10 μM. High salt concentration (1M NaCl) was also tested as this was reported to dissociate porcine p42P4 from the membrane fraction (Stricker et al. 1997). Histocytochemical evidence and confocal microscopy indicated that PIP3-BP and human centaurin-α1 are expressed in the
nucleus and cytosol (Tanaka et al. 1999; Venkateswarlu and Cullen 1999). To investigate the possible DNA interaction of centaurin-α2, DNaseI in 5 mM MgCl₂ was added. The western blot showed that 1% Triton caused dissociation of centaurin-α2 from the sucrose cushion pellet fraction (Figure 4.23), however the other conditions tested had no detectable effect.

4.3.5 Comparison of β-tubulin and Glut4 Subcellular Localisation with that of Centaurin-α2

The first of the confocal microscopy images using the centaurin-α2 antibody revealed a staining pattern similar to that reported for microtubules in cardiomyocytes (Park et al. 1984) (Section 4.4; Figure 4.26). To ascertain the distribution of the microtubules in adipocyte subcellular fractions, western blot analysis was performed using monoclonal anti-β-tubulin antibodies. The intense β-tubulin band in the cytosolic fraction might result from the detection of unpolymerised monomers (Figure 4.24).

The distribution of Glut4 in adipocyte subcellular fractions has been well documented (Weber et al. 1988), however we used anti-Glut4 antibodies to test for the presence of this integral membrane protein in the sucrose cushion pellet. Plasma membrane samples isolated from basal and insulin-treated adipocytes were included as controls to demonstrate the increase in Glut4 at the PM following insulin-stimulation (Figure 4.25). A concurring decrease of Glut4 in the LDM would normally be observed, however the HDM, LDM and cytosol fractions were not separated in order to reduce the time required for the fractionation procedure. The 45 kDa Glut4 band was not detected in the sucrose cushion pellet fraction on the film shown (Figure 4.25), but a faint band was visible on an overexposed film (data not shown). An intense Glut4 band was detected in the wash pellet fraction. However, it is important to note that an equal amount of protein (20 µg) was loaded for each fraction on the gel, hence the protein ratios are not representative of cellular conditions.
Figure 4.22. All components of the adipocytes subcellular fractionation were analysed using western blot analysis with anti-centaurin-α2. Rat adipocytes were fractionated in the normal way (Section 2.5.2). In addition to the four subcellular fractions (PM, purified plasma membrane; HDM, high-density microsomes; LDM, low-density microsomes; CYT, cytosol) an aliquot of the crude plasma membrane pellet (cPM), the sucrose cushion pellet (SP) and the pellet from the plasma membrane washes (WP) were retained. Cellular fractions (20 μg) and 250 ng recombinant wild-type centaurin-α2 were resolved on a 10% SDS-PAGE gel. Western blotting was performed in the absence of Tween-20 using affinity purified antiserum B was diluted 1:500 in 1% BSA-TBS. The positions of the molecular weight markers are shown. Results are from a single experiment.

Figure 4.23. Analysis of the sucrose cushion pellet. The interaction of centaurin-α2 with the sucrose cushion pellet was assessed by incubating the resuspended pellet with different reagents. (1) control, using water in placed of reagent, (2) 10 μM Ins 1,3,4,5-P4, (3) 1M NaCl, (4) 1% Triton and (5) DNaseI in 5 mM MgCl₂. These reagents were added to the protein fraction to give the final concentrations indicated. The reactions were incubated on ice for 1 h, with the exception of reaction 5 which was incubated at 25°C. All reactions were centrifuged at 17,500 x g for 20 min and the pellet was resuspended in HES buffer to a final volume of 50 μl. The supernatant, pellet fractions and 250 ng recombinant wild-type centaurin-α2 were resolved on a 10% SDS-PAGE gel. Western blotting was performed in the absence of Tween-20 using affinity purified antiserum B was diluted 1:500 in 1% BSA-TBS. The positions of the molecular weight markers are shown. Results are from a single experiment.
Figure 4.24. Western blot analysis of β-tubulin in adipocytes subcellular fractions. Rat adipocytes were fractionated in the normal way (Section 2.5.2). The four subcellular fractions (PM, HDM, LDM and cytosol) and the additional fractions (crude PM pellet; sucrose cushion pellet; wash pellet from the plasma membrane washes) were resolved on a 10% SDS-PAGE gel (20 μg of each fraction). Western blotting was performed using β-tubulin monoclonal antibody diluted 1:1,000 in 0.2% BSA-TBS. The positions of the molecular weight markers are shown.

Figure 4.25. Western blot analysis of Glut4 distribution in rat adipocytes subcellular fractions. The plasma membrane (PM) samples from the basal and insulin-stimulated adipocytes shown the increased levels of Glut4 in the insulin-stimulated PM sample. A separate subfractionation of basal adipocytes was performed and omitting centrifugation steps b and c so that the HDM, LDM and cytosol reamined in a single fraction (Figure 2.4). A sample of the crude plasma membrane was retained. The sucrose cushion pellet was investigated as was the wash pellet from the purification of the plasma membrane. All fractions (20 μg) were resolved on a 10% SDS-PAGE gel. Western blotting was performed using affinity Glut4 antibody diluted 1:4,000 in 1% BSA-TBS-T. The positions of the molecular weight markers are shown.
4.4 Subcellular Localisation of Centaurin-α2 in Rat Cardiomyocytes as Revealed by Confocal Microscopy

Due to its high level of expression in heart, the subcellular localisation of centaurin-α2 in isolated cardiomyocytes was investigated using immunofluorescence microscopy. In preliminary experiments cardiomyocytes were fixed by incubation in 4% paraformaldehyde (Section 2.4.7), followed by permeabilisation with 0.1% saponin. Cells were incubated with purified rabbit anti-centaurin-α2 antibodies for 1 h, and then washed extensively before incubation with fluorescein-conjugated anti-rabbit antibodies. Following this incubation the cells were washed again, mounted and viewed using a Zeiss laser scanning microscope. Control samples, with the omission of the primary antibodies, were performed in parallel to examine the non-specific staining of the secondary antibodies (data not shown). The settings were optimised to yield distinct staining for the centaurin-α2 antibody and all subsequent images were captured using these settings. The depth of field at which the image was captured was also kept constant so that the image contained the sectional plane of the cell with the nuclei clearly visible. Over 80% of rat cardiomyocytes are binucleate (Dow et al. 1981), and the two nuclei are clearly visible in the cells visualised using the transmissible lens (Figure 4.26, A). No centaurin-α2 staining was detected in the nuclei, which were clearly discernible as two black circular areas (Figure 4.26, B). Centaurin-α2 displayed punctate staining in a pattern that followed the striations of the myofibrils and was concentrated in the region extending longitudinally from the poles of the nuclei through the length of the cell.

4.4.1 Dual Labelling of Centaurin-α2 and β-tubulin

To further investigate the possible interaction with microtubules, the distribution of centaurin-α2 and β-tubulin in cardiomyocytes was analysed by confocal microscopy. Dual labelling was performed with monoclonal mouse anti-β-tubulin antibodies and the purified anti-centaurin-α2 antiserum. Rhodamine-conjugated anti-mouse antibodies were used to detect the β-tubulin antibodies. Examination of the cells revealed the β-tubulin to be concentrated at the periphery of the cell (Figure 4.27, A and B), and not
Figure 4.26. **Indirect immunofluorescence analysis of centaurin-α2 distribution in cardiomyocytes.** Isolated rat cardiomyocytes were fixed in 4% paraformaldehyde and permeabilised with 0.1% saponin. Cells were incubated with rabbit anti-centaurin-α2 antibodies (affinity purified antiserum B, 1:60 final dilution) for 1 h, which was then detected using fluorescein-conjugated anti-rabbit polyclonal antibodies. (A) Image obtained with the transmissible lens; and (B) anti-centaurin-α2 staining. The two nuclei are shown.
Figure 4.27. Indirect immunofluorescence analysis of centaurin-α2 and β-tubulin distribution in cardiomyocytes. Isolated rat cardiomyocytes were fixed in 4% paraformaldehyde and permeabilised with 0.1% saponin. Cells were incubated with rabbit anti-centaurin-α2 antibodies (affinity purified antiserum B, 1:60 final dilution) and monoclonal anti-β-tubulin antibodies (1:220 final dilution). The primary antibodies were detected using anti-rabbit and anti-mouse polyclonal antibodies coupled to fluorescein and rhodamine, respectively. (A) Cross section through the centre of the cell, two nuclei visible; (B) section at the surface of the same cell. The results shown are representative of 10 cells selected at random.
located throughout the cell as expected. Changing the permeabilisation conditions from 0.1% saponin to incubation in methanol at -20°C (Section 2.4.7) appeared to improve the penetration of the β-tubulin antibodies, without affecting the staining of the centaurin-α2 antibodies. Although the pattern of β-tubulin staining observed was very similar to that of centaurin-α2, the dual labelling showed only limited co-localisation (Figure 4.28, A and B). No staining was detected in the negative control that tested the non-specific binding of the two secondary antibodies (Figure 4.28, C and D).

### 4.4.2 The Effect of Insulin-Stimulation and Colchicine Treatment on the Distribution of Centaurin-α2

Previous confocal immunofluorescence studies performed on cardiomyocytes in our laboratory by Dr A Gillingham, revealed that in the basal state, Glut4 was located in latitudinal striations with intense staining concentrated in the perinuclear region (TGN). Although both Glut4 and centaurin-α2 staining patterns are punctate, the distributions appear to be quite different. Nevertheless the abundance of centaurin-α2 in insulin-responsive tissues (Section 4.3.3) prompted the investigation of insulin-stimulation on centaurin-α2 distribution. In addition the microtubule depolymerising drug, colchicine, was used to investigate the effect of microtubule network disruption on centaurin-α2 distribution.

Cardiomyocytes were isolated, left to recover and divided into two aliquots, one fraction remained untreated while the other was treated with 2 μM colchicine and both were incubated at 37°C for 30 min. The two cell suspensions were further divided, one pool from each was left untreated (basal) and the other was stimulated with 30 nM insulin for a further 30 min. The four cell suspensions (basal cells ± colchicine treatment and insulin-stimulated cells ± colchicine treatment) were examined using anti-centaurin-α2 and anti-β-tubulin antibodies (Figure 4.29).

All images were collected using identical settings on the laser microscope to enable direct comparison between the different treatment conditions. Upon comparison of a number of cells from each condition it was clear that there was no distinct difference in
Isolated rat cardiomyocytes were fixed in 4% paraformaldehyde and permeabilised by incubation in methanol at -20°C for 5 min (Section 2.4.7). The cells were then washed and incubated with rabbit anti-centaurin-α2 antibodies (affinity purified antiserum B, 1:60 final dilution) and monoclonal anti-β-tubulin antibodies (1:220 final dilution). The primary antibodies were detected using anti-rabbit and anti-mouse polyclonal antibodies coupled to fluorescein and rhodamine, respectively. (A) Dual labelling; (B) higher magnification (as indicated by the scale bar); (C) control sample with no primary antibodies performed to examine the non-specific staining of the secondary antibodies, (D) same cell as (C) observed with the transmissible lens. The results shown are representative of at least five cells selected at random.
Figure 4.29. Indirect immunofluorescence analysis of centaurin-α2 and β-tubulin distribution following insulin-stimulation and colchicine treatment. Isolated cardiomyocytes were divided into two cell suspensions, one was left untreated (A and B) and the other was treated with 2 μM colchicine (C and D) for 30 min. The two sets of cells were further divided. One sample from each set was left untreated (basal; A and C) and the other stimulated with 30 nM insulin (B and D) for an additional 30 min. The four groups of cardiomyocytes were washed and fixed as normal (Section 2.4.7). The cells were incubated with rabbit anti-centaurin-α2 antibodies (affinity purified antiserum B, 1:60 final dilution) and monoclonal anti-β-tubulin antibodies (1:220 final dilution). Primary antibodies were detected using anti-rabbit and anti-mouse polyclonal antibodies coupled to fluorescein and rhodamine, respectively. (A) Basal; (B) insulin-stimulated; (C) basal and colchicine-treated; (D) insulin-stimulated and colchicine-treated. The results shown are representative of at least 10 cells selected at random.
(C) Basal and colchicine-treated

(i) ![Image of basal and colchicine-treated cell](image1)

(ii) ![Image of basal and colchicine-treated cell](image2)

(D) Insulin-stimulated and colchicine-treated

(i) ![Image of insulin-stimulated and colchicine-treated cell](image3)

(ii) ![Image of insulin-stimulated and colchicine-treated cell](image4)
the localisation of centaurin-α2 in the basal and the insulin-stimulated cells (*Figure 4.29, A and B*). Some of the insulin-stimulated cardiomyocytes appeared to have more intense centaurin-α2 staining, particularly concentrated in the perinuclear region. However this finding was not indicative of all cells and was not reproducible in repeat experiments.

As expected, colchicine appeared to disrupt the microtubule network quite considerably, reducing the intensity of β-tubulin staining throughout the cell. However, the strong staining surrounding the nuclear membrane remained fairly intense (*Figure 4.29, C and D*). The level of centaurin-α2 and β-tubulin staining varied notably between cells. Hence, the selection of a single cardiomyocyte as a representative of the cell population was difficult. Consequently two cells of differing signal intensities were selected for the colchicine treated cardiomyocytes.

In the cells with very weak β-tubulin staining a concomitant reduction in centaurin-α2 signal was observed, for both the basal and insulin-stimulated cardiomyocytes (*Figure 4.29, Ci and Di*). In these cells, little or no centaurin-α2 staining was present in the region between the nuclei.
cDNA libraries from a number of tissues were utilised in the generation of the full-length centaurin-α2 sequence. The partial centaurin-α2 clone was isolated from an adipocyte library, whereas a liver library was used for 5′RACE and the full-length clone was amplified from adipocyte and brain libraries. Consequently the tissue distribution of centaurin-α2 was expected to be more widespread than that observed for centaurin-α and centaurin-α1 (Hammonds-Odie et al. 1996; Tanaka et al. 1997; Venkateswarlu and Cullen 1999). Our prediction was confirmed with the ubiquitous expression of the centaurin-α2 mRNA transcripts.

The major mRNA transcript (4.0 kb) was larger than that expected for the size of the centaurin-α2 cDNA clone. The size was considerably greater than the 2.5 kb centaurin-α transcript and was more befitting to that expected for the larger multidomain centaurin proteins of the centaurin-β, -δ and -γ families. The 4.0 kb centaurin-α2 transcript was detected using both the radioactively-labelled partial clone (Figure 4.6, A) and the RNA probe (encoding the 5′RACE product) with the DIG detection system (Figure 4.12, A). In addition the presence of an in-frame stop codon in the human centaurin-α2 cDNA, located 5′ of the proposed ATG start codon, strengthened the predicted position of the start codon. Thus, there was no clear explanation for the unexpected transcript size apart from the presence of a large 5′ untranslated region that was absent from the centaurin-α2 cDNA clone.

Interestingly a 4.0 kb minor transcript was detected in brain when using the centaurin-α probe, as previously reported by Theibert and colleagues (Hammonds-Odie et al. 1996). Although the centaurin-α and centaurin-α2 4.0 kb bands were superimposable in brain, they displayed distinct tissue distributions and therefore presumably do not result from cross-hybridisation of the two probes. In addition to the 2.5 and 4.0 kb transcripts already reported for centaurin-α (Hammonds-Odie et al. 1996), our findings indicated an additional minor transcript of approximately 3.1 kb (Figure 4.12, B). Minor transcripts were not reported for human centaurin-α1, which was detected as single 2.5 kb transcript in brain (at a relatively high level) and peripheral blood leukocytes (Venkateswarlu and Cullen 1999). However, on examination of the published rat
centaurin-α northern blot (Hammonds-Odie et al. 1996), the 2.5 kb band was rather diffuse and might represent unresolved the 2.5 kb and 3.1 kb transcripts.

Although the signal intensity of different probes can not be compared directly, it should be noted that the centaurin-α2 signal was considerably weaker than centaurin-α. This result was seen using the probes from both detection systems. Thus, centaurin-α2 might be expressed at relatively low levels, which could explain the difficulty in detecting the transcript from total RNA.

In contrast to the tissue distribution of mRNA, western blot analysis suggested that centaurin-α2 protein was highest in the insulin-sensitive tissues of heart, adipose tissue and skeletal muscle, with similar levels of expression also present in kidney. Consistent with the northern blot results, western blot analysis revealed centaurin-α2 expression in all of the tissues examined, although long exposure period was required for detection in lung. Due to the high abundance in brain, centaurin-α and centaurin-α1 were considered to be brain specific. However, Theibert and colleagues reported a 46 kDa protein (centaurin-α or related protein) that was specifically photolabelled with \([^3H]BZDC-Ins 1,3,4,5-P_4\) in the heparin-agarose fractions from the soluble fraction of rat heart (Hammonds-Odie et al. 1996). This protein was absent from the detergent-extracted membrane fractions. This was the first evidence of a centaurin-α or related protein in non-neuronal tissues. However, it is unlikely that this photlabelled band was centaurin-α2, as our western blot studies were unable to detect centaurin-α2 in the soluble fraction.

Normally the non-ionic detergent Tween-20 is added to the TBS buffer at a final concentration of 0.1% to reduce non-specific, low-affinity binding of antibodies during western blot analysis. Antibodies specifically recognise epitopes and bind to the antigen via non-covalent and hydrophobic interactions. Tween-20 appeared to affect the binding specificity of anti-centaurin-α2. It is plausible that this detergent disrupts the paratopes of the antibodies, thus increasing the breadth of specificity of this primary antibody. However, the mechanisms of this occurrence remain ambiguous. Nevertheless, in the absence of Tween-20 the antibody appeared to be highly specific recognising a single protein band of the correct size from total cell homogenates and the recombinant 6xHis-centaurin-α2.
Surprisingly, western blot analysis of adipocyte subcellular fractions revealed centaurin-α2 to be concentrated in the pellet at the base of the sucrose cushion. The distribution of the major cellular organelles among the adipocyte subcellular fractions have been reported and this pellet was found to consist of mitochondria, nuclei and cell debris (Simpson et al. 1983). Following incubation with the non-ionic detergent Triton (1%), which solubilises cellular membranes, centaurin-α2 was detected in the soluble fraction. Conditions of high ionic strength and DNase I and Ins 1,3,4,5-P4 addition did not alter the distribution of centaurin-α2. These findings indicated that centaurin-α2 was not retained in this fraction via ionic interaction, association with DNA or by Ins 1,3,4,5-P4 binding. Membrane solubilisation released the centaurin-α2 and therefore a nuclear or mitochondrial localisation is plausible. The initial confocal microscopy results dismiss the possibility of nuclear localisation and imply a possible association with cytoskeletal elements such as microtubules. The pattern of centaurin-α2 staining resembles the description of that of microtubules in cardiomyocytes which are interspersed between the myofibrils and encircle the nucleus (Park et al. 1984). In most animal cells, microtubules radiate from a prominent centrosomal focus, the microtubule organising centre. However, striated muscle, such as skeletal muscle and cardiomyocytes, possess a circumnuclear distribution of the microtubule-organising centre as seen in Figure 4.29 (Kronebusch and Singer 1987). The Golgi is reported to share this circumnuclear distribution and is usually concentrated at the ends or ‘poles’ of the elongated nucleus (Rambourg et al. 1984). However, there was no evidence from the western blot results to indicate that β-tubulin fractionates with centaurin-α2. Furthermore very little co-localisation of centaurin-α and β-tubulin was observed in the dual-labelling studies. Nevertheless, colchicine treatment did appear to reduce the intensity of centaurin-α2 staining along with that of β-tubulin.

It should be noted however, that the specificity of the anti-centaurin-α2 antisera has not been verified. It is possible that the centaurin-α2 antisera could interact with other proteins of similar size to centaurin-α2. The results of the western blot experiments described earlier suggest that the centaurin-α2 antisera does not cross-react with centaurin-α or centaurin-α1, as bands of this size were not detected in the cytosolic fractions. Nevertheless it cannot be assumed that the centaurin-α2 antisera is specific and therefore western blotting and confocal microscopy results described herein may
not represent the true properties of endogenous centaurin-α2. Further characterisation of the antisera would be useful. For example, if another antibody raised to a distinct region of centaurin-α2 was available it might be possible to immunoprecipitate centaurin-α2 with one antibody and use the second antibody for verification of the protein. Alternatively it may be beneficial to perform immunofluorescence microscopy studies using cell lines transiently transfected with constructs expressing tagged-centaurin-α2.

The various groups that have analysed the distribution for centaurin-α and centaurin-α1 and found them to be both in cytosolic and membrane associated. However, on closer examination of the methods employed, comparison with our results for centaurin-α2 reveal some interesting similarities and distinctions. Western blot analysis indicated that p42\textsubscript{IP4} was cytosolic and membrane associated, with a ratio of 75% of the binding activity of the brain homogenate was present in the particulate fraction and the remaining 25% in the soluble fraction (Strieker \textit{et al.} 1997). However, the preparation of membranes from pig cerebella homogenate involved four sequential centrifugation steps at 100,000 x g for 30 min each. It can therefore be assumed that the nuclei, mitochondria and cell debris were present in this membrane pellet. Similarly in the assessment of rat centaurin-α partitioning, the membrane-associated fraction generated from a single centrifugation step at 45,000 x g for 15 min would also contain the nuclei, mitochondria and cell debris (Hammonds-Odie \textit{et al.} 1996). Interestingly use of histocytochemical analysis led to detection of PIP3-BP in the nucleus of neuronal cells (Tanaka \textit{et al.} 1999). In support of this finding, the same group showed that in COS-7 cells transfected with constructs for expression of PIP3-BP and constitutively active PtdIns 3-kinase, PIP3-BP was found in the membrane and cytosolic fractions. Whereas in the absence of the active PtdIns 3-kinase, PIP3-BP was located in the nucleus (Tanaka \textit{et al.} 1999). Subsequently, confocal microscopy indicated a similar distribution pattern for human centaurin-α1 (Venkateswarlu and Cullen 1999).

A number of phospholipids including PtdIns 4-P and PtdIns 4,5-P\textsubscript{2} have been detected in the nuclear matrix and membrane along with the enzymes required for synthesising and metabolising these compounds. Interestingly PtdIns 3,4,5-P\textsubscript{3} production has also been detected by labelling studies in isolated nuclei (reviewed in D'Santos \textit{et al.} 1998). Furthermore, CKI\textalpha has been found to be associated with cytosolic vesicles, the mitotic
spindle and structures within the nucleus (Gross and Anderson 1998). Thus, although there is growing evidence to support a possible function for the centaurin-α1 proteins in the nucleus, this does not appear to be analogous with centaurin-α2. Furthermore, in contrast to centaurin-α1, the subcellular localisation of centaurin-α2 did not appear to be affected by insulin-stimulation. So despite the conclusion that centaurin-α1 and centaurin-α2 have distinct subcellular distributions, the precise localisation of centaurin-α2 remains unresolved.
5.0 INVESTIGATION OF THE BINDING SPECIFICITY OF THE CENTAURIN-α2 PH DOMAINS

5.1 Introduction

In the endeavour to understand the cellular functions of the centaurin-α proteins, several experimental approaches have been used to investigate the binding specificity of their PH domains. For instance the benzophenone photoaffinity label [3H]BZDC-Ins 1,3,4,5-P₄ was employed by Theibert and colleagues to efficiently label a 46 kDa protein in fractionated brain extracts (Hammonds-Odie et al. 1996). Binding specificity was determined using displacement of the photoaffinity label with increasing concentrations of unlabelled inositol phosphate or phosphoinositide. Rat centaurin-α was shown to have highest affinity for Ptdlns 3,4,5-P₃, whereas Ins 1,3,4,5-P₄ and PtdIns 4,5-P₂ bound with 5- and 12-fold lower affinity, respectively (Hammonds-Odie et al. 1996). An alternative approach, the Ins 1,3,4,5-P₄ displacement assay, was used to examine the binding specificity of native and recombinant p42IP₄ (Hanck et al. 1999; Strieker et al. 1997) and GST-tagged centaurin-α1 (Venkateswarlu and Cullen 1999). The PtdIns 3,4,5-P₃ analogue column used for the purification of PIP3-BP was also utilised to investigate its binding specificity (Tanaka et al. 1997). The binding was efficiently blocked using PtdIns 3,4,5-P₃, but not with Ins 1,3,4,5-P₄ or PtdIns 4,5-P₂. This finding differs from the other centaurin-α1 proteins, which were found to have high affinity for both Ins 1,3,4,5-P₄ and PtdIns 3,4,5-P₃ (Strieker et al. 1997; Venkateswarlu and Cullen 1999).

Mutagenesis studies using PIP3-BP indicated that both PH domains are required for binding to the PtdIns 3,4,5-P₃ analogue resin (Tanaka et al. 1997). A conserved arginine residue corresponding to that required for PtdIns 3,4,5-P₃ binding in the Btk PH domain was replaced with a cysteine in each of the PH domains of PIP3-BP. Mutation of the equivalent arginine residue in the PH domain Gap1ᵐ greatly reduced binding of Ins 1,3,4,5-P₄ (Fukuda and Mikoshiba 1996). More recently, the PtdIns 3,4,5-P₃ dependent recruitment of GFP-centaurin-α1 to the plasma membrane was reduced by mutagenesis of this conserved arginine residue in a single PH domain, while
mutation of both PH domains resulted in complete inhibition (Venkateswarlu et al. 1999b; Venkateswarlu and Cullen 1999).

Thus far, centaurin-α2 appears to have distinct tissue distribution and subcellular localisation from that of the centaurin-α1 proteins and it will be interesting to see if these differences are also observed with the binding properties. In order to investigate the binding characteristics of rat centaurin-α2, recombinant protein was expressed using the pET15b expression system (Figure 5.1). A 6xHis tag was incorporated onto the N-terminus, which enabled simple purification using the Talon Metal Affinity Resin. Single point mutations (Arg→Cys) were introduced into the PH domains of centaurin-α2 using site-directed mutagenesis. These mutated proteins were then utilised in Ins 1,3,4,5-P4 competition studies and BZDC-PtdInsPn photolabelling experiments to aid investigation of the centaurin-α2 PH domains.

5.2 Production of Recombinant Centaurin-α2

5.2.1 Cloning of Centaurin-α2 into pET15b

It was not possible to directly subclone centaurin-α2 DNA from pT7Blue (cloning vector) into the pET15b expression vector. Despite the presence of NdeI and BamHI sites in suitable positions in both pET15b and pT7Blue, the insert was in the wrong orientation for the expression of centaurin-α2. Consequently PCR primers were designed to amplify centaurin-α2 and incorporate NdeI and XhoI sites at the 5’ and 3’ ends, respectively (Figure 5.1, C). Amplification was performed using Advantage DNA polymerase and the following cycle parameters; an initial step of 94°C for 30 sec, 30 cycles of 94°C for 30 sec, 60°C for 1 min and 72°C for 4 min, with a final elongation step at 72°C for 10 min. Restriction digestion of the amplified cDNA and the pET15b vector, using NdeI and XhoI, generated compatible ends for ligation using T4 DNA ligase. The ligation reactions were then transformed into Singles (competent cells from the pT7Blue cloning kit). Recombinant clones were selected from which overnight cultures were prepared and the purified DNA was then analysed by restriction digestion with NdeI and XhoI to confirm the presence of an insert of the correct size (data not shown).
Figure 5.1. The cloning of rat centaurin-α2 into the pET15b expression vector. (A) Vector map of pET15b; (B) pET15b cloning/expression region; and (C) the sequences of the primers used to amplify and incorporate the NdeI site (CA'TATG) and XhoI site (GAGCTC) onto the 5’ and 3’ ends of centaurin-α2. The start methionine residue (ATG) and the stop codon (antisense of TGA) are shown in bold. The vector map and cloning region diagrams were reproduced with the kind permission of Novagen, Inc.
5.2.2 Introduction of Point Mutations in the cDNA

The conserved arginine of the PH-N and PH-C domains of centaurin-α2 (residues 151 and 275) were mutated to cysteine residues using the method employed by the QuikChange Site-Directed Mutagenesis Kit (Section 2.2.6; Figure 5.2, A). Oligonucleotide primers, each complementary to opposite strands of the double-stranded centaurin-α2 DNA were designed to contain the desired point mutations, R151C and R275C (Figure 5.2, B). The purified pET15b-centaurin-α2 recombinant plasmid was used as template DNA in the PCR reaction. Each of the primers annealed to the template and was extended during thermal cycling using PfuTurbo DNA polymerase. Following temperature cycling, treatment with DpnI digested the parental strand leaving the mutated plasmid. The amplified DNA was examined by agarose gel electrophoresis revealing a band of the expected size (approximately 6.9 kb; Figure 5.3). Following transformation into XL1-Blue competent cells the nicks in the mutated plasmid are repaired by the E.coli cells.

Plasmid DNA was purified and sequenced to confirm the introduction of the desired point mutation. A fraction of the R151C plasmid DNA was then subjected to a second round of mutagenesis (R275C) to generate centaurin-α2 containing point mutations in both PH domains (R151C/R275C).

5.3 Expression and Purification of Recombinant 6xHis-Centaurin-α2 Proteins

The plasmid DNA of the four centaurin-α2 pET15b constructs, wild-type (non mutated), R151C (arginine→cysteine mutation in PH-N domain), R275C (arginine→cysteine mutation in PH-C domain) and R151C/R275C (arginine→cysteine mutation in both PH domains) was purified. These constructs were then transformed into the E.coli strain BL21(DE3)pLysS. These cells contain a chromosomal copy of the gene for T7 RNA polymerase that is under the control of a promoter inducible by IPTG. Growing cultures were treated with IPTG, thus inducing T7 RNA polymerase, which in turn transcribed the target DNA in the pET15b vector (Section 2.4.8).
Figure 5.2. Site-directed mutagenesis of centaurin-α2. (A) Overview of the site-directed mutagenesis process. Oligonucleotides containing the target mutation anneal to the plasmid DNA. Using the non-strand displacing action of PfuTurbo DNA polymerase, extension and incorporation of the mutagenic oligonucleotides results in nicked circular DNA. The methylated non-mutated DNA template was digested with DpnI. Following transformation into XL1-Blue competent cells the nicks in the mutated plasmid were repaired. (B) The sense and antisense oligonucleotide primers that contain the desired point mutations for the PH-N (R151C) and PH-C (R275C) domains of centaurin-α2. The mutated residues are indicated in red.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Orientation</th>
<th>Oligonucleotide Sequence</th>
</tr>
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<tbody>
<tr>
<td>R151C</td>
<td>Sense</td>
<td>C CAG TTC CTG AGG TGC AGG TTT GTC CTT CTG TCA AGG Q F L R C R F V L L S R</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>CCT TGA CAG AAG GAC AAA CCT GCA CCT CAG GAA CTG G</td>
</tr>
<tr>
<td>R275C</td>
<td>Sense</td>
<td>CCC TTC AAG AAA TGC TGG TTT GCC TTG GAT CC P F K K C W F A L D</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>GG ATC CAA GGC AAA CCA GCA TTT CTT GAA GGG</td>
</tr>
</tbody>
</table>
Figure 5.3. Agarose gel electrophoresis of site-directed mutagenesis products. Site-directed mutagenesis amplification using oligonucleotide primers containing the desired mutation. R151C (arginine→cysteine mutation in PH-N domain), R275C (arginine→cysteine mutation in PH-C domain) and R151C/R275C (arginine→cysteine mutation in both PH domains). Molecular weight markers (MW) were resolved alongside the samples and the sizes of the appropriate bands are indicated.
The recombinant proteins were then purified from the cleared bacterial cell lysates using Talon Metal Affinity Resin (*Section 2.4.8*), which consists of electropositive transition metal ions linked to a Sepharose resin via a metal chelator. Histidine residues exhibit highly selective binding that can be reversed with the addition of an elution buffer containing imidazole, which has a structure identical to the side chain of histidine.

Wild-type centaurin-α2 was the first protein to be expressed and purified. Samples were retained from each purification step for examination on SDS-PAGE gels stained with Coomassie Blue. The fractions that were analysed included the supernatant and pellet from the bacterial cell lysate, supernatant following incubation with the Talon resin, 10 mM imidazole wash and the five 1 ml eluted fractions (data not shown). The majority of centaurin-α2 appeared to be present in the soluble fraction and not in inclusion bodies, thus evading the need for denaturing purification conditions.

Subsequently all four centaurin-α2 constructs were expressed and purified, and the eluted fractions and 10 mM imidazole wash fraction were examined by SDS-PAGE and Coomassie staining (*Figure 5.4*). For all four proteins the 10 mM imidazole wash appeared to successfully remove the weak binding non-specific proteins and a single band at approximately 45 kDa was present in the eluted fractions. The majority of the 45 kDa protein appears to be eluted in the first two 1 ml fractions. Consequently the first three fractions were pooled and placed inside Slide-A-Lyser cassettes and dialysed overnight in lysis buffer. Spectrophotometric analysis (A$_{280}$) for the determination of protein yield revealed that approximately 2 mg of each centaurin-α2 protein per litre of culture were purified. The proteins were then stored in 50% (v/v) glycerol at -20 °C.

Following the addition of glycerol the protein levels of the four centaurin-α2 proteins were compared by SDS-PAGE and staining with Coomassie Blue. Protein molecular weight markers of known concentration were run along side the protein samples to aid comparison of concentration as well as size (*Figure 5.5, A*). Purified 6xHis-centaurin-α2 was detectable as a single 45 kDa band using both monoclonal anti-pentaHis and the affinity purified anti-centaurin-α2 antiserum, thus confirming unequivocally the identity of the recombinant protein (*Figure 5.5, B and C*).
Figure 5.4. SDS-PAGE analysis of the recombinant centaurin-α2 proteins. The four 6xHis-centaurin-α2 proteins (wild-type and the three mutated proteins R151C, R275C and R151C/R275C) were purified from the cleared bacterial cell lysate using Talon Metal Affinity Resin. Weakly binding proteins were washed from the column using 10 mM imidazole in lysis buffer (5 bed volumes, 10 ml). The 6xHis-tagged proteins were then eluted using 100 mM imidazole in lysis buffer. Fractions of 1 ml were collected (eluate 1-3). 10 mM imidazole wash and the fractions (20 μl of each) were analysed on a 10% SDS-PAGE gel stained with Coomassie brilliant blue. Molecular weight markers (MW) were run alongside the samples, with appropriate band sizes indicated.
Figure 5.5. SDS-PAGE analysis of 6xHis-centaurin-α2. (A) Approximately 20 mg of the 6xHis-centaurin-α2 proteins (in 50% (v/v) glycerol) were resolved on a 10% SDS-PAGE gel. This was then stained with Coomassie brilliant blue to allow comparison of the protein quantities. (B) The supernatant following incubation with the Talon resin (unbound fraction) and the eluted purified protein were resolved on a 10% SDS-PAGE gel. Western blotting was performed using affinity purified anti-centaurin-α2 (serum B) diluted 1:2,000 in 1% BSA-TBS-T. (C) Cell lysate supernatant and the eluted purified protein were resolved on a 10% SDS-PAGE gel. Western blotting was performed using a monoclonal anti-pentaHis antibody diluted 1:2,000 in 1% BSA-TBS-T. Molecular weight markers (MW) were run alongside the samples, with appropriate band sizes indicated.
5.4 Investigation of the Binding Specificity of Centaurin-\(\alpha_2\) using \[^3\text{H}\]\text{Ins} 1,3,4,5-\(P_4\)

Long before the cloning of p42\(^{IP}\), Reiser and coworkers had detected \[^3\text{H}\]\text{Ins} 1,3,4,5-\(P_4\) binding in pig cerebellar membrane fractions (Donié and Reiser 1989). The \text{Ins} 1,3,4,5-\(P_4\) binding assay was adapted over time and was recently used to determine the binding specificity of recombinant p42\(^{IP}\) (Hanck \textit{et al}. 1999). In brief, 70-500 ng purified recombinant protein was incubated for 20 min on ice in a total volume of 280 \(\mu\)l of binding buffer (25 mM sodium acetate/25 mM potassium phosphate, pH 5.0, 0.05\% (w/v) Brij58, 1 mM EDTA) containing approximately 1 nM \[^3\text{H}\]\text{Ins} 1,3,4,5-\(P_4\). The bound and free ligands were separated by gel filtration using BioGel P-4 columns.

Cullen and Irvine employed a \text{Ins} 1,3,4,5-\(P_4\) binding assay for the investigation of GAP1\(^{IPdBp}\), however different buffer conditions were used and the bound and unbound ligand were separated by PEG precipitation (Cullen \textit{et al}. 1995a). This group had previously reported the presence of different types of \text{Ins} 1,3,4,5-\(P_4\) binding sites distinguished by acidic and neutral pH optima (Cullen and Irvine 1992). The \text{Ins} 1,3,4,5-\(P_4\) binding specificity of GAP1\(^{IPdBp}\) was shown to decline from pH 5.5 to pH 9.0 (Cullen \textit{et al}. 1995b). Likewise a sharp pH-dependence was detected for p42\(^{IP}\), with maximum \text{Ins} 1,3,4,5-\(P_4\) binding detected in the acetate/phosphate buffer at pH 5.0 (Strieker \textit{et al}. 1997). A 50\% reduction in binding was observed when the buffer was switched to 20 mM NaCl/100 mM KCl at either pH 7.0 or 5.5. Furthermore, no binding was detected using the low salt buffer at pH 7.4 (25 mM Tris-HCl, 1 mM EDTA, 0.5 mM phosphate and 0.5 mM pyrophosphate), the buffer that was used in the photoaffinity labelling studies for rat centaurin-\(\alpha\), (Hammonds-Odie \textit{et al}. 1996; Strieker \textit{et al}. 1997).

5.4.1 Development of the \textbf{Ins} 1,3,4,5-\(P_4\) Displacement Assay

For centaurin-\(\alpha_2\), preliminary binding assays were performed using two buffer conditions: 25 mM sodium acetate/25 mM potassium phosphate, pH 5.0, 0.05\% (w/v) Brij 58, 1 mM EDTA (Strieker \textit{et al}. 1996) and 100 mM KCl, 20 mM NaCl, 10 mM HEPES, pH 7.0, 1 mM EDTA (Cullen \textit{et al}. 1995a). Duplicate reactions were prepared using 100 ng recombinant protein, 1 nM \[^3\text{H}\]\text{Ins} 1,3,4,5-\(P_4\) in a final volume of 280 \(\mu\)l.
Bound ligand was separated from free ligand by centrifugation through BioGel P-4 resin columns (1.2 ml packed resin) and measured by liquid scintillation counting. High levels of radioactivity were detected in all samples including a sample with no protein (data not shown). The assay was repeated using 1 µg recombinant protein and an increased reaction volume of 500 µl. Once more the bound fraction contained the same high level of radioactivity in all samples including the negative control (no protein). The separation method appeared to be unsuccessful and consequently, separation by PEG precipitation was tested.

For the pH 5.0 and pH 7.0 buffers described above, reactions containing 1 µg recombinant protein and 1 nM [3H]Ins 1,3,4,5-P_4 were prepared in a final volume of 500 µl. In addition, a control reaction without protein was prepared for each buffer condition. Following equilibration, protein-ligand complexes were precipitated by the addition of 500 µg γ-globulin (as carrier protein) and 1 ml 25% (w/v) PEG_{3350} as described by Cullen et al. (1995a) (Section 2.6.1). Centaurin-α2 appeared to bind Ins 1,3,4,5-P_4 when using the acetate/phosphate buffer at pH 5.0, whereas levels equivalent to the non-specific control sample levels of binding (no protein) were observed with the pH 7.0 buffer (Figure 5.6). Hence, all subsequent reactions were performed using the acetate/phosphate buffer at pH 5.0 and with separation of bound ligand from free ligand by PEG precipitation.

Binding of [3H]Ins 1,3,4,5-P_4 to centaurin-α2 was reversible and specific as shown by the following results. Firstly, the addition of a 1000-fold molar excess of D/L-Ins 1,3,4,5-P_4 inhibited [3H]Ins 1,3,4,5-P_4 binding by approximately 95% (Figure 5.7). Secondly, the arginine to cysteine mutation introduced into both PH-N and PH-C domains of centaurin-α2 (R151C/R275C) resulted in almost complete loss of [3H]Ins 1,3,4,5-P_4 binding (Figure 5.7).

The Ins 1,3,4,5-P_4 binding increased proportionally with protein concentration with maximal binding at 500 ng, 220 nM (Figure 5.8). Subsequent reactions were performed using 500 ng recombinant protein for the investigation of centaurin-α2 binding specificity (the optimised assay is detailed in Section 2.6.1).
Figure 5.6. Comparison of [3H]Ins 1,3,4,5-P₄ binding using different buffer conditions. Binding reactions were performed using two buffer conditions: 25 mM sodium acetate/25 mM potassium phosphate, pH 5.0, 0.05% (w/v) Brij 58, 1 mM EDTA (Stricker et al. 1996) and 100 mM KCl, 20 mM NaCl, 10 mM HEPES, pH 7.0, 1 mM EDTA (Cullen et al. 1995a). In brief, duplicate reactions contained 1 µg recombinant centaurin-α2 (wild-type), binding buffer, 1 nM [3H]Ins 1,3,4,5-P₄. Non-specific binding was determined with no protein present. Data taken from a single assay.

Figure 5.7. Preliminary [3H]Ins 1,3,4,5-P₄ binding results. Binding reactions were performed as described above using the pH 5.0 acetate/phosphate buffer and 1 µg wild-type centaurin-α2 (WT, performed in duplicate). One assay was prepared with the addition of 1000-fold excess (1 µM) unlabelled Ins 1,3,4,5-P₄ (WT + IP₄). Another was prepared using 1 µg R151C/R275C in place of wild-type centaurin-α2. Non-specific binding was determined in a control with no protein present. Values shown are the total binding, which was corrected for non-specific binding. Data taken from a single assay.
Figure 5.8. $[^3]H$Ins 1,3,4,5-P$_4$ binding with increasing protein concentrations. Binding reactions were performed as before but with varying concentrations of wild-type centaurin-α2: 0, 44 nM (100 ng), 110 nM (250 ng), 220 nM (500 ng), 330 nM (750 ng) and 440 nM (1 μg). Data taken from a single assay.
5.4.2 Determination of Phosphoinositide Binding Specificity of Centaurin-α2 using the Ins 1,3,4,5-P₄ Displacement Assay

Increasing concentrations of unlabelled inositol phosphates and phosphoinositides were included in the Ins 1,3,4,5-P₄ binding assay to determine their ability to displace [³H]Ins 1,3,4,5-P₄ from wild-type centaurin-α2 (Figure 5.9). The most effective ligand at displacing [³H]Ins 1,3,4,5-P₄ was D/L-Ins 1,3,4,5-P₄. This unlabelled ligand inhibited 50% of [³H]Ins 1,3,4,5-P₄ binding (IC₅₀) at a concentration of 55 nM. The next most potent of the ligands tested were diC₁₀PtdIns 3,4,5-P₃ and diC₁₀PtdIns 4,5-P₂, which had similar IC₅₀ values of 154 nM and 160 nM, respectively. DiC₁₀PtdIns 3,4-P₂ and diC₁₀PtdIns 3,5-P₂ however, only competed at higher concentrations, with 940 nM and 1.9 µM being required to give 50% displacement respectively. Interestingly Ins 1,4,5-P₃ (the inositol phosphate corresponding to PtdIns 4,5-P₂) was unable to displace [³H]Ins 1,3,4,5-P₄, even when added at concentration of 50 µM (50,000-fold molar excess, data not shown).

5.4.3 Determination of Phosphoinositide Binding Specificity of the Mutant Centaurin-α2 Proteins

Mutation of the conserved arginine residue in the PH-N domain (R151C), resulted in almost complete loss of [³H]Ins 1,3,4,5-P₄ binding, similar to that observed when both PH domains are mutated, R151C/R275C (Figure 5.10, A). Whereas the comparative mutation in the PH-C domain appeared to have little or no effect on [³H]Ins 1,3,4,5-P₄ binding. The displacement curve with unlabelled Ins 1,3,4,5-P₄ revealed the binding activity of R275C to be essentially similar to that of wild-type centaurin-α2 (Figure 5.10, B). Thus, it would appear that Ins 1,3,4,5-P₄ was binding predominantly to the PH-N domain of centaurin-α2. As both PtdIns 3,4,5-P₃ and PtdIns 4,5-P₂ were able to displace Ins 1,3,4,5-P₄ with high affinity, PH-N appeared to have specificity for these ligands in addition to Ins 1,3,4,5-P₄.
Figure 5.9. Displacement of $[^3H]${ins}1,3,4,5-P$_4$ binding from recombinant wild-type centaurin-$\alpha$2. Unlabelled inositol phosphates and phosphoinositide were added to the binding assay at increasing concentrations. The concentration at which 50% of $[^3H]${ins}1,3,4,5-P$_4$ binding was displaced (IC$_{50}$) was determined for each ligand. The rank order of affinity and their IC$_{50}$ values are Ins 1,3,4,5-P$_4$ (55 nM) > diC$_{16}$PtdIns 3,4,5-P$_3$ (154 nM) > diC$_{16}$PtdIns 4,5-P$_2$ (160 nM) > diC$_{16}$PtdIns 3,4-P$_2$ (940 nM) > diC$_{16}$PtdIns 3,5-P$_2$ (1.9 $\mu$M). Ins 1,4,5-P$_3$ was unable to displace $[^3H]${ins}1,3,4,5-P$_4$ when added to concentrations of 50 $\mu$M. Each value represents the mean ± SEM of at least three independent assays.
Figure 5.10. Characterisation of [$^3$H]Ins 1,3,4,5-P$_4$ to the mutant 6xHis centaurin-α2 proteins. (A) Comparison of total of [$^3$H]Ins 1,3,4,5-P$_4$ binding to wild-type, the two single mutant (R151C and R275C) and the double mutant (R151C/R275C) centaurin-α2 proteins. Binding values are expressed as a percentage of Ins 1,3,4,5-P$_4$ binding to wild-type and each value represents the mean ± S.E.M of at least four assays. (B) Comparison of the displacement of [$^3$H]Ins 1,3,4,5-P$_4$ from wild-type and from the PH-C mutant (R275C) by unlabelled Ins 1,3,4,5-P$_4$. Each value represents the mean ± SEM from two independent assays.
5.5 Photoaffinity Labelling of Centaurin-α2 with $[^3\text{H}]$BZDC-PtdIns 3,4,5-P$_3$ and -PtdIns 3,4-P$_2$

Benzophenone photoaffinity labels have previously been used successfully to label several PtdInsP$_n$ and InsP$_n$ binding proteins including synaptotagmin C2 domains, AP-3, COPI, CAPS, Gcs1 and centaurin-α (Blader et al. 1999; Chaudhary et al. 1998; Hammonds-Odie et al. 1996; Hao et al. 1997; Loyet et al. 1998; Mehrotra et al. 1997). The benzophenone group has largely replaced earlier photoprobes as it is chemically more stable and does not decompose in ambient light. Activation at wavelengths of 350-360 nm causes the photoprobe to react with hydrophobic regions in the protein (Dorman and Prestwich 1994). The covalent attachment of the photoaffinity label enables the protein fractions to be denatured and resolved by SDS-PAGE and the $[^3\text{H}]$-label can then be detected by autoradiography.

5.5.1 Development of the Photoaffinity Labelling Assay

Initially photoaffinity labelling reactions were performed using 3 μg recombinant centaurin-α2 and $[^3\text{H}]$BZDC-PtdIns 3,4-P$_2$ or $[^3\text{H}]$BZDC-PtdIns 3,4,5-P$_3$ (0.1 μCi, 47 μM) using a modification of protocols described elsewhere (Chaudhary et al. 1998; Hammonds-Odie et al. 1996). Samples were equilibrated on ice for 10 min and exposed to UV-irradiation (350 nm) for six 5-min periods with 5-min intervals. The photolabelled proteins were then resolved on 10% SDS-PAGE gels and stained with Coomassie Blue. Exposing the gels to film for long periods of time was inconvenient, consequently the gel was cut into slices and the radioactivity determined by liquid scintillation counting as described previously by Holman et al. (1990). Wild-type recombinant centaurin-α2 bound both the $[^3\text{H}]$BZDC-PtdIns 3,4-P$_2$ and -PtdIns 3,4,5-P$_3$ photoaffinity probe (Figure 5.11). This binding was displaced by the addition of 1000-fold molar excess of the respective unlabelled ligand (Figure 5.11). The centaurin-α2 double mutant R151C/R275C was unable to bind $[^3\text{H}]$BZDC-PtdIns 3,4-P$_2$, thus indicating that with wild-type centaurin-α2 the photoaffinity label was binding specifically to the PH domains.
Figure 5.11. Photoaffinity labelling of centaurin-α2 using \(^{3}\text{H}\)BZDC-PtdIns 3,4-P\(_2\) and \(-\text{PtdIns 3,4,5-P}\(_3\)\) probes. (A) Wild-type and R151C/R275C centaurin-α2 (3 µg) were labelled using \(^{3}\text{H}\)BZDC-PtdIns 3,4-P\(_2\) (0.1 µCi, 47 µM). A second reaction was prepared for wild-type (WT) centaurin-α2 with the addition of 1000-fold molar excess of unlabelled PtdIns 3,4-P\(_2\). Samples were equilibrated on ice for 10 min and exposed to UV-irradiation (350 nm) for six 5-min periods with 5-min intervals. The samples and molecular weight markers were resolved on 10% SDS-PAGE gels and stained with Coomassie Blue. The gel was cut into slices, which were dried and solubilised prior to liquid scintillation counting. The position of the 29, 45 and 66 kDa markers are indicated. The values represent counts per minute (CPM) with the non-specific level subtracted. (B) The total binding was calculated from the area under the peak at 45 kDa (peak area). The values for the displacement with unlabelled ligand, and R151C/R275C are expressed as a percentage of the wild-type binding. All values are from a single experiment.
Optimisation of the protein concentration showed the bound \[^3H\]BZDC-Ptdlns 3,4-P\(_2\) to increase proportionally with protein concentration (Figure 5.12). The buffer conditions were tested by using lysis buffer (used for recombinant protein purification that was used in the previous photoaffinity labelling reactions) and the acetate/phosphate buffer at pH 5.0 employed in the \[^3H\]Ins 1,3,4,5-P\(_4\) binding assay. However, the pH dependent binding that was observed for \[^3H\]Ins 1,3,4,5-P\(_4\) was not detected for photoaffinity labelling using either probe (Figure 5.13). The effect of glycerol was also investigated as the storage of centaurin-\(\alpha\)2 in 50% (v/v) glycerol resulted in a final percentage of up to 15% in the photoaffinity labelling reactions. Glycerol did not appear to affect the binding activity (Figure 5.14). Finally the optimal irradiation time-course was established using \[^3H\]BZDC-Ptdlns 3,4-P\(_2\). Samples were irradiated for 5, 10 and 30 min (i.e. one, two or six 5-min periods with 5-min intervals). The increase in bound label was proportional when the irradiation time was doubled from 5 to 10 min, but not when increased from 10 to 30 min (Figure 5.15). All further experiments were performed using the optimised assay with 3 \(\mu\)g recombinant centaurin-\(\alpha\)2 in lysis buffer, pH 8.0, and irradiation for 20 min as described in Section 2.6.2.

5.5.2 Photoaffinity Labelling of Mutant Centaurin-\(\alpha\)2 Proteins

The centaurin-\(\alpha\)2 double mutant R151C/R275C was unable to bind \[^3H\]BZDC-Ptdlns 3,4,5-P\(_3\) or -Ptdlns 3,4-P\(_2\) (Figure 5.16, A and B). However, the single mutants R151C and R275C produced differing results. The binding of \[^3H\]BZDC-Ptdlns 3,4,5-P\(_3\) was unaltered by mutation of PH-C (R275C), and R151C displayed 50% reduction in binding (Figure 5.16, A). By contrast the mutation of PH-N had no effect on \[^3H\]BZDC-Ptdlns 3,4-P\(_2\) binding with R151C having similar activity to wild-type centaurin-\(\alpha\)2 and the mutation of PH-C greatly reduced binding (Figure 5.16, B).

The mutant centaurin-\(\alpha\)2 proteins have enabled us to determine some binding characteristics of the individual PH domains. Firstly the Ins 1,3,4,5-P\(_4\) binding studies suggest that Ins 1,3,4,5-P\(_4\) might bind exclusively to PH-N and the photoaffinity labelling experiments show similar binding preference for Ptdlns 3,4,5-P\(_3\), whereas Ptdlns 3,4-P\(_2\) appears to bind predominantly to PH-C.
Figure 5.12. Relationship between wild-type centaurin-α2 protein concentration and photoaffinity labelling with [³H]BZDC-PtdIns 3,4-P₂. Photolabelling reactions were performed with a range of wild-type centaurin-α2 concentrations (1.3 μM, 3 μg – 0.4 nM, 1 ng) using [³H]BZDC-PtdIns 3,4-P₂. All values represent total binding expressed as the peak area (at 45 kDa) from a single experiment.

Figure 5.13. Comparison of photoaffinity labelling of centaurin-α2 with [³H]BZDC-PtdIns 3,4-P₂ using different buffer conditions. Photolabelling reactions were performed with wild-type centaurin-α2 (1.3 μM, 3 μg) using two different buffer conditions, pH 5.0 (25 mM sodium acetate/25 mM potassium phosphate, pH 5.0, 0.05% (w/v) Brij 58, 1 mM EDTA) and pH 8.0 (20 mM Tris-HCl, pH 8.0, 100 mM NaCl). All values represent total binding expressed as the peak area (at 45 kDa) from a single experiment.
Figure 5.14. The effect of glycerol on $[^3]H$BZDC-PtdIns 3,4-P$_2$ photoaffinity labelling. Aliquots of the wild-type centaurin-α2 from the same purification were either stored in glycerol at -20°C or without glycerol at 4°C. Photoaffinity labelling reactions were prepared with wild-type centaurin-α2 (1.3 μM, 3 μg) ± glycerol. The final glycerol concentration in + glycerol reaction was 14.5% (v/v). All values represent total binding expressed as the peak area (at 45 kDa) from a single experiment.

Figure 5.15. Irradiation time course optimisation. Wild-type centaurin-α2 and $[^3]H$BZDC-PtdIns 3,4-P$_2$ reactions were irradiated for 5, 10 or 30 min (i.e. one, two or six 5-min periods with 5-min intervals). All values represent total binding expressed as the peak area (at 45 kDa) from a single experiment.
Figure 5.16. Photoaffinity labelling of centaurin-α2 mutants. Recombinant centaurin-α2 wild-type and mutant proteins (3 μg) were labelled with (A) $[^3H]$BZDC-PtdIns 3,4,5-P$_3$ or (B) $[^3H]$BZDC-PtdIns 3,4-P$_2$ and irradiated for 20 min. R151C (PH-N mutant), R275C (PH-C mutant) and R151C/R275C (mutations in both PH domains). All values represent the total binding ± S.E.M from at least three independent experiments, and are expressed as a percentage of the total ligand binding to wild-type centaurin-α2.
5.6 Discussion

A number of the studies investigating the binding properties of the centaurin-α1 proteins used heterogeneous membrane fractions isolated from brain homogenates. For example, photoaffinity labelling experiments were performed on brain homogenate purified using a heparin-agarose column, which is thought to act as a pseudo-affinity resin for inositol phosphate binding proteins (Hammonds-Odie \textit{et al.} 1996). Several proteins of different sizes were specifically photolabelled with $[^3\text{H}]$BZDC-Ins 1,3,4,5-P$_4$ using this membrane fraction. Although the photolabelled band at 46 kDa was assumed to correspond to centaurin-α, it may not be homogenous and could result from other proteins of similar size. The authors also reported the discovery of a specifically photolabelled 46 kDa band in fractionated heart extracts (purified using heparin-agarose), however the centaurin-α transcript was absent from the northern blot analysis (Hammonds-Odie \textit{et al.} 1996). These findings further strengthen the possibility of an additional protein of similar size being present in the heart and brain membrane extracts, both of which were specifically photolabelled. Another example was the use of cerebellum membranes to establish the Ins 1,3,4,5-P$_4$ binding activity of pig p42$^{IP4}$. This membrane fraction might contain other inositol phosphate and phospholipid binding proteins or enzymes that could convert or metabolise the phospholipids, which may well interfere with the results of the binding assay (Strieker \textit{et al.} 1997). Thus, in our study we have utilised bacterially expressed recombinant centaurin-α2 protein to eliminate the possible contamination of associated mammalian proteins in the phospholipid binding assays.

During the optimisation of the Ins 1,3,4,5-P$_4$ binding assay, centaurin-α2 was found to display pH dependent Ins 1,3,4,5-P$_4$ binding similar to that shown for p42$^{IP4}$, with high levels of binding at pH 5.0, but not at pH 7.0 (Strieker \textit{et al.} 1996). The pH dependence of Ins 1,3,4,5-P$_4$ binding has been addressed previously by Cullen and Irvine (1992). These authors detected specific Ins 1,3,4,5-P$_4$ binding in rat cerebellar microsomes and bovine adrenal-cortical microsomes at both pH 5.5 and 7.0 (100 mM KCl, 20 mM NaCl, \textit{see Section 5.4.1}). However, when assayed using a low ionic strength acetate buffer at pH 5.0, cerebellar microsomes retained their binding specificity, whereas bovine adrenal microsomes displayed less specific binding. The addition of 25 mM phosphate to the assay buffer had a small inhibitory effect on the amount of specific Ins...
1,3,4,5-P_{4} binding at pH 5.5 compared with that at pH 7.0, therefore a more acidic pH is favoured for binding in these assay conditions (Cullen and Irvine 1992). Of the two buffers, pH 5.0 and 7.0, initially used for the Ins 1,3,4,5-P_{4} binding assay with centaurin-α2, only the pH 5.0 buffer contained 25 mM acetate/25 mM phosphate. Therefore the lack of detectable binding at pH 7.0 cannot be explained by this rationale.

The mutation of arginine residues at positions 151 and 275 of centaurin-α2 (corresponding to Arg-28 of Btk) affected Ins 1,3,4,5-P_{4}, PtdIns 3,4,5-P_{3} and PtdIns 3,4-P_{2} binding to centaurin-α2. The application of these mutated centaurin-α2 proteins in Ins 1,3,4,5-P_{4} binding studies lead to the hypothesis that this ligand might bind exclusively to the PH-N domain, as mutation of R151C resulted in almost complete loss of Ins 1,3,4,5-P_{4} binding, whereas R275C had little or no effect. Furthermore, PtdIns 3,4,5-P_{3} and PtdIns 4,5-P_{2} displace [^{3}H]Ins 1,3,4,5-P_{4} at low concentrations, indicating that these also bind to PH-N. PtdIns 3,4,5-P_{3} appears to bind to both PH domains, but preferentially to PH-N. Mutation of this domain reduced binding by 50 %, whereas the corresponding mutation in PH-C did not inhibit binding. However, if PtdIns 3,4,5-P_{3} was binding solely to PH-N then the amount of binding observed for R151C should be consistent with that of R151C/R275C. Mutational studies have shown PtdIns 3,4,5-P_{3} to bind to both PH domains of PIP3-BP and centaurin-α1 (Tanaka et al. 1997; Venkateswarlu and Cullen 1999). In this latter study the recruitment of GFP-centaurin-α1 to the plasma membrane was reduced with both single mutants, and completely inhibited with the double mutant.

Interestingly PtdIns 3,4-P_{2} appeared to bind predominantly to PH-C, as indicated by the diminished binding of R275C in the photoaffinity labelling studies. PtdIns 3,4-P_{2} was able to displace [^{3}H]Ins 1,3,4,5-P_{4} although with 6-fold lower affinity than PtdIns 3,4,5-P_{3} and PtdIns 4,5-P_{2}. A model of centaurin-α2 PH domain binding specificity is shown in Figure 5.17. The PH-N domain of centaurin-α2 displayed low affinity for PtdIns 3,5-P_{2} compared to Ins 1,3,4,5-P_{4}, PtdIns 3,4,5-P_{3} and PtdIns 4,5-P_{2}. However, from the results of this study it was not possible to determine whether PtdIns 3,5-P_{2} binds to the PH-C domain of centaurin-α2.

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1,3,4,5-P₄ binding at pH 5.5 compared with that at pH 7.0, therefore a more acidic pH is favoured for binding in these assay conditions (Cullen and Irvine 1992). Of the two buffers, pH 5.0 and 7.0, initially used for the Ins 1,3,4,5-P₄ binding assay with centaurin-α2, only the pH 5.0 buffer contained 25 mM acetate/25 mM phosphate. Therefore the lack of detectable binding at pH 7.0 cannot be explained by this rationale.

The mutation of arginine residues at positions 151 and 275 of centaurin-α2 (corresponding to Arg-28 of Btk) affected Ins 1,3,4,5-P₄, PtdIns 3,4,5-P₃ and PtdIns 3,4-P₂ binding to centaurin-α2. The application of these mutated centaurin-α2 proteins in Ins 1,3,4,5-P₄ binding studies lead to the hypothesis that this ligand might bind exclusively to the PH-N domain, as mutation of R151C resulted in almost complete loss of Ins 1,3,4,5-P₄ binding, whereas R275C had little or no effect. Furthermore, PtdIns 3,4,5-P₃ and PtdIns 4,5-P₂ displace [³H]Ins 1,3,4,5-P₄ at low concentrations, indicating that these also bind to PH-N. PtdIns 3,4,5-P₃ appears to bind to both PH domains, but preferentially to PH-N. Mutation of this domain reduced binding by 50 %, whereas the corresponding mutation in PH-C did not inhibit binding. However, if PtdIns 3,4,5-P₃ was binding solely to PH-N then the amount of binding observed for R151C should be consistent with that of R151C/R275C. Mutational studies have shown PtdIns 3,4,5-P₃ to bind to both PH domains of PIP3-BP and centaurin-α1 (Tanaka et al. 1997; Venkateswarlu and Cullen 1999). In this latter study the recruitment of GFP-centaurin-α1 to the plasma membrane was reduced with both single mutants, and completely inhibited with the double mutant.

Interestingly PtdIns 3,4-P₂ appeared to bind predominantly to PH-C, as indicated by the diminished binding of R275C in the photoaffinity labelling studies. PtdIns 3,4-P₂ was able to displace [³H]Ins 1,3,4,5-P₄ although with 6-fold lower affinity than PtdIns 3,4,5-P₃ and PtdIns 4,5-P₂. A model of centaurin-α2 PH domain binding specificity is shown in Figure 5.17. The PH-N domain of centaurin-α2 displayed low affinity for PtdIns 3,5-P₂ compared to Ins 1,3,4,5-P₄, PtdIns 3,4,5-P₃ and PtdIns 4,5-P₂. However, from the results of this study it was not possible to determine whether PtdIns 3,5-P₂ binds to the PH-C domain of centaurin-α2.
Figure 5.17. A model of centaurin-α2 PH domain binding specificity. The preliminary binding data suggests that Ins 1,3,4,5-P₄, PtdIns 3,4,5-P₃, and PtdIns 4,5-P₂ bind preferentially to PH-N, whereas PtdIns 3,4-P₂ binds to the PH-C of centaurin-α2.
One important point to consider is that Ins 1,3,4,5-P₄ displacement studies were performed using wild-type centaurin-α2. Therefore, if PtdIns 3,4-P₂ bound preferentially to PH-C the amount of free ligand competing with [³H]Ins 1,3,4,5-P₄ at PH-N would be considerably lower than the concentration of competitor added to the reaction. Hence it might be beneficial to express the PH domains individually, and determine the binding activities independently to corroborate these proposals.

There are advantages and disadvantages of expressing the PH domains separately as mentioned above. Firstly, when investigating proteins with multiple PH domains it simplifies the interpretation of the data if each PH domain is investigated separately. The introduction of a point mutation may only inhibit binding of certain phosphoinositides while others may be able to bind as normal, thus furthering the complexity of elucidation. In contrast, the isolated PH domain may not retain the binding characteristics of the integral protein. Other domains may interact and influence the PH domain. For example, the PH domain of cytohesin-1 is followed by a 17-residue polybasic sequence, which has been shown to be required for high affinity binding to PtdIns 3,4,5-P₃ (Nagel et al. 1998). Furthermore, only a small number of proteins possess two PH domains and the significance of which remains unknown. An interesting study led to the discovery that the PH-N domain of TAPP1 does not bind phospholipids, but mutation of the conserved arginine (R28L) in this domain affected the binding affinity of PH-C, which displayed selectively for PtdIns 3,4-P₂. Furthermore, the isolated PH-C domain bound PtdIns 3,4-P₂ with 5-fold lower affinity than the full length protein, suggesting that PH-N contributes to the affinity with which PH-C binds to PtdIns 3,4-P₂ (Dowler et al. 2000).

The results from the [³H]Ins 1,3,4,5-P₄ displacement assay provide an indication of the binding selectivity of centaurin-α2's PH-N domain. However the IC₅₀ values should be considered with some care, as there was a significant amount of ligand depletion in these assays. Furthermore, the phosphoinositides employed in all assays in this study were dipalmitoyl (C₁₆), whereas dioctanoyl (C₈) derivatives are generally preferred as they are more water-soluble. Alternatively, some assays use the water-soluble inositol phosphate head groups rather than the phosphoinositide (Stricker et al. 1997). More recently these authors have compared the binding of the different lipids to p42¹₉⁴ and found Ins 1,3,4,5-P₄ and diC₈PtdIns 3,4,5-P₃ to be equipotent, whereas diC₁₆PtdIns 3,4,5-P₃ appeared to have an IC₅₀ value 30-fold higher (Hanck et al. 1999).
An anomaly in our study is the inconsistency between Ins\textsubscript{1,4,5-P\textsubscript{3}} and PtdIns\textsubscript{4,5-P\textsubscript{2}} in the displacement of [\textsuperscript{3}\text{H}]Ins\textsubscript{1,3,4,5-P\textsubscript{4}}. If PtdIns\textsubscript{4,5-P\textsubscript{2}} binds to the PH domain in the opposite orientation to PtdIns\textsubscript{3,4,5-P\textsubscript{3}}, as shown in the crystal structures of PLC\textgreek{6} and Btk (Baraldi \textit{et al.} 1999; Ferguson \textit{et al.} 1995), it is possible that the 1-phosphate of Ins\textsubscript{1,3,4,5-P\textsubscript{4}} and Ins\textsubscript{1,4,5-P\textsubscript{3}} are binding at distinct sites. If this is so, then the 1-phosphate of Ins\textsubscript{1,4,5-P\textsubscript{3}} may not be accommodated in the PH domain of centaurin-\textalpha{}2, but PtdIns\textsubscript{4,5-P\textsubscript{2}} can bind without difficulty.

If we were to assign the individual centaurin-\textalpha{}2 PH domains to one of the four PH domain groups shown in \textit{Figure 1.4} on the basis of the binding characteristics, the PH-C domain would be in Group III, affinity for PtdIns\textsubscript{3,4-P\textsubscript{2}} and PtdIns\textsubscript{3,4,5-P\textsubscript{3}}. PH-N would probably be placed in Group II, affinity for PtdIns\textsubscript{4,5-P\textsubscript{2}} as well as PtdIns\textsubscript{3,4,5-P\textsubscript{3}}. As PH-N binds PtdIns\textsubscript{3,4,5-P\textsubscript{3}} with high affinity it might be expected to fit the sequence models for PtdIns\textsubscript{3,4,5-P\textsubscript{3}} binding proteins. Sequence analysis of the \textbeta{}1 and \textbeta{}2 regions of the centaurin-\textalpha{}2 PH domains revealed that the PH-N domain does not conform to the PPBM sequence or the more detailed "Signature Motif for 3-Phosphoinositide Binding" (\textit{Figure 5.18}). The centaurin-\textalpha{}2 PH-N domain contains a hydrophobic leucine residue (L149) in place of the conserved basic arginine/lysine residue in \textbeta{}2. Interestingly, the leucine residue (L149) is conserved in the PH-N domains of centaurin-\textalpha{}1. This arginine/lysine is one of four strictly conserved residues within the Signature Motif. However, structural analysis revealed this lysine residue of GRP1 contributes ionic and water mediated interactions with the 1-phosphate of Ins\textsubscript{1,3,4,5-P\textsubscript{4}} and therefore does not appear to confer specificity for 3-phosphate phosphoinositides (Lietzke \textit{et al.} 2000). Centaurin-\textalpha{}2 lacks the insertion in the \textbeta{}6/\textbeta{}7 loop that is shared by the PtdIns\textsubscript{3,4,5-P\textsubscript{3}} binding GRP1, cytohesin-1 and ARNO. Furthermore, in the \textbeta{}1/\textbeta{}2 region both centaurin-\textalpha{}2 PH domains contain a single glycine residue that corresponds to the diglycine of GRP1, which is PtdIns\textsubscript{3,4,5-P\textsubscript{3}} selective, and triglycine of ARNO and cytohesin-1, which bind PtdIns\textsubscript{3,4,5-P\textsubscript{3}} and PtdIns\textsubscript{4,5-P\textsubscript{2}} (Klarlund \textit{et al.} 2000).

If the centaurin-\textalpha{}1 proteins bind Ins\textsubscript{1,3,4,5-P\textsubscript{4}} at only one of the PH domains, as we have shown to be the case for centaurin-\textalpha{}2, the binding data will be dependent on the specificity of a single PH domain. Consistent with this possibility, Scatchard analysis
Signature Motif for 3-Phosphoinositide Binding

\[
[LVIMF]-X-K-X-[GASP]-X_m-[WFA]-(K/R)-X-R-X-[FL]-X-[LM]-X_n-[LIF]-X-Y
\]

PH-N domain

\[
\begin{align*}
&----L----W--K--R--G--RDNSQ----F-------L----R--R--R--F--V--L--LSREGL--K--Y \\
&140 \quad 149 \quad 151 \quad 164
\end{align*}
\]

PH-C domain

\[
\begin{align*}
&----M----E--K--T--G--PKHREP--F-------K--K--R--W--A--L--DPQERR--L--L--Y \\
&263 \quad 273 \quad 275 \quad 288
\end{align*}
\]

Figure 5.18. Sequence comparison of centaurin-α2 PH-domains and the Signature Motif for 3-Phosphate Binding. Signature Motif for PH domains known and/or predicted to bind 3-phosphoinositides with high affinity. The list of residues in the square brackets represents the observed range of sequence variability at a given position. X denotes any residue; m = 5-10; n = 6-13. The expanded motif spans the β1-β3 strands and consists of four invariant residues (red). For centaurin-α2 the residues corresponding to the conserved residues are also indicated in red and the amino acid residue number is indicated below.
of [3H]Ins 1,3,4,5-P_4 binding suggested a one binding site model for p42^{IP} and human centaurin-α1 (Stricker et al. 1997; Venkateswarlu and Cullen 1999). In view of the proposed binding model of centaurin-α2, some of the apparent anomalies in the literature can be explained. For example, Tanaka et al. reported that Ins 1,3,4,5-P_4 did not inhibit PIP3-BP binding to the PtdIns 3,4,5-P_3 analogue resin and therefore concluded that this compound had at least a ten-fold lower binding affinity than PtdIns 3,4,5-P_3 (Tanaka et al. 1997). Similarly, it was concluded that Ins 1,3,4,5-P_4 had a five-fold lower affinity for centaurin-α than PtdIns 3,4,5-P_3 based on photoaffinity labelling experiments (Hammonds-Odie et al. 1996). Explanations for these data that are consistent with our model for centaurin-α2 can be postulated. Firstly, Ins 1,3,4,5-P_4 may not displace PIP3-BP from the PtdIns 3,4,5-P_3 resin if it only competes for the binding of one of the PH domains (PH-N) while the other PH domain (PH-C) remains bound to the resin. Secondly, rat centaurin-α differs from the other centaurin-α1 proteins and centaurin-α2 in the PH-N domain region and therefore may exhibit a distinct binding specificity. Although the dual specificity model can account for data on the binding properties of centaurin-α1, we can not rule out the possibility that centaurin-α2 has a fundamentally different binding interaction compared with the centaurin-α1 proteins.

Interestingly centaurin-α2 appeared to have similar affinity for PtdIns 3,4,5-P_3 and PtdIns 4,5-P_2, which is distinct from the centaurin-α1 proteins. Considering the relative cellular levels of the different inositol phosphates and phosphoinositides it could be postulated that PtdIns 4,5-P_2 is the most likely ligand for the PH-N domain of centaurin-α2. In some proteins, such as ARNO/cytohesin-1/GRPl and human centaurin-α1 the PH domains have been shown to target the protein to the plasma membrane in response to PtdIns 3-kinase activation (Langille et al. 1999; Nagel et al. 1998; Venkateswarlu et al. 1998b; Venkateswarlu et al. 1999a). In addition to targeting the protein, PH domains have also been shown to be responsible for activation of a proteins catalytic domain. For example, PtdIns 4,5-P_2 has been shown to activate the ARF-GAP activity of ASAP1 (Kam et al. 2000). Further characterisation of centaurin-α2 binding, with emphasis on the properties of the individual PH domains, is required before the functional significance can be resolved.
6.0 CONCLUSIONS

The initial aim of this study was to establish whether centaurin-α related proteins were present in insulin-sensitive tissues. The full-length centaurin-α2 clone was amplified from an adipocyte cDNA library, and in contrast to the brain specific centaurin-α, the mRNA of centaurin-α2 appeared to be ubiquitously expressed. Interestingly the western blot data indicated centaurin-α2 to be most abundant in the insulin-sensitive tissues, adipose tissue, heart and skeletal muscle.

Despite sharing 51% homology with centaurin-α and between 57-59% homology with the centaurin-α1 proteins, the investigations of this study have revealed quite distinct characteristics for centaurin-α2. Although the specificity of the anti-centaurin-α2 antisera has not been determined, if the results shown herein reflect those of centaurin-α2, then it would appear that centaurin-α2 was located in the particulate fraction of all tissues and not in the cytosolic fraction, which contrasts the distribution of centaurin-α1. Further analysis of the rat adipocyte subcellular fractions suggested that centaurin-α2 was concentrated in the mitochondria and nuclei fraction. However, confocal microscopy clearly indicated the staining of the anti-centaurin-α2 antisera was not located in the nuclei. The staining pattern was indicative of microtubule distribution, but co-localisation was not detected using dual labelling for β-tubulin and centaurin-α2. As well as having a dissimilar subcellular localisation to centaurin-α1, the distribution of centaurin-α2 appears to be unaffected by insulin-stimulation, which causes plasma membrane recruitment of centaurin-α1 (Venkateswarlu and Cullen 1999). Interestingly the difference in subcellular localisation can be rationalised by the preliminary binding data of centaurin-α2. Although both centaurin-α1 and centaurin-α2 display high-affinity binding for PtdIns 3,4,5-P_3 and Ins 1,3,4,5-P_4, the centaurin-α2 PH-N domain was found to have similarly high-affinity for PtdIns 4,5-P_2. This finding suggests that centaurin-α2 might be constitutively associated with internal membranes, which might explain the lack of response to insulin. Furthermore, Ins 1,4,5-P_3 was unable to displace Ins 1,3,4,5-P_4 in the binding assay and therefore hydrolysis of PtdIns 4,5-P_2 would probably not induce concomitant repartitioning to cytosol as displayed by PLCδ (Hurley and Misra 2000). This preliminary binding data
has implicated centaurin-α2 to be distinct from the other centaurin-α proteins, however it would be beneficial to perform more extensive binding analysis using the centaurin-α2 PH domains expressed independently in conjunction with a more extensive range of phosphoinositides and inositol phosphates.

Interestingly ARF-GAP activity has not been demonstrated for centaurin-α or the centaurin-α1s. Yet centaurin-α1 was found to functionally complement the yeast ARF-GAP Gcs1, suggesting a possible ARF regulatory function for centaurin-α1 (Venkateswarlu et al. 1999b). Despite these investigations and the identification of CK1α as the first protein partner for centaurin-α1, the role of centaurin-α1 is unresolved. The potential ARF-GAP activity was not investigated in this study of centaurin-α2, however this would be very interesting and might help ascertain the cellular function of this novel centaurin-α protein. Identification of interacting proteins for centaurin-α2 might also highlight its functional importance. Extensive research is required in order to establish a cellular function for the centaurin-α family of proteins.
7.0 REFERENCES


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