# Protein-Protein Interactions in GnRH Receptor Signalling

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# Contents

# Tables and Figures

# Declaration

I hereby declare that the work presented within this thesis was carried out by myself during the course of my PhD and has not been submitted for any other degree or qualification. Where I have used the work of others, the sources of the information have detailed explicitly in the presentation.

Lindsay Davidson

# Acknowledgements

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## Publications and presentation

The majority of the data presented in this thesis has been pulished in two papers in the Journal of Biological Chemistry:

Lindsay Davidson, Adam J. Pawson, Robert P. Millar and Stuart Maudsley (2004). Cytoskeletal reorganisation dependence of signalling by the Gonadotropin-releasing hormone receptor. *Journal of Biological Chemistry* **279** 1980 – 1993.

Lindsay Davidson, Adam J. Pawson, Rakel Lopez de Maturana, Sarah H. Freestone, Perdita Barran, Robert P. Millar and Stuart Maudsley (2004). Gonadotropin-releasing hormone induced activation of diacylglycerol kinaseζ and its association with active Src. *Journal of Biological Chemistry* **279** 11906 – 11916.

Data obtained during this PhD was also presented in two posters at the 85$^{th}$ Endocrine Society meeting in Philadelphia in 2003, and at the Biochemical Society meeting Biosciences 2004 in Glasgow.

# Abbreviations

| | |
|---|---|
| ADP | Adenosine diphosphate |
| AP | Alkaline phosphatase |
| APS | Ammonium persulphate |
| ATP | Adenosine triphosphate |
| C- | Carboxyl- |
| C1 | C kinase homology 1 |
| C2 | C kinase homology 2 |
| CaMKII | $Ca^{2+}$/calmodulin dependent kinase II |
| CH | Calponin homology domain |
| CHAPS | 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate |
| CK2 | Caesin kinase 2 |
| CRIB | Cdc42/Rac interactive binding |
| Csk | C-terminal Src kinase |
| DGK | Diacylglycerol kinase |
| DH | Double homology |
| DNA | Deoxyribonucleic acid |
| DMEM | Dulbecco's modified Eagle's medium |
| DMSO | Dimethyl sulphoxide |
| DTT | Dithiothreitol |
| ECF | Enhanced chemifluorescence |
| EDTA | Ethylenediaminetetraacetic acid |
| EF | Elongation factor |
| EGF | Epidermal growth factor |
| EGFP | Enhanced green fluorescent protein |
| EGFR | Epidermal growth factor receptor |
| EGTA | Ethyleneglycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid |
| ERK | Extracellular-signal regulated kinase |
| FAK | Focal adhesion kinase |
| FITC | Fluorescein isothiocyanate |
| FRNK | FAK related non-kinase |
| FSH | Follicle stimulating hormone |

| | |
|---|---|
| G-protein | Heterotrimeric guanine nucleotide binding protein |
| GAP | GTPase activating protein |
| GDI | GDP dissociation inhibitor |
| GDP | Guanine diphosphate |
| GEF | Guanine nucleotide exchange factor |
| GnRH | Gonadotrophin releasing hormone |
| GST | Glutathione-s-transferase |
| GPCR | G-protein coupled receptor |
| Grb2 | Growth factor receptor bound 2 |
| GTP | Guanine triphosphate |
| HEK | Human embryonic kindney |
| HEPES | N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) |
| HGF | Hepatocyte growth factor (Scatter factor) |
| IB | Immuno (Western) blot |
| IP | Immune precipitation |
| $Ins(4,5)P_2$ | Inositol (4,5) bisphosphate |
| $Ins(1,4,5)P_3$ | Inositol (1,4,5) trisphosphate |
| $Ins(1,3,4,5)P_4$ | Inostiol (1,3,4,5) tetrakisphosphate |
| IPTG | Isopropyl β-D-1 thiogalactopyranoside |
| Kd | Dissociation constant |
| KSR | Kinase supressor of Ras |
| LB | Luria broth |
| LH | Luteinizing hormone |
| LIM | Lin-11, Isl-1, Mec-3 |
| MALDI-ToF | Matrix assisted laser desorption-ionisation time of flight |
| MARCKS | Myristoylated alanine rich C kinase substrate |
| MEK | MAPK and ERK kinase |
| MMP | Matrix metalloproteinase |
| MOWSE | Molecular weight search |
| N- | Amino- |
| N-WASp | Neuronal Wiscott-Aldrich syndrome protein |
| NP-40 | Nonident P-40 |
| PBS | Phosphate buffered saline |

| | |
|---|---|
| PCR | Polymerase chain reaction |
| PDGF | Platelet derived growth factor |
| PDK-1 | Phosphoinositide dependent kinase-1 |
| PH | Pleckstrin homology |
| PtdIns | Phosphatydlinositol |
| PI-3 kinase | Phosphoinositide-3 kinase |
| PIP-5 kinase | Phosphatydlinositol (4) phosphate-5 kinase |
| PIPES | Piperazine-N,N'-bis-2-ethanesulfonic acid |
| PKA | Protein kinase A (A kinase) |
| PKC | Protein kinase C (C kinase) |
| PLC | Phospholipase C |
| PLC-β1 | Phospholipase C beta 1 |
| PMSF | Phenylmethanesulphonyl fluoride |
| pSer (pS) | Phosphoserine |
| PTB | Phosphotyrosine binding |
| PtdIns(4)P | Phosphatydlinositol (4) phosphate |
| PtdIns(4,5)$P_2$ | Phosphatydlinositol (4,5) bisphosphate |
| PtdIns(3,4,5)$P_3$ | Phosphatydlinositol (3,4,5) trisphosphate |
| PTEN | Phosphatase and tensin homologue deleted on Chromosome 10 |
| pThr (pT) | Phosphothreonine |
| PTPα | Protein tyrosine phosphatase alpha |
| PTP1B | Protein tyrosine phosphatase 1 B |
| pTyr (pY) | Phosphotyrosine |
| PVDF | Polyvinylidene fluoride |
| RACK | Receptor for activated C kinase |
| rdg | Retinal degeneration |
| R-G-D-S | Arginine-glycine-aspartic acid-serine |
| R-G-E-S | Arginine-glycine-glutamic acid-serine |
| RGS | Regulator of G-protein signalling |
| RNA | Ribonucleic acid |
| RNAi | RNA interference |
| RSK | p90 ribosomal S6 kinase |
| SDS | Sodium dodecyl sulfate |

| | |
|---|---|
| SDS-PAGE | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| SH1 | Src homology 1 |
| SH2 | Src homology 2 |
| SH3 | Src homology 3 |
| Sos | Son-of-sevenless |
| STAT | Signal transducers and activators of transcription |
| TEMED | N-N-N'-N'-Tetramethylethylethylenediamine |
| Tiam1 | T-lymphoma invasion and metastasis inducing protein 1 |
| Tris | Tris-(hydroxymethyl)-aminomethane |
| v-Src | Oncogene encoded by the Rous avian sarcoma virus |
| v-Fps | Oncogene encoded by the Fujinami avian sarcoma virus |
| VEGF | Vascular endothelial growth factor |
| WASp | Wiscott-Aldrich syndrome protein |

## Abbreviations of amino acids

| Amino acid | Three letter code | One letter code |
|---|---|---|
| Alanine | Ala | A |
| Arginine | Arg | R |
| Asparagine | Asn | N |
| Aspartic acid | Asp | D |
| Cysteine | Cys | C |
| Glutamine | Gln | Q |
| Glutamic acid | Glu | E |
| Glycine | Gly | G |
| Histidine | His | H |
| Isoleucine | Ile | I |
| Leucine | Leu | L |
| Lysine | Lys | K |
| Methionine | Met | M |
| Phenylalanine | Phe | F |
| Proline | Pro | P |
| Serine | Ser | S |
| Threonine | Thr | T |
| Tryptophan | Trp | W |
| Tyrosine | Tyr | Y |
| Valine | Val | V |

# Abstract

Many signal transduction pathways in eukaryotic cells are controlled by the reversible assembly of proteins into signalling complexes that integrate and transmit signals from cell surface receptors to the cytoplasm and nucleus. Protein phosphorylation on serine, threonine and tyrosine residues plays a key role in these events by creating binding sites for modular protein-interaction domains to allow proteins to associate with one another, and regulating the activitiy of many enzymes and transcription factors.

The extracellular signal regulated kinase (ERK) cascade is one of the most intensively studied signalling pathways in mammalian cells. ERK 1 and 2 have been shown to regulate gene expression programmes in response to extracellular signals, including gonadotrophin releasing hormone (GnRH), which binds to a G-protein coupled receptor (GPCR). However, many questions remain to be answered regarding the proximal events that lead to the activation of the ERK cascade by GnRH.

A HEK 293 cell line, stably expressing the rat GnRH receptor, was used to investigate the mechanism of ERK activation by GnRH. ERK activation was found to be dependent on cell adhesion to the extracellular matrix, and required an intact actin cytoskeleton. Through the use of specific pharmacological inhibitors and by expression of dominant negative cDNA constructs, ERK activation was found to be mediated by the Rho family GTPase Rac1, and the non-receptor tyrosine kinases focal adhesion kinase (FAK) and Src. FAK was found to function as a tyrosine phosphorylated scaffold upon which key components of the ERK cascade assembled.

Having established a role for Src in the activation of ERK, a proteomics study was undertaken to identify novel Src binding proteins that may be involved in the regulation of GnRH receptor signalling. Through a combination of immune precipitation, two-dimensional gel electrophoresis and matrix assisted laser desorption ionisation-time of flight (MALDI-ToF) mass spectrometry, Src was found to associate with the lipid kinase

diacylglycerol kinase ζ (DGKζ). This interaction was found to be required for GnRH to stimulate DGKζ enzyme activity. By phosphorylating the second messenger molecule diacylglycerol to produce phosphatidic acid, DGKζ may play an important role in regulating GnRH receptor signalling.

In this thesis, a potential mechanism of ERK activation is described for the GnRH receptor, with Src playing a key role in this pathway. In addition, Src was found to be involved in the activation of DGKζ, and is therefore implicated in the regulation of diacylglycerol signalling. This is the first report of an interaction between Src and DGKζ.

# Literature review

## Introduction

This literature review is divided into two parts. In the first part, modular protein- and lipid-interaction domains are introduced, and their role in controlling the activity and substrate specificity of protein kinases are discussed. Src and the conventional and novel protein kinase C (PKC) isozymes are given as examples. In the second part, Gonadotrophin releasing hormone (GnRH) physiology is introduced and GnRH receptor signalling discussed. Emphasis is placed on the role of interaction domains and protein kinases in this process.

## Signal transduction in eukaryotic cells

Virtually every aspect of cellular function within a metazoan organism is controlled by external signalling molecules, either in the form of soluble hormones or proteins anchored to the membrane of adjacent cells or the extracellular matrix. These factors exert their effects by binding to receptors on the surface of the cell and activating a myriad of intracellular signal transduction pathways. Over the past two decades, considerable advances have been made in the field of signal transduction and have demonstrated that many signalling pathways in eukaryotic cells are regulated by the reversible assembly of protein complexes, which integrate and transmit signals from receptors to the cytoplasm and nucleus (Pawson and Scott, 1997).

Many proteins involved in intracellular signalling are constructed in a modular fashion from domains that have enzyme activity or mediate molecular interactions. These interaction domains target proteins to specific subcellular localisations, provide a means for recognition of post-translational modifications or chemical second messengers, nucleate the formation of signalling complexes, and control the conformation, activity, and substrate specificity of many enzymes (Pawson and Nash, 2003). Thus, they assist in

maintaining the fidelity of signal transduction. As a result mutations in these domains contribute to a number of pathological conditions, including cancer (Tartaglia *et al.*, 2001; He *et al.*, 2002).

**Modular interaction domains**

Modular interaction domains often fold independently with their N- and C-termini closely apposed in space, allowing them to be readily incorporated into proteins in a manner that leaves their ligand-binding surface available (Pawson and Nash, 2003). This feature is thought to have facilitated the evolution of complex signalling pathways in eukaryotic cells, as they can be incorporated into proteins to create new connections and bestow new functions. Indeed, several interaction domains are present in hundreds of copies in the genome of mammals (Venter *et al.*, 2001). Evolution therefore appears to have used a limited set of domains, joined together in a combinatorial fashion, to direct the actions of many enzymes (Pawson and Scott, 1997; Pawson and Nash, 2003).

Modular interaction domains can usually be identified through their consensus amino acid sequences, allowing the binding properties of proteins to be predicted (Copley *et al.*, 2002). They recognise exposed sites on their protein partners or bind the charged head groups of phospholipids, with dissociation constants in the low nanomolar to high micromolar range. Typically, a protein-interaction domain recognises a core determinant with flanking residues providing additional contacts and an element of specificity (Songyang *et al.*, 1993; Gustafson *et al.*, 1995). Post-translational modifications often complete binding sites, allowing precise control of ligand binding, enzyme activity, and the assembly of signalling complexes. The most frequently used modification is the phosphorylation of serine, threonine or tyrosine residues. However, hydroxylation, acetylation, methylation, ubiquitination and sumoylation of proteins can also function to control modular interactions (Min *et al.*, 2002; Jacobson *et al.*, 2000; Bannister *et al.*, 2001; Polo *et al.*, 2002, Melchior, 2000). In many cases, the affinity of a single domain

for a peptide motif appears sufficient for a specific interaction to occur between two proteins *in vitro*. However, tertiary interactions, sub-cellular localisation and structural organisation of interacting proteins are also likely to contribute to selectivity *in vivo* (Pawson and Nash, 2003).

In the following discussion emphasis is placed on Src homology 2 (SH2), Src homology 3 (SH3), C kinase homology 1 (C1) and C kinase homology 2 (C2) domains, which regulate the activity of many enzymes and are therefore important modulators of signal transduction. SH2 and SH3 domains were among the first protein-interaction modules to be described, and have been shown to recognise phosphotyrosine motifs and proline rich sequences in their protein partners, respectively (Anderson *et al.*, 1990; Ren *et al.*, 1993). They are frequently found together in the non-receptor tyrosine kinases, where they regulate the activity of the catalytic domain and its interaction with target molecules (Pellicena *et al.*, 1998; Schaller *et al.*, 1999). C1 and C2 domains were first identified in conventional and novel PKC isozymes, and were found to bind diaclglycerol and phosphatidylserine, respectively (Newton, 1997). Subsequently, they have been identified in a wide range of cytoplasmic proteins and additional ligands have been discovered. Numerous structural and biochemical studies have provided many details of the mechanisms of ligand binding for these important domains.

**SH2 domains**

The SH2 domain was originally defined in the retroviral oncoprotein v-Fps as a sequence of approximately 100 amino acids that regulated the function of the catalytic domain and its interaction with target proteins in the host cell (Sadowski *et al.*, 1986). Related sequences were identified in Src family kinases, leading to the 'Src homology domain' nomenclature, with the catalytic domain designated SH1. Subsequently, SH2 domains were identified in many proteins involved in signal transduction and were found to bind

phosphotyrosine motifs in receptor tyrosine kinases and cytoplasmic proteins alike (Anderson *et al.*, 1990; Mayer *et al.*, 1991).

The SH2 fold (Figure 1) consists of a seven-stranded β-meander that is sandwiched on either side by an α-helix (Waksman *et al.*, 1992). The spine of the domain comprises an anti-parallel β-sheet formed from strands βA, βB, βC, βD and βG, which divide it into two functionally distinct sides. One side, flanked by helix αA, binds the phosphotyrosine moiety, while the other side, flanked by helix αB and the EF and BG loops, provides residues that interact with the side chains of the peptide ligand C-terminal to the phosphotyrosine (Waksman *et al.*, 1992; Kuriyan and Cowburn, 1997). In most SH2-ligand structures, the phosphopeptide binds in an extended conformation and lies across the surface of the domain perpendicular to the axis of the central β-sheet. The SH2-ligand interaction is of moderate strength (Kd ~ 0.1 μM – 1.0 μM) and analyses of binding kinetics have shown that the on and off rates are quite high, allowing rapid association and dissociation of the ligand (Felder *et al.*, 1993). This feature is common to a number of interaction domains that recognise phosphorylated motifs and may therefore be physiologically important given the transient nature of many signal transduction events, as the balance shifts between the activity of protein kinases and phosphatases (Yaffe and Elia, 2001; Pawson and Nash, 2003).

Although the specifics of ligand binding vary between individual SH2 domains, the overall nature of the interaction is well conserved. Phosphotyrosine recognition elements are provided by residues in αA, βB, βD, and the loop connecting strands βB and βC. The phosphotyrosine sits in a moderately deep pocket; the backbone of the residue is held in position by residues in the βD strand, and the phosphate group enters into ionic interactions with ArgβB5 (Waksman *et al.*, 1992; Kuriyan and Cowburn, 1997). This interaction is strictly conserved in all SH2 domains, and mutation of the arginine residue to alanine significantly inhibits ligand binding (Mayer *et al.*, 1992). The location of

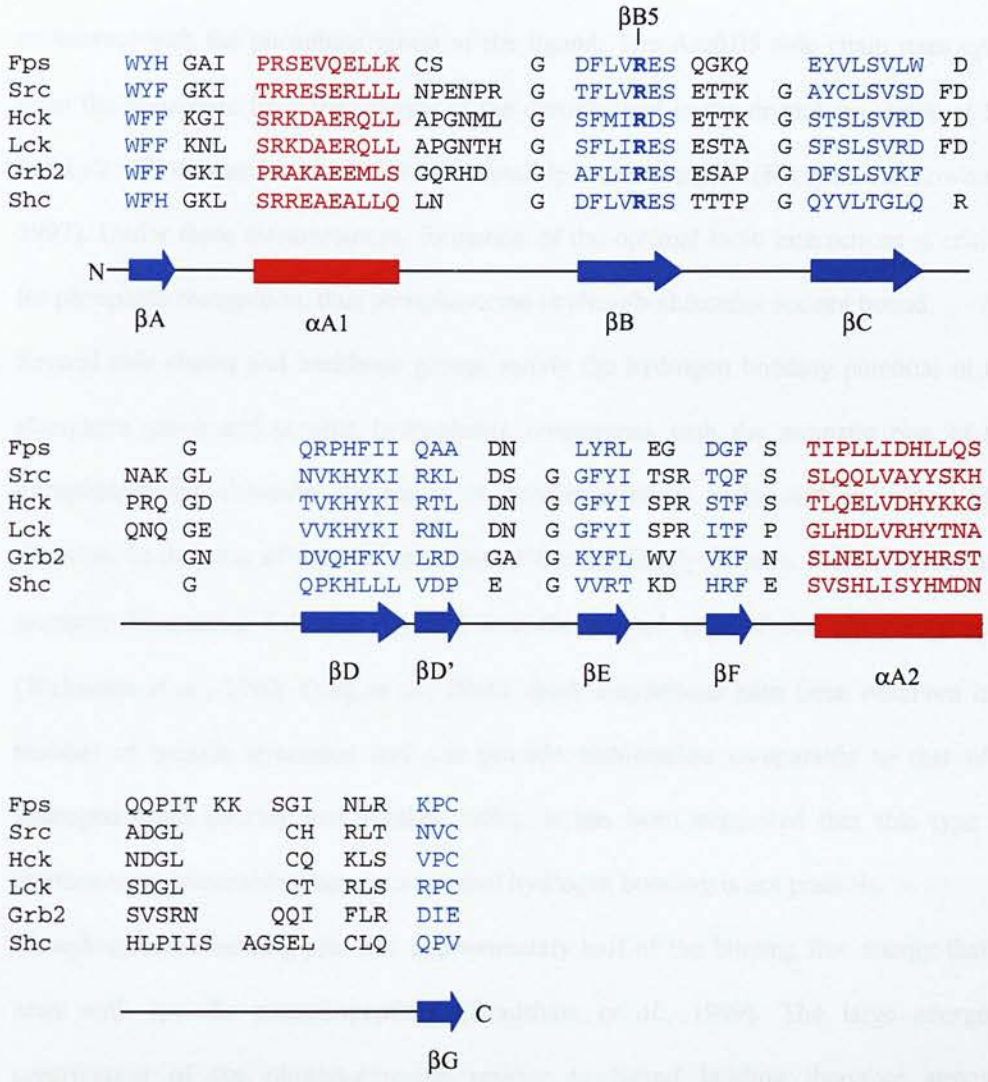


**Figure 1: Sequence alignment of SH2 domains**. Several SH2 domains from non-receptor tyrosine kinases and adapter proteins are shown, red and blue boxes indicate the positions of α-helices and β-strands, respectively. The conserved arginine residues at the βB5 position, which plays a critical role in phosphotyrosine recognition, is highlighted in bold. Adapted from Kuriyan and Cowburn (1997).

ArgβB5 is such that in the fully extended conformation, its side chain is just long enough to interact with the phosphate group of the ligand. The ArgβB5 side chain rises up to meet the phosphate from the interior of the domain, and in the crystal structures of Src and Lck SH2 domains it is not solvent accessible in this complex (Kuriyan and Cowburn, 1997). Under these circumstances, formation of the optimal ionic interactions is critical for phosphate recognition, thus phosphoserine or phosphothreonine are not bound.

Several side chains and backbone groups satisfy the hydrogen bonding potential of the phosphate group and provide hydrophobic interactions with the aromatic ring of the phosphotyrosine. However, the nature of these interactions varies with individual SH2 domains. In the case of the SH2 domains of the Src family kinases, there is an amino-aromatic interaction between ArgαA2 and the phenol ring of the phosphotyrosine (Waksman *et al.*, 1992; Tong *et al.*, 1996). Such interactions have been observed in a number of protein structures and can provide stabilisation comparable to that of a hydrogen bond (Burley and Petsko, 1986). It has been suggested that this type of interaction is favourable when conventional hydrogen bonding is not possible.

Phosphotyrosine binding provides approximately half of the binding free energy that is seen with specific phosphopeptides (Bradshaw *et al.*, 1999). The large energetic contribution of the phosphotyrosine residue to ligand binding therefore strongly discriminates between phosphorylated and non-phosphorylated sequences. The remaining free binding energy comes from a network of interactions involving adjacent residues (Bradshaw and Waksman, 1999). Using phosphopeptide libraries, many SH2 binding specificities have been identified (Cantley and Songyang, 1994). This pioneering work established that the three residues immediately C-terminal to the phosphotyrosine are key determinants of specificity, and that sequence preferences in the targets could be correlated with particular residues in the SH2 domain. The clearest example of this correlation is provided for by the residue at the βD5 position, which contacts the side

chains of the ligand at the pY+1 and pY+3 positions. The SH2 domains of the Src family kinases have aromatic side chains at βD5 and preferentially bind peptides containing polar side chains at the pY+1 position. In contrast, the SH2 domains of PLCγ have hydrophobic residues at βD5 and preferentially select hydrophobic amino acids at the pY+1 position (Cantley and Songyang, 1994). Furthermore, by mutating this critical amino acid, it is often possible to switch the binding specificity of individual SH2 domains (Marengere *et al.*, 1994; Songyang *et al.*, 1995a).

Because SH2 domains play an important role in controlling protein interactions, mutations in them have been implicated in a number of pathological conditions. For example, point mutations in the SH2 domain of Bruton's tyrosine kinase cause some cases of X-linked agammaglobulinaemia (Vihinen *et al.*, 1999). These mutant proteins cannot interact with their tyrosine phosphorylated substrates during B cell development (Mattson *et al.*, 2000), resulting in severe B cell deficiency due to the failure of precursor cells to differentiate in the bone marrow (Noordzij *et al.*, 2002). Point mutations in the SH2 domain of SAP, a T-cell specific adapter protein that mediates signalling through the SLAM receptor (Latour *et al.*, 2001), cause X-linked lymphohyperproliferative syndrome, which is characterised by impaired cytotoxic activities of T cells (Sharifi *et al.*, 2004) and extreme susceptibility to infection by Epstein-Barr virus (Purtilo *et al.*, 1975). These studies emphasise the important role played by SH2 domains in maintaining the fidelity of signal transduction pathways involving the tyrosine phosphorylation of proteins, and in the physiology of the whole organism.

**SH3 domains**

SH3 domains are small (55 – 70 amino acid) protein-interaction modules that are found in many signalling proteins (Pawson, 1995). In addition, they are also present in a number of proteins involved in the regulation of the cytoskeleton, and may therefore represent a link between signal transduction pathways and the structural organisation of

the cell (Musacchio *et al.*, 1992a). The SH3 fold (Figure 2) is highly conserved and consists of a two-stranded β-sheet and a three-stranded β-sheet packed together approximately at right angles to form a β-barrel structure (Musacchio *et al.*, 1992b). The inner face of the β-sheets form a hydrophobic core lined with conserved aromatic residues, which maintain the structure of the binding site and make contacts with the peptide ligand (Yu *et al.*, 1992). Determination of SH3-ligand structures by X-ray crystallography established that the ligand forms a left-handed type II polyproline helix (Musacchio *et al.*, 1994). Polypeptides with this conformation adopt an extended molecular scaffold that is well suited to the long shallow groove of the SH3 domain (Yu *et al.*, 1994). In comparison, an α-helix with an equivalent number of residues would be approximately half the length. However, the most striking feature of the polyproline type II helix is its highly regular structure and approximately three-fold symmetry. The helix has three residues per turn and amino acids at position n and n+3 lie on the same face of the helix (Adzhubei and Sternberg, 1993).

Polyproline type II helices are ideal mediators of protein-protein interactions as they are frequently found on the surface of globular proteins (Adzhubei and Sternberg, 1993). This is because the structure is not internally hydrogen bonded but depends on backbone solvation for stability (Adzhubei and Sternberg, 1993). The bound peptide interacts with the SH3 domain by intercalating its side chains into the ladder of aromatic residues that line the binding site (Feng *et al.*, 1994). Screening combinatorial libraries of proline rich peptides has identified optimal ligands for a number of SH3 domains (Yu *et al.*, 1994). Optimal ligands for the SH3 domain of PI-3 kinase have the consensus sequence R-X-L-P-P-R-P-X-X, where X represents any amino acid other than cysteine, while ligands for the SH3 domain of Src have a similar consensus sequence, R-X-L-P-P-L-P-R-ϕ, where X represents any amino acid other than cysteine and ϕ represents a hydrophobic residue.

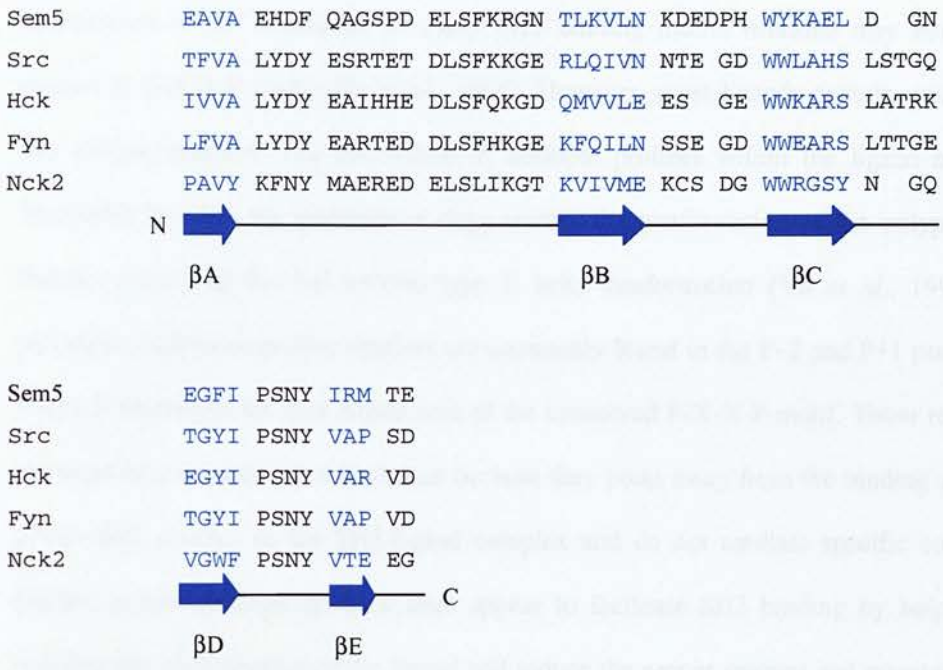


**Figure 2: Sequence alignment of SH3 domains**. The SH3 domains from several non-receptor tyrosine kinases and adapter proteins are shown, blue boxes indicate the position of β-strands. Adapted from Kuriyan and Cowburn (1997).

Comparison of the sequences of many SH3 binding motifs revealed they contain a conserved P-X-X-P motif (Yu *et al.*, 1994). However, most ligands contain more than two proline residues. The occurrence of multiple prolines within the ligand may be favourable because the pyrrolidone rings restrict the conformation of the polypeptide, thereby promoting the left handed type II helix conformation (Yu *et al.*, 1994). In particular, additional proline residues are commonly found in the P–2 and P+1 positions, where P represents the first amino acid of the conserved P-X-X-P motif. These residues are especially suitable for substitution because they point away from the binding surface of the SH3 domain in the SH3-ligand complex and do not mediate specific contacts. Rather, proline residues at these sites appear to facilitate SH3 binding by helping to stabilise the conformation of the ligand and induce the proper spacing and orientation of key contact residues (Yu *et al.*, 1994). Other amino acids at these positions tend to be hydrophobic, possibly reflecting packing interactions between the helix and the rest of the SH3 binding protein (Yu *et al.*, 1994). An unusual feature of the SH3 domain is that ligands appear to be able to bind in two orientations (Yu *et al.*, 1994; Feng *et al.*, 1994). The direction in which the peptide binds is determined primarily by charge pairing between arginine residues in the peptide and acidic residues on the surface of the domain (Lim *et al.*, 1994).

Because SH3 domains bind to their targets with a relatively small interaction surface, with limited hydrogen bonding, SH3-ligand interactions tend to be quite weak ($K_d \sim 1 - 10$ μM). The reliance of binding affinity on hydrophobic interactions, which are generally less specific than those involving hydrogen bond formation, also results in relatively promiscuous peptide recognition. Therefore, for many SH3 domains, specificity requires multiple binding sites and/or tertiary interactions. For example, in many of the non-receptor tyrosine kinases, SH3 domains compliment SH2 domains in target recognition, and thereby function to increase the affinity of the kinase for its

substrates. As a result, mutations in SH3 domains can contribute to a number of pathological conditions. For example, deletion of the C-terminal 21 amino acids of the SH3 domain of Bruton's tyrosine kinase causes some cases of X-linked lymphohypoproliferative syndrome (Zhu *et al.*, 1994). The deletion of these amino acids causes the domain to adopt a random coil conformation rather than the normal β-barrel structure (Chen *et al.*, 1996), which prevents it form binding its cognate ligands.

**C1 domain**

C1 domains are small (approximately 50 amino acid) lipid-interaction modules that are found in many proteins involved in cell signalling, notably the PKCs, diacylglycerol kinases (DGKs), kinase supressor of Ras (KSR) and Raf-1. They contain the conserved sequence motif H-$X_{10-12}$-C-$X_2$-C-$X_{11-19}$-C-$X_2$-C-$X_4$-H-$X_{2-4}$-C-$X_{5-9}$-C (Zhou *et al.*, 2002) that is responsible for co-ordinating two $Zn^{2+}$ ions that are required for the proper folding of the domain (Quest *et al.*, 1992). The C1 domain (Figure 3) belongs to the zinc finger fold, and consists of two small β-sheets and a very short C-terminal α-helix. The first β-sheet is formed from strands β1, β4 and β5, and the second from strands β2 and β3. The C-terminal helix packs against the β1 strand to form one of the $Zn^{2+}$ binding sites, while the other is formed from residues in the loop connecting strand β5 and the C-terminal helix (Mott *et al.*, 1996; Zhou *et al.*, 2002). The two histidine residues and five of the conserved cysteine residues are absolutely required for the co-ordination of the $Zn^{2+}$ ions, and mutation of any of these residues has a detrimental effect on the structural integrity of the domain and consequently inhibits ligand binding (Kazanietz *et al.*, 1995).

Biochemical and mutational studies have shown that C1 domains bind to membranes via a combination of electrostatic and hydrophobic interactions. Basic residues around the

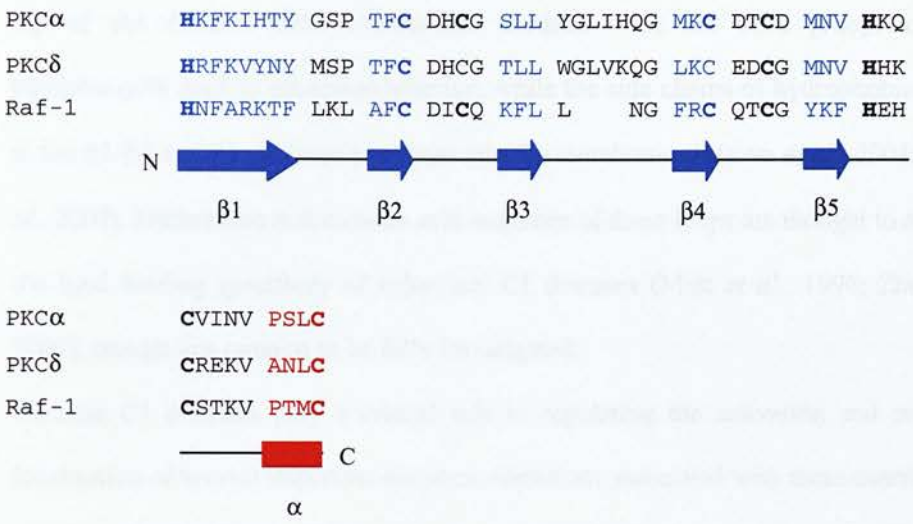


**Figure 3: Sequence alignment of C1 domains**. The C1 domains of PKCα, PKCδ and Raf-1 are shown. Red and blue boxes indicate the positions of α-helices and β-strands, respectively. The six conserved cysteine residues and the two conserved histidine residues are highlighted in bold. Adapted from Zhang *et al.* (1995) and Mott *et al.* (1996).

top of the domain make electrostatic contacts with the head groups of acidic phospholipids such as phosphatidylserine, while the side chains of hydrophobic residues in the β1-β2 and β3-β4 loops penetrate into the membrane (Bittova *et al.*, 2001; Zhou *et al.*, 2002). Differences in the amino acid sequence of these loops are thought to determine the lipid binding specificity of individual C1 domains (Mott *et al.*, 1996; Zhou *et al.*, 2002), though this remains to be fully investigated.

Because C1 domains play a crucial role in regulating the activation and subcellular localisation of several important enzymes, mutations associated with them contribute to a number of pathological conditions. For example, missense mutations in the C1 domains of PKCγ cause some cases of spinocerebellar ataxia, a severe and debilitating hereditary neurodegenerative disorder that is characterised by progressive loss of gait and motor function, and by speech and eye movement disturbances (Chen *et al.*, 2003). However, only neurones in the central nervous system are affected in this disorder, due to the restricted tissue distribution of PKCγ.

**C2 domain**

C2 domains are a second class of lipid-interaction modules that are found in many proteins involved in cell signalling, including the PKCs, phospholipase Cs (PLCs), phospholipase A2 (PLA2), and the synaptotagmins. The C2 fold consists of a compact anti-parallel β-sandwich composed of eight β-strands connected by three loops at the top of the domain and four loops at the bottom (Sutton *et al.*, 1995; Essen *et al.*, 1996; Shao *et al.*, 1996). There is a high degree of structural homology between C2 domains in their core β-sandwiches, however, the sequences of the top loops, which are involved in ligand binding, are much more variable (Rizo and Südhof, 1998).

Classically, the C2 domains have been divided into two groups based on their mode of ligand binding. The first group, which includes the C2 domains of the conventional

PKCs, bind acidic phospholipids in a $Ca^{2+}$-dependent manner. Five conserved aspartate residues in the top loops and at the top of the β-strands (Figure 4) are responsible for co-ordinating a cluster of two or three $Ca^{2+}$ ions at the tip of the domain (Rizo and Südhof, 1998). In the absence of $Ca^{2+}$, the negative charges of these aspartate residues repel the negatively charged head groups of acidic phospholipids, thereby preventing membrane binding. However, when $Ca^{2+}$ binds, it causes a major change in the electrostatic potential of the domain and allows basic residues in the top loops to make electrostatic contacts with acidic phospholipids (Shao *et al.*, 1997; Ubach *et al.*, 1998). The co-ordination spheres of the $Ca^{2+}$ ions are incomplete when bound to the C2 domain and are fulfilled by interacting with the phosphate head group of phospholipids. Thus, the $Ca^{2+}$ ions also act as a bridge between the C2 domain and phospholipids (Verdaguer *et al.*, 1999). Mutation of the conserved aspartate residues to asparagine prevents the co-ordination of $Ca^{2+}$ ions and abolishes membrane binding (Bolsover *et al.*, 2003).

The second group of C2 domains, which includes those of the novel PKCs, bind phospholipids in a $Ca^{2+}$-independent manner. These domains lack some or all of the five aspartate residues that are found in the top loops of the $Ca^{2+}$-dependent C2 domains and consequently do not bind $Ca^{2+}$. These domains appear to bind to membranes through a combination of electrostatic and hydrophobic interactions (Ochoa *et al.*, 2001). In the case of the C2 domain of PKCε, Arg26, Arg32 and Arg50 make electrostatic contacts with acidic phospholipids, while the hydrophobic side chains of Trp23, Ile89 and Tyr92 insert into the membrane (Ochoa *et al.*, 2001).

Mutations in C2 domains are also found in a number of pathological conditions, where they cause the miss-location of the affected protein. For example, mutations in the C2 domain of the lipid phosphatase PTEN, which prevent it from efficiently binding to the plasma membrane, are found in many sporadic primary cancers, including endometrial carcinomas and glioblastoma multiform (Eng, 2003).

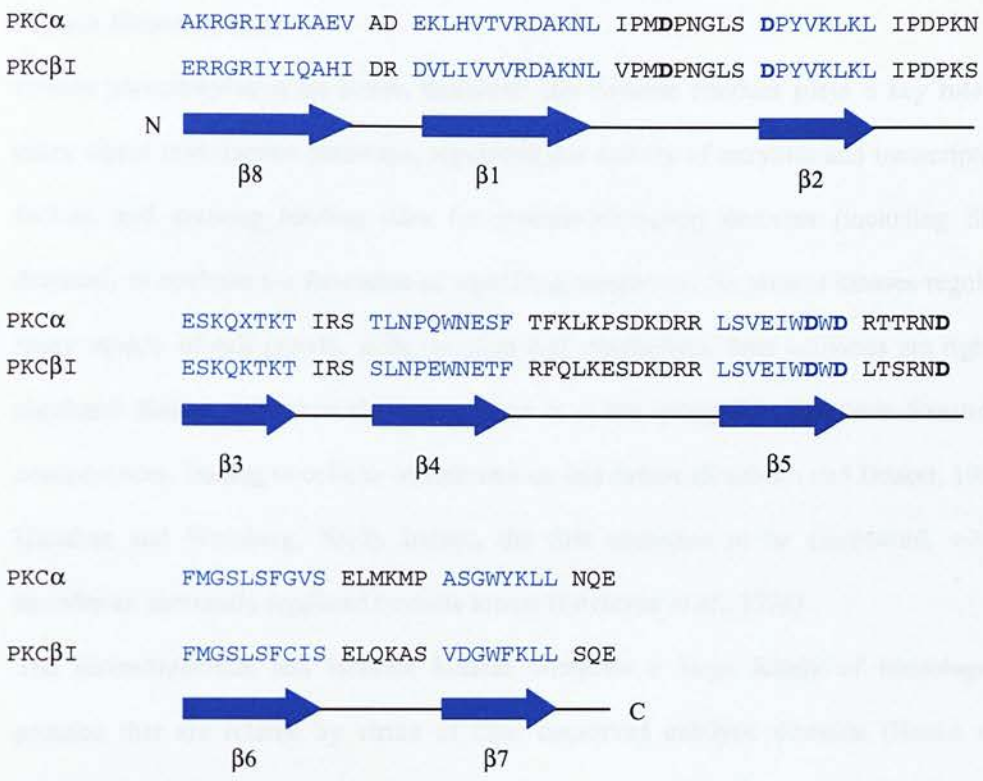


**Figure 4: Sequence alignment of $Ca^{2+}$-dependent C2 domains.** The C2 domains of PKCα and PKCβI are shown. Blue boxes indicate the position of β-sheets. The five conserved aspartate residues responsible for the co-ordination of $Ca^{2+}$ ions are highlighted in bold. Adapted from Ochoa *et al.* (2001).

**Protein kinase domain**

Protein phosphorylation on serine, threonine and tyrosine residues plays a key role in many signal transduction pathways, regulating the activity of enzymes and transcription factors, and creating binding sites for protein-interaction domains (including SH2 domains) to nucleate the formation of signalling complexes. As protein kinases regulate many aspects of cell growth, differentiation and metabolism, their activities are tightly regulated. Kinase activity in the wrong place or at the wrong time can have disastrous consequences, leading to cellular transformation and cancer (Kolibaba and Druker, 1997; Hanahan and Weinberg, 2000). Indeed, the first oncogene to be discovered, v-Src, encodes an aberrantly regulated tyrosine kinase (Levinson *et al.*, 1978).

The serine/threonine and tyrosine kinases comprise a large family of homologous proteins that are related by virtue of their conserved catalytic domains (Hanks and Hunter, 1995), which catalyse the transfer of the γ-phosphate of adenosine triphosphate (ATP) to the hydroxyl group of serine, threonine or tyrosine, generating adenosine diphosphate (ADP) (Figure 5). Receptor kinases are activated by their cognate ligands binding to their extracellular domains, while non-receptor kinases are activated by a wide variety of intracellular signals downstream of cell surface receptors. Efficient substrate phosphorylation requires the precise co-ordination of ATP in complex with divalent cations, usually $Mg^{2+}$ or $Mn^{2+}$, and substrate. Comparison of the crystal structures of the catalytic domains of several protein kinases reveals they adopt a similar conformation in the active state, reflecting the chemical constraints that must be met for phosphotransfer (Jeffrey *et al.*, 1995, Canagarajah *et al.*, 1997; Hubbard, 1997; Huse and Kuriyan, 2002). However, they may adopt different conformations in the inactive state, reflecting the diverse mechanisms that have evolved to regulate the activity of individual kinases (Huse and Kuriyan, 2002). Structural and biochemical studies indicate that these regulatory elements determine kinase activity by impinging upon just a few key components of the

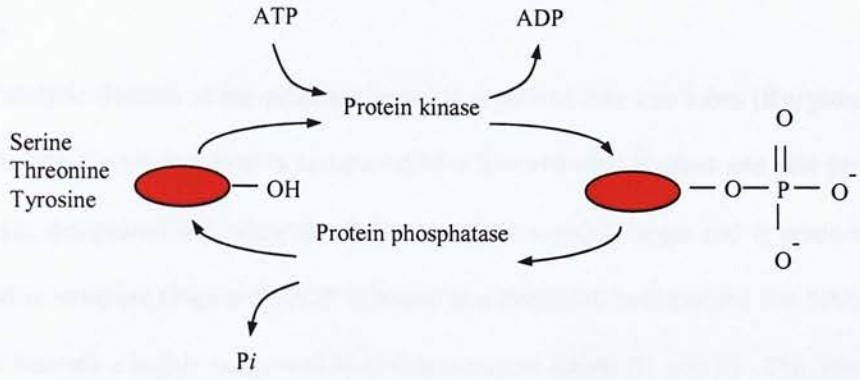


**Figure 5: The protein phosphorylation reaction**. Protein kinases catalyse the reversible transfer of the γ-phosphate group of ATP to the hydroxyl group of serine, threonine or tyrosine residues. The reaction is opposed by protein phosphatases, which catalysed the removal of the phosphate group.

catalytic domain (Morgan and De Bondt, 1994; Johnson *et al.*, 1996; Hubbard *et al.*, 1998).

The catalytic domain of the protein kinases is separated into two lobes (Knighton *et al.*, 1991). The N-terminal lobe is composed of a five-stranded β-sheet and one prominent α–helix, designated αC, while the C-terminal lobe is much larger and is predominantly helical in structure (Figure 6). ATP is bound in a deep cleft between the two lobes where it sits beneath a highly conserved loop that connects stands β1 and β2. This phosphate-binding loop contains a conserved glycine rich sequence motif, G-X-G-X-ϕ-G, where ϕ is usually an aromatic amino acid. The glycine residues approach the α- and β-phosphate groups of ATP very closely and help co-ordinate them via backbone interactions, while the conserved aromatic side chain caps the site of phosphate transfer (Hanks and Hunter, 1995; Huse and Kuriyan, 2002). The adenine ring of the nucleotide is enclosed in a hydrophobic pocket formed by surrounding residues (Hanks and Hunter, 1995).

Peptide substrates bind to the catalytic domain in an extended conformation across the front of the nucleotide-binding pocket, and are brought into close proximity to the γ-phosphate group of ATP. A centrally located loop, called the activation loop, provides a platform for the peptide substrate and interacts with residues adjacent to the phosphoacceptor. In most protein kinases, this loop is phosphorylated when the catalytic domain is active. Phosphorylation has been shown to stabilise the activation loop in an open conformation that is permissive to substrate binding (Yamaguchi and Hendrickson, 1996), however, the number and exact placement of phosphorylation sites varies with individual kinases. In the unphosphorylated state, the activation loop often collapses back into the active site to block the binding of ATP and substrate, and is therefore an important autoinhibitory element for many protein kinases (Hubbard *et al.*, 1994; Xu *et al.*, 1997).

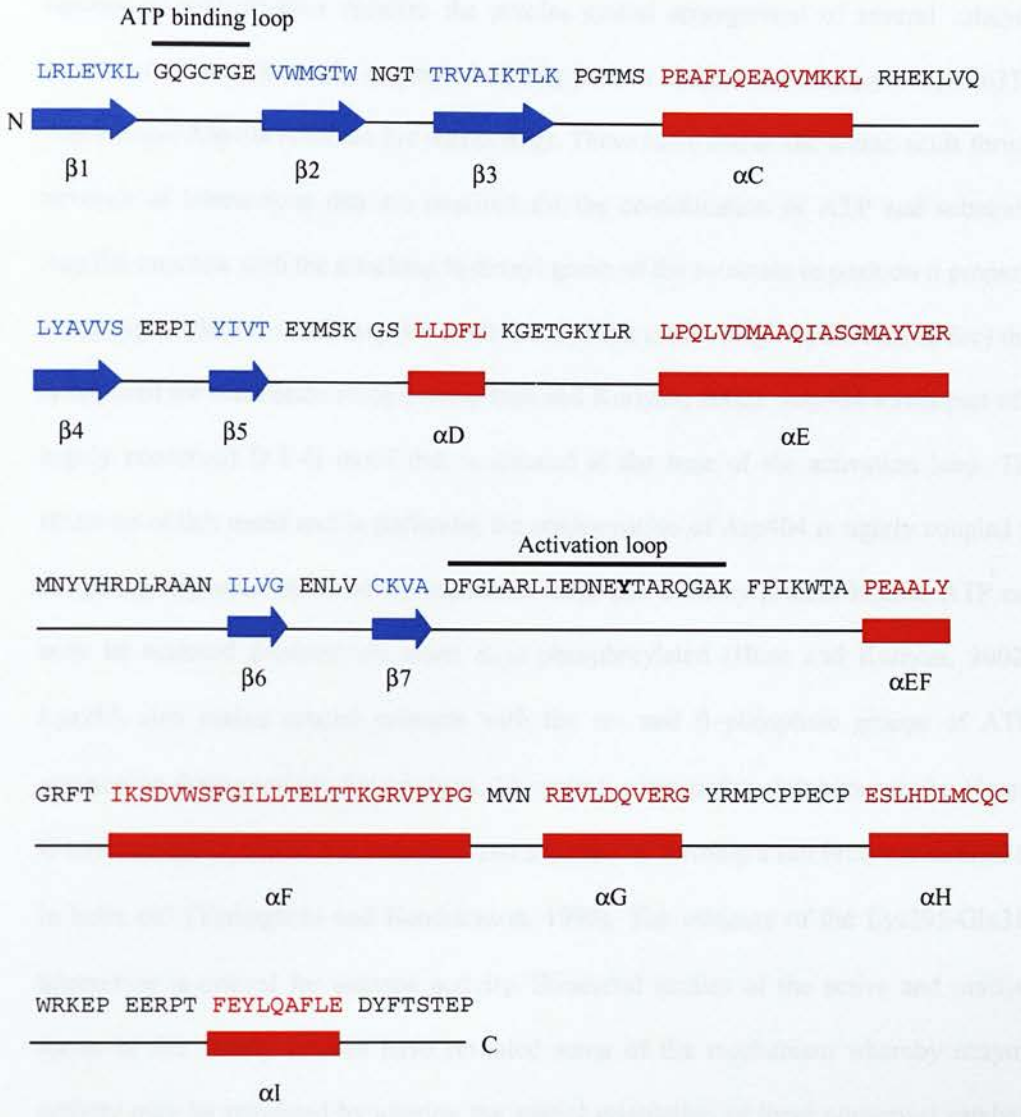


**Figure 6: Organisation of the catalytic domain of Src**. The catalytic domain of Src is shown, red and blue boxes indicate the positions of α-helices and β-strands, respectively. The N-terminal lobe is comprised of strands β1, β2, β3, β4, β5 and helix αC. The C-terminal lobe is comprised of strands β7, β8 and helices αD, αE, αEF, αF, αG, αH and αI. The ATP binding loop and activation loop are highlighted. Adapted from Xu *et al.* (1997).

Optimal phosphotransfer requires the precise spatial arrangement of several catalytic residues that are absolutely conserved among protein kinases, namely Lys295, Glu310, Asp386 and Asp404 (chicken Src numbering). These basic and acidic amino acids form a network of interactions that are required for the co-ordination of ATP and substrate. Asp386 interacts with the attacking hydroxyl group of the substrate to position it properly for phosphorylation, while Asp404 binds the divalent cation ($Mg^{2+}$ in the case of Src) that is required for nucleotide recognition (Huse and Kuriyan, 2002). Asp404 forms part of a highly conserved D-F-G motif that is situated at the base of the activation loop. The structure of this motif and in particular the conformation of Asp404 is tightly coupled to the phosphorylation status of the activation loop, and in many protein kinases ATP can only be oriented productively when it is phosphorylated (Huse and Kuriyan, 2002). Lys295 also makes crucial contacts with the α– and β–phosphate groups of ATP, positioning them properly for catalysis. This residue lies in the cleft between the N- and C-terminal lobes, where it is stabilised and oriented by forming a salt bridge with Glu310 in helix αC (Yamaguchi and Hendrickson, 1996). The integrity of the Lys295-Glu310 interaction is critical for enzyme activity. Structural studies of the active and inactive forms of Src family kinases have revealed some of the mechanism whereby enzyme activity may be regulated by altering the spatial orientation of these conserved catalytic residues (Xu *et al.*, 1997; Yamaguchi and Hendrickson, 1996).

**Regulation of the Src family kinases**

The Src family non-receptor tyrosine kinases play an important role in signal transduction by many cell surface receptors, including receptor tyrosine kinases, integrins, and GPCRs. To date, nine family members have been cloned in mammals (Src, Yes, Yrk, Fyn, Fgr, Lyn, Hck, Lck and Blk). While Src, Fyn and Yes are ubiquitously expressed, the other family members are much more restricted in their tissue distribution. Each shares a common modular structure consisting of a unique N-terminal domain that

contains a myristoylation sequence, an SH3 domain, an SH2 domain, a catalytic domain, and a short C-terminal tail that includes an important conserved tyrosine residue (Tyr527 in chicken Src) (Figure 7).

Myristoylation of Src family kinases allows them to associate with cell membranes. All N-myristoylated proteins begin with the sequence Met-Gly. The initiating methionine is removed by methionine amino peptidase and myristate, a 14 carbon saturated fatty acid moiety, is attached to the α-amino group of the N-terminal glycine residue via a stable amide link in a reaction that is catalysed by N-myristoyl transferase (Gordon *et al.*, 1991). Studies of myristoylated proteins have established that the addition of myristate occurs co-translationally and that the N-terminal glycine is absolutely required for this reaction, however, no strict consensus sequence has been identified (Wilcox *et al.*, 1987). Covalent modification by fatty acid acylation occurs on a wide variety of signalling proteins and plays a key role in controlling their subcellular localisation and therefore interaction with substrates and regulatory molecules. Indeed, non-myristoylated, activated mutant forms of Src are incapable of transforming cells (Kamps *et al.*, 1985), indicating that membrane targeting is crucial for enzyme function.

Because the Src family kinases are potent oncogenes, they are under tight control and have low basal enzyme activity. Structural and biochemical studies have shown that they are maintained in an assembled inactive conformation by intramolecular interactions involving their SH2 and SH3 domains (Eck *et al.*, 1994; Williams *et al.*, 1997; Xu *et al.*, 1997). In quiescent cells, the conserved tyrosine residue in the C-terminal tail is phosphorylated by C-terminal Src kinase (Csk) (Nada *et al.*, 1991; Okada *et al.*, 1991), thereby creating a binding site (pY-Q-P-G) for the SH2 domain. When the SH2 domain binds to the phosphorylated tail, it causes the molecule to adopt a closed conformation in which the N- and C-terminal lobes of the catalytic domain are not properly aligned for catalysis. This is further stabilised by the interaction of the SH3 domain with a proline

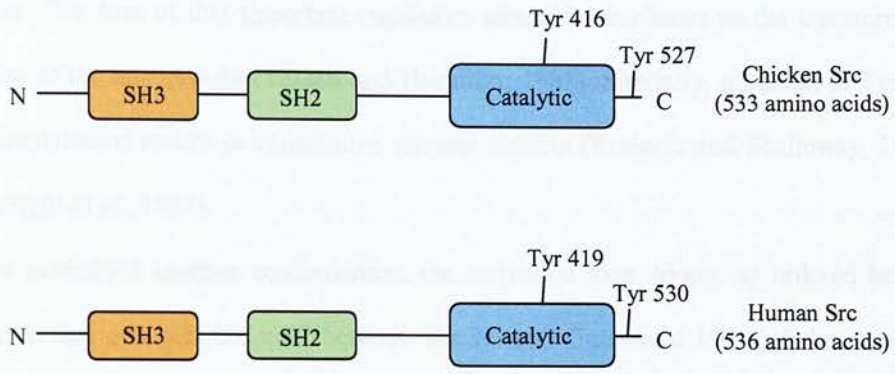


**Figure 7: Structural organisation of chicken and human Src.** Src has a modular structure comprising an SH3 domain, an SH2 domain and a conserved catalytic domain. The N-terminus contains a myristoylation sequence. The C-termius contains two regulatory tyrosine residues, Tyr416 and Tyr527 in chicken Src, and Tyr419 and Tyr 530 in human Src. Tyr416/419 lie within the activation loop of the catalytic domain.

rich sequence in the linker between the SH2 and catalytic domains (Superti-Furga *et al.*, 1993). In v-Src, there is a C-terminal truncation that deletes the conserved tyrosine residue. The loss of this important regulatory element contributes to the transforming activity of the oncoprotein (Takeya and Hanafusa, 1983). Similarly, mutation of Tyr527 to phenylalanine results in constitutive enzyme activity (Kmiecik and Shalloway, 1987; Cartwright *et al.*, 1987).

In the assembled inactive conformation, the activation loop adopts an ordered helical structure that occupies the cleft between the N- and C-terminal lobes of the catalytic domain (Xu *et al.*, 1999). This prevents substrate binding in two ways; the helix physically occupies the space where the peptide should sit, and it stabilises the activation loop in a conformation in which the peptide recognition site is not fully formed (Xu *et al.*, 1997). The regulatory phosphorylation site in the activation loop, Tyr416, intercalates into a hydrophobic pocket that is formed by Phe278, Ile411, Phe424 and Pro425 and thus is unavailable for phosphorylation (Xu *et al.*, 1999). The interactions around Tyr419 also stabilises the closed conformation of the catalytic domain by helping to fix the relative orientation of the N- and C-terminal lobes (Xu *et al.*, 1999). Residues at the base of the activation loop interact closely with the N-terminal lobe, causing helix αC to swing away from the active site, which prevents Glu310 from interacting with Lys295 (Xu *et al.*, 1997). In the inactive conformation, Glu310 is stabilised by forming a salt bridge with Arg409 and by interactions with Tyr382, while Lys295 forms a salt bridge with Asp404 (Xu *et al.*, 1999). This results in a non-productive alignment of the phosphate groups of ATP.

During Src activation, the C-terminal regulatory tyrosine is dephosphorylated or displaced from the SH2 domain by a high affinity ligand. Protein tyrosine phosphatase α (PTPα) and protein tyrosine phosphatase 1B (PTP1B) have been proposed to regulate Src activity by dephosphorylating pTyr527. Evidence for this has been provided from studies

of fibroblasts from PTPα and PTP1B knockout mice, which have reduced levels of Src activity *in vitro* (Ponniah *et al.*, 1999; Dadke and Chernoff, 2003). Binding of the SH2 domain of Src to high affinity phosphopeptide ligands in receptor tyrosine kinases such as the epidermal growth factor receptor (EGFR), and focal adhesion kinase (FAK) also leads to Src activation (Oude *et al.*, 1994; Eide *et al.*, 1995). The release of the inhibitory intramolecular interactions allows Src to adopt an open conformation in which the N- and C-terminal lobes of the catalytic domain are aligned for catalysis and the activation loop is disordered. Helix αC rotates inwards, allowing Lys295 to form a salt bridge with Glu310, thereby freeing Asp404 (Xu *et al.*, 1999). Lys295 and Asp404 can then orient the phosphate groups of ATP correctly for nucleophilic attack. Once these changes have taken place, the catalytic domain becomes active and autophosphorylates Tyr416 (Kmiecik *et al.*,, 1988). The phosphorylated activation loop then packs against the C-terminal lobe of the kinase domain to form the substrate recognition site and is held in place by co-ordination of the phosphorylated tyrosine by two conserved arginine residues, Arg385 and Arg409 (Yamaguchi and Hendrickson, 1996; Xu *et al.*, 1999). The catalytic domain can then bind and phosphorylate substrate molecules containing the consensus sequence Y*-I/V-E/D-E/D (Songyang *et al.*, 1995b), where the residue marked by an asterix represents the phosphoacceptor. However, as the catalytic domain is not very stringent, the SH2 and SH3 domains also play a key role in substrate selection (Pellicena and Miller, 2002). Src is inactivated by the dephosphorylation of pTyr416, by an as yet unidentified protein tyrosine phosphatase, and by phosphorylation of Tyr527 by Csk.

Many Src substrates are phosphorylated at multiple sites *in vitro*. In general, multiple phosphorylations of a protein can proceed by either a processive or a distributive mechanism (Scott and Miller, 2000). In a distributive mechanism, each phosphorylation results form a separate collision between enzyme and substrate. In a processive

mechanism, however, the kinase remains bound to its substrate until it completes all of the phosphorylations; therefore only one collision between enzyme and substrate is required. The presence of ligands for Src SH2 and/or SH3 domains in substrate molecules promotes their stable association with Src and allows processive phosphorylation to occur (Scott and Miller, 2000; Pellicena and Miller, 2001). Therefore, modular protein-interaction domains also regulate the kinetics of Src activity.

**Regulation of PKCs**

Protein phosphorylation and modular interaction domains also play an important role in regulating the activity of the PKC isozymes, though the mechanism involved is somewhat different from that described for the Src family kinases. The PKCs comprise a family of ten related serine/threonine kinases that have been divided into three groups named conventional, novel and atypical, based on their domain structure and mechanism of activation (Figure 8) (Newton, 1997). The conventional PKCs ($\alpha$, $\beta$I, $\beta$II, $\gamma$ ) were the first to be identified and consist of a conserved catalytic domain, two C1 domains (designated C1A and C1B), a $Ca^{2+}$-dependent C2 domain, and an auto-inhibitory pseudosubstrate domain. They are activated by the $Ca^{2+}$-dependent binding of the C2 domain to phosphatidylserine, and by the C1 domains binding diacylglycerol. The novel PKCs ($\eta$, $\varepsilon$, $\delta$, $\theta$) have a similar domain structure, consisting of a conserved catalytic domain, two C1 domains (also designated C1A and C1B), a $Ca^{2+}$-independent C2 domain, and a pseudosubstrate domain. They are activated by the $Ca^{2+}$-independent binding of the C2 domain to phosphatidylserine, and by the C1 domains binding diacylglycerol. The atypical PKCs ($\iota$, $\zeta$) are smaller molecules, consisting of a conserved catalytic domain, a single C1 domain and a pseudosubstrate domain. They are activated by the C1 domain binding phosphatidylserine, however, they shall not be discussed further in this section. Recent data indicates that the two C1 domains of the conventional

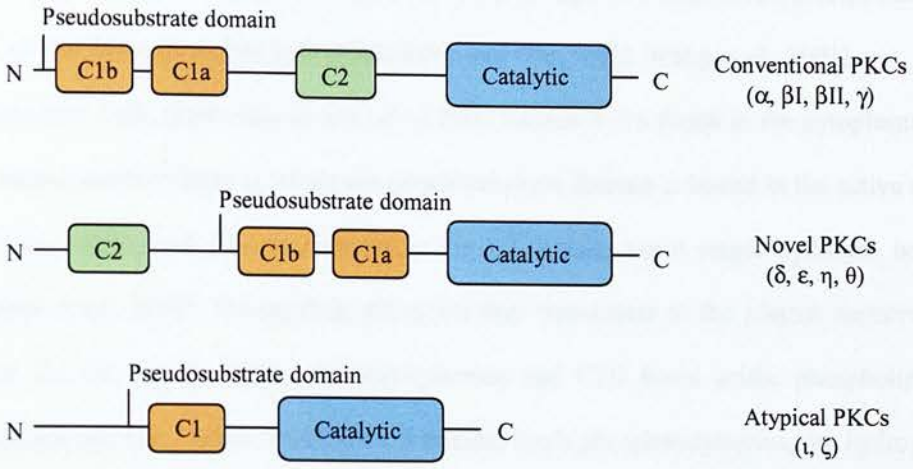


**Figure 8: Structural organisation of the PKC isozymes**. The PKCs have been organised into three groups named conventional, novel and typical based on their domain structure and mechanism of activation. The conventional PKC consist of a conserved catalytic domain, two C1 domains (designated a and b), a $Ca^{2+}$-dependent C2 domain and a pseudosubstrate domain. The sequence of the pseudosubstrate domain fits the consensus sequence for the catalytic domain but lacks a phosphorylatable serine or threonine residue. The novel PKCs consist of a conserved catalytic domain, two C1 domains, a $Ca^{2+}$-independent C2 domain and a pseudosubstrate domain. The atypical PKCs consist of a conserved catalytic domain, a C1 domain and a pseudosubstrate domain.

and novel PKCs are not equivalent. *In vitro* studies have shown that C1A has a much higher affinity for diacylglycerol than C1B, and they appear to play different roles during PKC activation as described below (Medkova and Cho, 1999; Wang *et al.*, 2001).

In quiescent cells, conventional and novel PKC isozymes are found in the cytoplasm in an inactive conformation in which the pseudosubstrate domain is bound in the active site (Newton, 1997), and C1A is tethered to the C2 domain via a single hydrogen bond (Bittova *et al.*, 2001). During their activation they translocate to the plasma membrane where the C2 domain binds phosphatidylserine and C1B binds acidic phospholipids (Medkova and Cho, 1999). When the C2 domain binds phosphatidylserine, the hydrogen bond that connects it to C1A is broken and the protein undergoes a conformational change that induces the insertion of C1A into the lipid bilayer (Bittova *et al.*, 2001). Membrane penetration allows the side chains of hydrophobic residues in the ligand binding surface of C1A to interact with diacylglycerol and drives the release of the pseudosubstrate domain form the active site (Newton, 1997; Medkowa and Cho, 1999; Bittova *et al.*, 2001). The catalytic domain can then phosphorylate target molecules containing the consensus sequence R/K-X-S*/T*-X-R/K, where X represents any amino acid, and the residue marked by an asterix represents the phosphoacceptor (Kennelly and Krebs, 1991).

The residence time of PKCs at the plasma membrane is relatively short and their activation is transient unless they are stabilised by other mechanisms. Activated, PKCs have been shown to bind to a family of intracellular proteins called receptor for activated C Kinase (RACK), which maintain them in an active conformation and target them to particular locations within the cell, where they interact with their substrates (Schechtman and Mochly-Rosen, 2001). Each PKC isozyme has its own specific RACK, which accounts for the differences observed in the subcellular localisations of the active enzymes (Kiley *et al.*, 1990; Mochly-Rosen *et al.*, 1990; Disatnik *et al.*, 1994). For

example, RACK1 binds PKCβII (Ron *et al.*, 1994) and targets it to integrins (Liliental and Chang, 1998), while RACK2 binds PKCε and targets it to the golgi (Csukai *et al.*, 1997). Therefore, the interaction of PKC isozymes with their respective RACKs plays an important role in regulating access of the active kinase to pools of substrate molecules and may ultimately determine the outcome of PKC activation (Mochly-Rosen, 1995). Binding of PKCε to RACK2 has also been reported to increase the efficiency of substrate phosphorylation (Csukai *et al.*, 1997). Therefore, interactions with RACKs may also regulate the kinetics of enzyme activity.

Before the conventional and novel PKC isozymes can be activated by extracellular signalling molecules, they must undergo a process of enzyme maturation. The earliest translational products are unphosphorylated and are found in association with the cytoskeleton (Shirai and Saito, 2002). The initial step in the maturation process is the phosphorylation of Thr500 (PKC-βII numbering) in the activation loop by phosphoinositde dependent kinase 1 (PDK-1) (Dutil *et al.*, 1998). This aligns the active site for catalysis, and permits subsequent autophosphorylation of Thr641 and Ser660 (Keranen *et al.*, 1995; Dutil *et al.*, 1998). Once these residues are phosphorylated, the enzyme assumes its mature conformation and it becomes cytoplasmic (Shirai and Saito, 2002). This series of phosphorylation events is required for the catalytic competence and correct intracellular localisation of the conventional and novel PKCs in quiescent cells and therefore represents one of the rate limiting steps for PKC activation. The exception is PKCδ, as phosphorylation of Thr505 in the activation loop is not required for its catalytic activity. This is because the negative charge of the phosphate group is compensated for by a glutamic acid residue (Glu500) that is adjacent to the phosphorylation site (Stempka *et al.*, 1997; Gschwendt, 1999).

**GnRH physiology**

GnRH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly.$NH_2$) plays a pivotal role in the control of reproduction in mammals. It is synthesised by a small population of neurones in the median basal hypothalamus whose axons project to the median eminence. GnRH is secreted in a pulsatile manner into the hypophyseal portal blood vessels, and is carried to the anterior pituitary where it binds to its cognate receptor on the surface of gonadotroph cells to stimulate the synthesis and secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH), which regulate gonadal function. Each pulse of GnRH stimulates a pulse of LH secretion, however, FSH pulses are less distinct and it appears to be constitutively released (Millar, 2002).

The gonadotrophins are structurally related glycoprotein hormones that are composed of a common α subunit (αGSU) and a hormone-specific β subunit (LHβ and FSHβ) that are non-covalently associated. In the rat, the gene encoding αGSU is located on chromosome 5, while the genes encoding LHβ and FSHβ are located on chromosomes 1 and 3, respectively. *In vivo* and *in vitro* studies have shown that the expression of each gonadotrophin subunit gene is differentially regulated by GnRH (Haisenleder *et al.*, 1991; Haisenleder *et al.*, 1993; Haisenleder *et al.*, 1997).

**The mammalian GnRH receptor**

The mammalian GnRH receptor is a member of the rhodopsin family of G-protein coupled receptors (GPCRs), which have a common structure consisting of an N-terminal extracellular domain and seven transmembrane domains, connected by three intracellular and three extracellular loops. However, the GnRH receptor is unique among the GPCR superfamily as it does not possess an intracellular C-terminal tail (Tsutsumi *et al.*, 1992; Eidne *et al.*, 1992). The cytoplasmic tails of GPCRs play a critical role in regulating signal transduction, and are phosphorylated by a number of serine/threonine kinases,

including caesin kinase 2 (CK2), protein kinase A (PKA), PKC and the G-protein coupled receptor kinases (GRKs) (Chaung *et al.*, 1996). Phosphorylation of the tail (and intracellular loops) causes uncoupling from downstream signalling, and targets the receptor for internalisation, a process referred to as desensitisation (Chaung *et al.*, 1996). Because the mammalian GnRH receptor lacks a cytoplasmic tail and is not phosphorylated, it is not subject to rapid (seconds to minutes) agonist induced desensitisation (Willars *et al.*, 1999). This feature may be physiologically important as gonadotroph cells are required to remain responsive to repeated pulses of GnRH, which would otherwise cause the down regulation of a rapidly desensitising receptor (Willars *et al.*, 1999).

The rat and mouse GnRH receptors have been shown to be glycosylated at Asn4 and Asn18, within the N-terminal domain, while the human receptor is glycoslated at Asn18 (Millar, 2002). Ligand binding studies have shown that glycosylation plays an important role in receptor expression and/or stability, and mutation of these conserved asparagine residues to glutamine decreases cell surface expression (Davidson *et al.*, 1995). The GnRH receptor is also stabilised by the formation of disulphide bonds between Cys14 in the N-terminal domain and Cys199 in extracellular loop 2, and between Cys144 in extracellular loop 1 and Cys195 in extracellular loop 2 (rat GnRH receptor numbering), and mutation of these residues to alanine also decreases cell surface expression (Cook and Eidne, 1997).

Though the three dimensional structure of the mammalian GnRH receptor is not known, molecular models have been generated based on the structure of bovine rhodopsin, the only GPCR to be crystallised to date (Palczewski *et al.*, 2000). The seven transmembrane domains are thought to form a tight bundle enclosing a hydrophilic pocket. GnRH is thought to bind within this pocket, where it makes contact with residues in the

extracellular loops and in the superficial regions of the transmembrane domains (Millar *et al.*, 2004).

**GnRH receptor-ligand interactions**

Short peptides such as GnRH are highly flexible in solution, and are thought to be able to adopt many different conformations (Momany, 1976). However, studies by Maliekal *et al.* (1997) indicate that GnRH binds to its receptor in a β-II' type conformation, in which the N- and C-termini of the peptide are closely apposed in space in a horseshoe structure. This conformation is stabilised by the formation of hydrogen bonds between pGlu1 and Gly10.NH2, and between Ser4 and Arg8 (Maliekal *et al.*, 1997). Mutagenesis studies have identified a number of putative sites of interaction between ligand and receptor. Arg8 of GnRH is thought to form an electrostatic interaction with Glu301 (in the human, the equivalent residue is Asp302) at the top of transmembrane domain 7, and mutation of this residue to glutamine reduces GnRH binding affinity by approximately fifty fold (Flanagan *et al.*, 1994). The interaction of Arg8 with this conserved acidic residue is thought to induce or stabilise the β-II' type conformation of the ligand (Fromme *et al.*, 2001), which facilitates the interaction of pGlu1 and His2 with Lys121 in transmembrane domain 3 (Zhou *et al.*, 1995; Millar, 2002), and Gly10.$NH_2$ with Asn102 in transmembrane domain 2 (Davidson *et al.*, 1996).

**GnRH receptor signalling**

When GnRH binds to its receptor, it is thought to bring about a change in the orientation of the transmembrane domains. These changes are in turn transmitted to the intracellular loops and thus uncover previously masked binding sites for G-proteins (Millar *et al.*, 2004), which are heterotrimeric molecules composed of an α, β and γ subunit (Hamm, 1998). The α subunit is responsible for binding and hydrolysing guanine nucleotides, while the β and γ subunits form a dimer that is not dissociable except by denaturation

(Hamm, 1998). The mammalian GnRH receptor has been shown to couple to Gq and G11 (Hsieh and Martin, 1992). Mutational studies have shown that Ala260 and Arg261, at the C-terminus of intracellular loop 3, are important sites of interaction with these proteins. Mutation of Ala260 to leucine, isoleucine, lysine, glutamate or phenylalanine causes uncoupling of the receptor from downstream signalling (Myburgh *et al.*, 1998). Similarly, mutation of Arg261 to glutamine also causes uncoupling. This mutation has been found in some cases of hypogonadotrophic hypogonadism in the human (de Roux *et al.*, 1999).

In the inactive state, the α subunit of the G-protein has guanine diphosphate (GDP) bound in the active site, and has a high affinity for the βγ dimer. When Gq or G11 interact with an activated GnRH receptor, the α subunits exchanges bound GDP for guanine trisphosphate (GTP) and undergoes a conformational change that reduces its affinity for the βγ dimer leading to its dissociation from the complex (Hamm, 1998). Both the α subunit and the βγ dimer can then interact with their effectors to initiate downstream signalling. Signalling is terminated by the intrinsic GTPase activity of the α subunit, which hydrolyses GTP to GDP. When the α subunit has GDP bound in the active site, it has a low affinity for its effectors, thereby promoting its re-association with the βγ dimer (Hamm, 1998). The GTPase activity of the α subunit is stimulated by members of the RGS family of proteins, which bind to it via their conserved RGS domain and stimulate GTP hydrolysis (Hamm, 1998).

Both Gαq and Gα11 have been shown to activate PLC-β1, which hydrolyses PtdIns(4,5)$P_2$ in the plasma membrane to generate the second messengers Ins(1,4,5)$P_3$ and diacylglycerol (Smrcka and Sternweis, 1993). PLC-β1 is a modular protein consisting of an N-terminal PH domain, four EF hand motifs, a catalytic domain, a $Ca^{2+}$-independent C2 domain, and a 400 amino acid long C-terminal tail. The PH domain binds inositol lipids and targets PLC-β1 to the plasma membrane (Wang *et al.*, 1999a), while the C2 domain binds Gαq, leading to the allosteric activation of the catalytic domain

(Wang *et al.*, 1999b). Interestingly, the C2 domains of the PLCs do not appear to bind phospholipids and therefore represent a novel protein-interaction module, with functions that are distinct from those of other C2 domains (Wang *et al.*, 1999b). The C-terminal tail of PLC-β1 also contacts Gαq and is required for the full activation of the catalytic domain. In addition, it functions as a GTPase activating protein, which stimulates the hydrolysis of GTP by Gαq, to terminate G-protein signalling (Paulssen *et al.*, 1996).

The $Ins(1,4,5)P_3$ that is generated by the PLC-β1 mediated hydrolysis of $PtdIns(4,5)P_2$ binds to its intracellular receptor in the membrane of organelles to trigger the release of $Ca^{2+}$ from non-mitochondiral stores, which stimulates LH secretion (Stojilkovic *et al.*, 1994). Studies of cultured rat gonadotroph cells have shown that GnRH preferentially stimulates the release of $Ca^{2+}$ from structures called subsurface cisternae, which lie just beneath the plasma membrane, close to the sites of hormone secretion (Tse *et al.*, 1997). $Ca^{2+}$ entry through voltage gated $Ca^{2+}$ channels does not appear to stimulate hormone exocytosis in these cells (Tse *et al.*, 1993). $Ca^{2+}$ has also been shown to stimulate gonadotrophin subunit gene transcription and therefore also plays a role in regulating gonadotrophin synthesis (Haisenleder *et al.*, 2001).

Many of the effects ascribed to $Ca^{2+}$ are mediated by the serine/threonine kinase $Ca^{2+}$/calmodulin dependent kinase II (CaMKII), which is activated by binding $Ca^{2+}$/calmodulin. CamKII exists as a holoenzyme complex composed of eight to twelve α, β, γ and δ subunits, mixed together at random (Bennet *et al.*, 1983; Kuret and Schulman, 1984). Each subunit contains a conserved catalytic domain, an autoinhibitory pseudosubstrate domain, and a C-terminal association domain that is necessary and sufficient for holoenzyme formation (Shen and Meyer, 1998; Hudmon and Schulman, 2002). They are organised into a ring like structure that allows them to behave independently of one another for enzyme activity and $Ca^{2+}$/calmodulin binding (Hudmon and Schulman, 2002). In quiescent cells, the pseudosubstrate domain is bound in the

active site, preventing phosphorylation of potential substrates. When a CaMKII subunit binds $Ca^{2+}$/calmodulin, it undergoes a conformational change that drives the release of the pseudosubstrate domain from the active site. Binding of $Ca^{2+}$/calmodulin (to the subunits of CaMKII) is 1:1 in solution (Meyer *et al.*, 1992), and maximal activation of the holoenzyme appears to require stoichiometric binding (Katoh and Fujisawa, 1991). The catalytic domain then autophosphorylates a conserved threonine residue (Thr286 in the α subunit, Thr287 in the β, γ and δ subunit), within the pseudosubstrate domain. As a result, the enzyme becomes autonomous in its activity and no longer requires $Ca^{2+}$/calmodulin to be bound (Hanson *et al.*, 1989; Waxham *et al.*, 1990). Subsequent dephosphorylation of this residue converts CaMKII back to its $Ca^{2+}$/calmodulin dependent state (Lai *et al.*, 1986; Schworer *et al.*, 1986). In cultured rat gonadotroph cells, GnRH has been shown to activate CaMKII by inducing the autophosphorylation of Thr286/287, and CamKII activity is required for the induction of αGSU, LHβ and FSHβ gene expression by GnRH (Haisenleder *et al.*, 2003). However, the substrates of CaMKII in these cells are currently unknown.

The second messenger activities of $Ins(1,4,5)P_3$ and $Ca^{2+}$ are tightly regulated in cells. $Ins(1,4,5)P_3$ signalling is terminated by the actions of an inositol-5-phosphatase, which dephosphorylates the D5 position of the inositol ring to produce $I(1,4)P_2$, and by an inositol-3-kinase which phosphorylates the D3 position of the inositol ring to produce $Ins(1,3,4,5)P_4$ (Shears, 1992). Neither of these molecules is able to bind the $Ins(1,4,5)P_3$ receptor or trigger $Ca^{2+}$ release from internal stores. $Ca^{2+}$ is pumped back into organelles or out of the cell by a membrane bound $Ca^{2+}$ pump, to return the cytoplasmic concentration of the ion to a low level of around 100 nM in resting cells (Alberts *et al.*, 1994).

The diacylglycerol that is generated by the PLC-β1 mediated hydrolysis of $PtdIns(4,5)P_2$ allosterically activates conventional and novel PKC isozymes as previously described.

PKC has been shown to play an important role in GnRH receptor signalling (Stojilkovic *et al.*, 1994). In cultured rat gonadotroph cells, activation of PKC with phorbol esters or the diacylglycerol analogue sn-1,2-dioctanoylglycerol stimulates αGSU and LHβ gene expression, and LH secretion (Haisenleder *et al.*, 1995). However, like CaMKII, the substrates of PKC in these cells are currently unknown.

The activity of conventional and novel PKC isozymes is negatively regulated by the DGKs, which phosphorylate diacylglycerol to produce phosphatidic acid. To date, nine isoforms of DGK have been identified in mammals (Topham and Prescott, 1999), which have been divided into five groups based on their structural organisation (Figure 9). All of the DGKs contain a bipartite catalytic domain that is responsible for phosphorylating diacylglycerol, and either two or three C1 domains (Topham and Prescott, 1999). The C1 domain closest to the catalytic domain is highly conserved and features a unique 15 amino acid extension that is required for efficient substrate phosphorylation. It has been proposed that this sequence helps the C1 domain to present diacylglyerol to the catalytic domain, though how this is achieved is currently not understood (van Blitterswijk and Houssa, 2000).

The catalytic domain of the DGKs shares some sequence similarity with those of the protein kinases, including a putative ATP binding loop that contains the conserved sequence motif G-X-G-X-X-G, where X represents any amino acid (Luo *et al.*, 2004). However, in several DGK isoforms the two lobes of the catalytic domain are separated by a 300 amino acid long serine/threonine rich insertion, indicating that they may function as two independent units for catalysis (Luo *et al.*, 2004a). Such long inserts are not seen in the catalytic domain of any known protein kinase. In addition, the DGKs feature a wide variety of protein- and lipid-interaction domains, including elongation factor (EF) hand

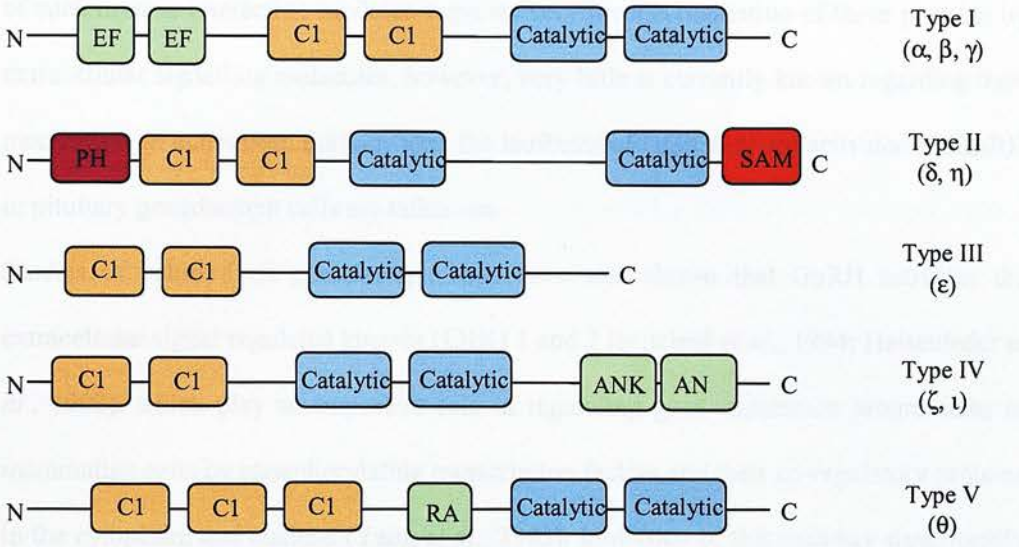


**Figure 9: Structural organisation of the diacylglycerol kinase isozymes**. Mammalian DGKs are divided into five families (Type I-V) according to their domain structure. All dicaylglycerol kinases share a common bipartite catalytic domain and two or three C1 domains. In addition, the posses a wide variety of modular interaction domains which define their class. Type I DGKs have two EF hands motifs (EF) that bind calcium. Type II DGK have a PH domain that binds phosphoinositides and a SAM domain that mediates homo- and hetero-oligomerisation of proteins. Type III DGKs are the simplest in structure and do not contain any other recognisable domains. Type IV DGKs have two tandem Ankyrin repeats (ANK) that may mediate protein-protein interactions. Type V DGKs have three C1 domain and an Ras association (RA) domain that is found in several proteins that interact with the small GTPase Ras.

motifs, ankyrin repeats, and pleckstrin homology (PH) domains (Figure 9). The presence of such diverse interaction modules suggests very precise regulation of these proteins by extracellular signalling molecules, however, very little is currently known regarding their mechanism of activation. Furthermore, the isoforms of DGK that are activated by GnRH in pituitary gonadotroph cells are unknown.

Studies of cultured rat gonadotroph cells have also shown that GnRH activates the extracellular signal regulated kinases (ERK) 1 and 2 (Mitchell *et al.*, 1994; Haisenleder *et al.*, 1998), which play an important role in regulating gene expression programmes in mammalian cells by phosphorylating transcription factors and their co-regulatory proteins in the cytoplasm and nucleus (Yang *et al.*, 2003). Inhibition of this pathway significantly inhibits αGSU and FSHβ gene transcription in these cells in response to GnRH (Haisenleder *et al.*, 1998), indicating that these kinases play an important role in the control of gonadotrophin synthesis.

ERK 1 and 2 are activated through a conserved cascade of protein kinases often operating downstream of the small GTPase Ras, which is targeted to the plasma membrane by fatty acid acylation and/or prenylation (Resh, 1996). Ras is activated by extracellular signalling molecules through the membrane recruitment of guanine nucleotide exchange factors such as Son-of-sevenless (Sos), which stimulate it to exchange bound GDP for GTP (Aronheim *et al.*, 1994). When Ras binds GTP, it undergoes a conformational change (Hu and Redfield, 1993) that allows it to interact with the serine/threonine kinase Raf-1 via its effector domain. Raf-1 is thereby recruited to the plasma membrane where it is activated. The mechanism of Raf-1 activation by extracellular signalling molecules is complex and remains poorly understood, however, protein phosphorylation by Src family kinases has been shown to be important in this process. Src phosphorylates Raf-1 at Tyr340 and Tyr341 (Marais *et al.*, 1995), which lie just N-terminal to the catalytic domain. Phosphorylation of these residues is thought to bring about a conformational

change in the molecule that contributes to its activation (Cutler *et al.*, 1998). Furthermore, mutation of these amino acids to phenylalanine has been shown to abolish Raf-1 activity *in vitro* (Marais *et al.*, 1995). In addition, Raf-1 can be activated independently of Ras by PKC (Seger and Krebs, 1995; Benard *et al.*, 2001), which has been shown to phosphorylate Ser499 within the activation loop of the catalytic domain (Kolch *et al.*, 1993). Mutation of this residue to alanine abolishes Raf-1 activation by phorbol esters (which activate PKC), but not by Ras and Src (Kolch *et al.*, 1993), indicating that several different signals may converge at the level of Raf-1 to activate the ERK signalling cascade in mammalian cells.

Once activated, Raf-1 phosphorylates the dual specificity serine/threonine and tyrosine kinases MAP and ERK kinase (MEK) 1 and 2 at Ser218 and Ser222 (MEK1 numbering) within their activation loops, leading to their activation. These kinases in turn activate ERK 1 and 2 by phosphorylating them at Thr183 and Tyr185 (ERK 2 numbering) within their activation loops. Phosphorylation of these residues correctly aligns the N- and C-terminal lobes of the catalytic domain for phosphotransfer and leads to the refolding of the activation loop to form the substrate binding pocket (Canagarajah *et al.*, 1997). ERK 1 and 2 then phosphorylate substrates containing the consensus sequence P-X-S*/T*-P, where X represents a neutral or basic amino acid, and the residue marked by an asterix represents the phosphoacceptor (Clark-Lewis *et al.*, 1991; Gonzalez *et al.*, 1991). In contrast, most other serine/threonine kinases actively exclude substrates with proline residues in the S*/T* +1 position (Songyang *et al.*, 1994).

Specificity of signalling through the ERK cascade is maintained by the direct interaction of the successive kinases; Raf-1 binds to MEK 1 and 2, which in turn bind to ERK 1 and 2 (Kolch, 2000). In addition, the scaffolding protein KSR has been shown to bind Raf-1, MEK and ERK *in vitro* and *in vivo*, and can assemble them into an organised signalling module (Therrien *et al.*, 1996; Michaud *et al.*, 1997; Yu *et al.*, 1998). While a low level

of KSR expression has been shown to facilitate ERK activation, over expression of this protein has an inhibitory effect (Cacace *et al.*, 1999). Such a dose-dependent reversal in effect is typical for a scaffolding protein that can only assemble its clients when present in an appropriate stoichiometric ratio, but disperses signalling complexes when over-expressed (Kolch, 2000).

Once activated, ERK 2 has been shown to homodimerise and translocate from the cytoplasm to the nucleus, where it can phosphorylate nuclear substrates (Khokhlatchev *et al.*, 1998). When phosphorylated at Thr183, ERK 2 undergoes a conformational change that exposes a hydrophobic patch on its surface promoting homodimerisation (Canagarajah *et al.*, 1997). Because the size of the ERK 2 dimer exceeds the limit for passive diffusion through the nuclear pore complex (approximately 40 KDa), it must be actively transported into the nucleus. Nuclear import is controlled by a family of proteins called importins, which recognise specific nuclear localisation sequences in target molecules (Hood and Silver, 1999). Because ERK 2 does not contain a recognisable nuclear localisation sequence, dimerisation may facilitate nuclear shuttling by allowing the association of the dimer with an additional transport protein. Alternatively, a functional nuclear localisation sequence may be created from two non-contiguous stretches of amino acids, however, this remains to be determined (Khokhlatchev *et al.*, 1998). The requirement for phosphorylation and dimersation of ERK 2 for nuclear translocation is reminiscent of the mechanism of activation of the signal transducers and activators of transcription (STAT) proteins by cytokine receptors (Sekimoto *et al.*, 1996), and is emerging as a common theme in the control of nucleo-cytoplasmic shuttling of proteins (Hood and Silver, 1999).

Studies in the αT3-1 gonadotroph cell line have shown that GnRH activates ERK 1 and 2 via PKC (Sim *et al.*, 1995; Sundaresan *et al.*, 1996; Reiss *et al.*, 1997), which can directly activate Raf-1 (Benard *et al.*, 2001), and via a protein tyrosine kinase that is required for

the Ras dependent activation of Raf-1 (Benard *et al.*, 2001). Protein tyrosine phosphorylation has been shown to be important for the activation of ERK 1 and 2 by many GPCRs (Della Rocca *et al.*, 1999), however, the identity of the tyrosine kinase(s) involved in the activation of this pathway by GnRH are uncertain. In αT3-1 cells GnRH has been reported to activate Src, and inhibition of Src family kinases with pharmacological inhibitors, or by over-expression of CSK, significantly inhibits ERK activation (Grosse *et al.*, 2000; Benard *et al.*, 2001). In addition, Grosse *et al.* (2000) have shown that the GnRH receptor can *trans*-activate the epidermal growth factor receptor (EGFR) in this cell line, and that EGFR kinase activity is also required for the activation of ERK 1 and 2 (by GnRH). However, several other studies have failed to show EGFR dependent activation of the ERK signalling cascade in αT3-1 cells, indicating that more than one protein tyrosine kinase may be involved (Benard *et al.*, 2001; Bonfil *et al.*, 2004). Benard *et al.* (2001) have reported that GnRH can activate focal adhesion kinase (FAK) in these cells, which has been shown to be involved in the activation of ERK 1 and 2 by many other neuropeptide hormones that bind Gq coupled receptors (Zachary *et al.*, 1992; Sinnett-Smith *et al.*, 1993). Activation of FAK by GnRH may therefore provide an alternative means for activating this signalling pathway, independently of the EGFR. However, as over-expression of FAK-related non-kinase (FRNK), which can function as a dominant negative inhibitor of FAK dependent signalling, did not significantly inhibit the activation of ERK 1 and 2 by GnRH in αT3-1 cells (Benard *et al.*, 2001), it remains to be demonstrated conclusively if FAK is indeed involved. The reason for the differences in results published by these groups is not clear, however, it is possible that under different growth conditions, populations of cells may be selected that exhibit different repertoires of signalling molecules, or preferences for particular pathways.

Several other studies have sought to identify the G-proteins involved in the activation of ERK 1 and 2 by the GnRH receptor. While an early study indicated that ERK activation

by GnRH was mediated by Gi in αT3-1 cells (Sim *et al.*, 1995), subsequent studies have failed to observe any effect of pertussis toxin, which ADP ribosylates and inactivates Gαi (Reiss *et al.*, 1997; Grosse *et al.*, 2000), and the activation of this pathway is generally thought to proceed via Gq/11 (Grosse *et al.*, 2000). Furthermore, a recent study in the LβT2 gonadotroph cell line has shown that ERK activation by GnRH can be blocked by cell permeable inhibitors of Gαq but not Gαi (Liu et al., 2002).

The *trans*-activation of the EGFR by Gq/11 coupled GPCRs has been shown to be mediated by a membrane metalloproteinase that cleaves heparin-binding epidermal growth factor (EGF) from the cell surface. The soluble ligand then acts in an autocrine manner on the EGFR (Prenzel *et al.*, 1999; Dong *et al.*, 1999). Cleavage of heparin-binding EGF is thought to be mediated by metalloproteinases and is sensitive to the broad range metalloproteinase inhibitor batimastat (Schafer *et al.*, 2004). GnRH has been shown to activate several matrix metalloproteinases (MMP), including MMP-2 and -9 (Roelle *et al.*, 2003).

The EGFR is a receptor tyrosine kinase, which belongs to the ErbB/HER family. When it binds ligand, it undergoes a conformational change that leads to receptor dimerisation, activation of the catalytic domain, and *trans*-autophosphorylation. Though the EGFR has emerged as an important drug target in cancer therapy, the mechanism of activation of the catalytic domain remains poorly understood. Unlike other receptor tyrosine kinases, it does not appear to require the phosphorylation of residues within the activation loop for its activity. In the unphosphorylated state, the activation loop assumes an open conformation that is permissive to substrate binding. This is due to the presence of four glutamic acid residues (Glu841, 842, 844 and 847) within the loop that compensate for the negative charge of the phosphate group (Stamos *et al.*, 2002). However, the catalytic domain is somehow maintained in an inactive conformation by the extracellular domain until it binds ligand. Evidence for this is provided for by the oncoprotein v-Erb, in which

truncation of the extracellular portion of the receptor results in constitutive kinase activity, and leads to cellular transformation and cancer (Downward *et al.*, 1984).

Autophosphorylation of the EGFR functions to recruit cytoplasmic proteins to the receptor that couple it to downstream signalling pathways, including the ERK cascade (Schlessinger, 2000). Autophosphorylation of Tyr1068 creates a high affinity binding site (pY-I-N-Q) for the SH2 domain of the adapter protein Growth factor receptor bound 2 (Grb2) (Batzer *et al.*, 1994), which binds Sos with its SH3 domain and thereby recruits it to the activated receptor (Egan *et al.*, 1994). In addition, autophosphorylation of the EGFR at Tyr1148 and Tyr1173 creates docking sites for the adapter protein Shc, which binds pTyr1173 (pY-L-R-V) via its SH2 domain (Batzer *et al.*, 1994), and pTyr1148 (N-P-D-pY) via its phosphotyrosine binding (PTB) domain (Sakaguchi *et al.*, 1998). PTB domains offer an alternative means of recognition of phosphotyrosine motifs in proteins and recognise the core sequence N-P-X-pY, where X represents any amino acid (Blaikie *et al.*, 1994; Gustafson *et al.*, 1995). Shc is then phosphorylated at Tyr317 by Src, which is also recruited to and activated by the EGFR, to create an additional binding site (pY-L-R-N) for the SH2 domain of Grb2 (Gotoh *et al.*, 1997). Once recruited to the EGFR, Sos then activates Ras at the membrane to initiate the activation of ERK 1 and 2.

Several other Gq/11 coupled GPCRs, including those of the neuropeptide hormones bombesin, bradykinin, endothelin, gastrin and vasopressin have been shown to induce the tyrosine phosphorylation of the non-receptor tyrosine kinase FAK (Zachary *et al.*, 1992; Sinnett-Smith *et al.*, 1993). FAK phosphorylation requires the activation of members of the Rho family of small GTPases (Rankin *et al.*, 1994), which play a key role in regulating the organisation of the actin cytoskeleton in eukaryotic cells (Hall 1998). The most extensively characterised members of this family are RhoA, Rac1 and Cdc42. Like Ras, these proteins act as molecular switches that cycle between GTP and GDP bound states. When they bind GTP they undergo a conformational change that allows them to

interact with effector molecules, which regulate actin dynamics. The intrinsic GTPase activity of the proteins then returns them to the inactive GDP bound state to terminate signal transduction (Van Aelst and D'Souza Schorey, 1997). Proper control of the actin cytoskeleton requires the precise spatial and temporal regulation of these proteins, which is orchestrated by a wide range of guanine nucleotide exchange factors and GTPase activating proteins, and by the GDP-dissociation inhibitor Rho-GDI (Bishop and Hall, 2000). In quiescent cells, the Rho family GTPases are found in the cytoplasm in association with Rho-GDI, which maintains them in the inactive (GDP bound) state (Ueda *et al.*, 1990). During activation by extracellular signalling molecules, they dissociate from this complex and translocate to the plasma membrane, where they are activated by their respective guanine nucleotide exchange factors, and can interact with effectors (Bokoch *et al.*, 1994).

Activation of RhoA typically leads to the formation of stress fibres (Ridley and Hall, 1992), which are contractile bundles of actin and myosin filaments (actinomyosin) that resemble tiny myofibrils found in muscle cells (Alberts *et al.*, 1994). The stress fibres are anchored to the plasma membrane at focal adhesions, where aggregated integrin receptors bind to the ECM on the outside of the cell and the actin microfilaments on the inside. Integrins are heterodimeric molecules that are assembled from a pool of 17 α subunits and 8 β subunits, to give rise to 23 different receptors that can bind a wide variety of extracellular matrix proteins (Darribère *et al.*, 2000). Most integrins involved in the formation of focal adhesions belong to the β1 and β3 families. For cells grown in serum, vitronectin is normally the extracellular matrix protein that is adsorbed to the surface of the dish, and focal adhesions form containing the αvβ3 integrin. For cells plated on surfaces coated with fibronectin, focal adhesions normally contain the α5β1 integrin (Burridge and Chrzanowska-Wodnicka, 1996). A large number of cytoplasmic proteins associate with integrins at focal adhesions. The structural proteins α-actinin, talin and

vinculin provide a physical link between the integrin and actin microfilaments. FAK is specifically targeted to focal adhesions by a unique 150 amino acid focal adhesion targeting domain located at its C-terminus (Hildebrand *et al.*, 1993), which contains sequences that are responsible for binding talin and another focal adhesion protein, paxillin (Chen *et al.*, 1995; Hayashi *et al.*, 2002).

The formation of stress fibres and focal adhesions following the activation of RhoA is mediated by Rho kinase, which inhibits the activity of myosin light chain phosphatase (Kimura *et al.*, 1996), and directly phosphorylates the light chains of non-muscle myosin-II (Amano *et al.*, 1996). Light chain phosphorylation induces a conformational change in myosin that promotes the formation of bipolar filaments containing 15 – 20 molecules. It also exposes an actin binding site, which promotes the formation of actinomyosin, and stimulates myosin ATPase activity. This leads to the contraction of actinomyosin, which generates tension within the filament (Alberts *et al.*, 1994). Myosin also cross-links adjacent filaments, and bundles them together to form the prominent stress fibres that are seen following the activation of RhoA. It is the bundling of actinomycin into stress fibres that leads to the aggregation of integrins at the plasma membrane to form focal adhesions (Burridge and Chrzanowska-Wodnicka, 1996).

In contrast, activation of Rac1 and Cdc42 by extracellular signalling molecules stimulates actin polymerisation at the cell cortex, leading to the formation of lamellipodia and filopodia, respectively (Ridley *et al.*, 1992; Nobes and Hall, 1995). These effects are regulated by Rac1 and Cdc42 through their interaction with members of the Wiscott-Aldrich Syndrome protein (WASp)/Scar family of proteins, which stimulate the Arp2/3 complex to nucleate the formation of new actin filaments (Machesky and Insall, 1998; Machesky *et al.*, 1999). Though actin is found at high concentrations in cells (up to 100 μM) it does not spontaneously polymerise due to the inherent instability of dimers and trimers. The Arp2/3 complex stimulates the formation of actin filaments by stabilising

actin trimers to allow the filament to grow beyond this critical length. Once nucleated, new filaments grow rapidly towards the plasma membrane until capped by proteins such as gelsolin and capping protein (Millard *et al.*, 2004). The Arp2/3 complex also binds to the sides and ends of existing actin filaments to stimulate filament branching, creating the dense cortical actin network that is seen in lamellipodia and filopodia (Amann and Pollard, 2001).

The mammalian WASp/Scar family consists of five members; WASp, N-WASp, and Scar-1, -2 and -3. The C-termini of these proteins contains a conserved module that is responsible for binding and activating the Arp2/3 complex (Machesky *et al.*, 1999; Suetsugu *et al.*, 1999). The N-termini of WASp and N-WASp contain a Cdc42/Rac interactive binding (CRIB) domain that binds Cdc42 (Higgs and Pollard, 2000; Rohatgi *et al.*, 2000). The N-termini of the Scar proteins contains a unique Scar homology domain of unknown function (Millard *et al.*, 2004).

In quiescent cells, WASp an N-WASp adopt an assembled inhibitory conformation in which the CRIB domain is bound to the C-terminus of the protein, preventing interactions with the Arp2/3 complex (Kim *et al.*, 2000). When Cdc42 is activated, it undergoes a conformational change that allows it to bind to the CRIB domain, which relieves this inhibitory interaction, and allows WASp and N-WASp to interact with the Arp2/3 complex (Kim *et al.*, 2000). In addition, Src has been shown to phosphorylate WASp at Tyr291, adjacent to the CRIB domain (Cory *et al.*, 2002). Phosphorylation of this residue correlates with increased rates of actin polymerisation *in vitro* and may help to stabilise the active conformation of WASp (Cory *et al.*, 2002). When Cdc42 hydrolyses GTP to GDP, it dissociates from WASp and N-WASP, allowing them to return to their autoinhibited state.

As the Scar proteins do not contain a CRIB domain, their activity is regulated in a very different manner by Rac1. In quiescent cells, Scar1 is found in the cytoplasm in

association with four other proteins, namely PIR121, Nap125, Abi-2 and HSPC300, which maintain it in an inactive state (Eden *et al.*, 2002). When Rac1 is activated by extracellular signalling molecules, it binds to this complex, causing the dissociation of Scar1 and HSPC300. Scar1 can then activate the Arp2/3 complex (Eden *et al.*, 2002).

In addition to stimulating actin polymerisation at the cell cortex, Rac1 and Cdc42 also induce the formation of focal complexes, small clusters of integrins and associated proteins, that provide anchor points for the cell as it extends its plasma membrane to form lamellipodia and filopodia (Nobes and Hall, 1995). Though smaller than focal adhesions, these structures contain many of the same components, including vinculin, paxillin and FAK (Nobes and Hall, 1995; Hotchin and Hall, 1995). Several studies indicate that the GnRH receptor can activate Rac (Levi *et al.*, 1998; Allen *et al.*, 2002), and that Rac is involved in the activation of ERK 1 and 2 by GnRH (Allen et al., 2002).

The aggregation of integrins in focal adhesions and focal complexes (collectively referred to as focal contacts) leads to the tyrosine phosphorylation of FAK, and the activation of ERK 1 and 2 (Chen *et al.*, 1994; Miyamoto *et al.*, 1995). It is thought that when integrins become aggregated in these structures, FAK forms dimers and oligomers that *trans*-autophosphorylate each other at Tyr397 (Leu and Maa, 2002; Katz *et al.*, 2002), though it remains unknown how oligomerisation may regulate the activity of the catalytic domain. Autophosphorylation of Tyr397 creates a high affinity binding site (pY-A-E-I) for the SH2 domain of Src, which recruits it to the focal contact (Eide *et al.*, 1995). Src also binds to FAK via its SH3 domain, which recognises the sequence R-A-L-P-S-I-P-K-L that is approximately twenty amino acids N-terminal to Tyr397 (Thomas *et al.*, 1998). The sequence of this motif is almost identical to the optimal ligand identified for the SH3 domain of Src by Yu *et al.* (1994). By binding to FAK at these sites, the inhibitory intramolecular interactions that maintain Src in the inactive conformation are relieved, leading to the activation of its catalytic domain. Src then phosphorylates FAK on a

number of additional tyrosine residues, including Tyr576, Tyr577, and Tyr925 (Schaller *et al.*, 1999). Tyr576 and Tyr577 lie within the activation loop and phosphorylation of these residues helps to stabilise the active conformation of the catalytic domain, which allows FAK to phosphorylate exogenous substrates (Calalb et al., 1995). Phosphorylation of Tyr925 creates a high affinity binding site (pY-E-N-V) for the SH2 domain of Grb2 (Schlaepfer *et al.*, 1994), which recruits Sos to the plasma membrane, where it activates Ras as described.

The use of either the EGFR or FAK by Gq/11 coupled receptors was originally though to represented functional redundancy within the system. However, recent studies suggest that this may not be the case. Activation of ERK by the EGFR typically results in the translocation of the active enzymes to the nucleus (Horgan and Stork, 2003; Luttrell and Luttrell, 2003). In contrast, ERK activated by FAK localises to focal contacts (Fincham *et al.*, 2000; Luttrell and Luttrell, 2003). Therefore, the mechanism of ERK 1 and 2 activation used by a GPCR may determine the localisation of the active enzymes within the cell and promote the phosphorylation of a particular subset of substrates.

**Aims**

Tyrosine kinases such as the EGFR and FAK play an important role in the activation of ERK 1 and 2 by many Gq/11 coupled GPCRs, creating dockings sites for Grb2, which recruits Sos to the plasma membrane. Because the molecular events that lead to the activation of ERK 1 and 2 by the GnRH receptor were not well understood when this project was started, the initial aim was to investigate the role of protein tyrosine phosphorylation in this pathway. Subsequently, it was decided to use proteomic techniques to identify novel substrates of tyrosine kinases that may be involved in other aspects of GnRH receptor signalling. As Src was found to play a key role in the activation of ERK 1 and 2, and features SH2 and SH3 domains that mediate specific molecular interactions, it was selected as the molecule upon which this study would be

based. A HEK 293 cell line stably expressing the rat GnRH receptor was used as a model for the majority of the experiments described in this thesis.

# Materials and Methods

This chapter details the methods and laboratory techniques used throughout this thesis. Unless otherwise indicated all chemicals and reagents were from Sigma Aldrich. For details of the antibodies and cDNA constructs used during the course of these studies readers are directed to appendices A and B, at the back of this thesis.

**Preparation of competent DH5α cells**

A glycerol stock of *E. coli* strain DH5α was streaked out on a LB-agar plate and grown overnight at 37°C. A single colony was picked, inoculated into 5 ml of LB medium, and incubated for 16 hours at 37°C with constant shaking. 1 ml was then used to inoculate 100 ml of LB medium containing 20 mM $MgSO_4$, and the culture grown until an absorbance (600 nm) of 0.4 – 0.6 was reached. The culture was then chilled on ice and bacteria harvested by centrifugation at 4,500 xg for 5 minutes at 4°C. All subsequent steps were performed in a cold room using pre-chilled solutions and plasticware, as competent cells are highly sensitive to elevated temperatures. The cell pellet was gently resuspended in 40 ml of 30 mM potassium acetate, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 100 mM RbCl, 15% glycerol (pH 5.8) and collected by centrifugation at 4,500 xg for 10 minutes at 4°C. Cells were then suspended in 4 ml of 10 mM PIPES, 75 mM $CaCl_2$, 10 mM RbCl, 15% glycerol (pH 6.5) and divided into 100 µl aliquots. The competent cells were frozen in a dry ice/ethanol bath and stored at –70°C until use.

**Transformation of competent cells**

Cells were thawed on ice and incubated with 10 ng of plasmid DNA for 30 minutes. They were then warmed to 42°C for 30 seconds and returned to ice for a further 2 minutes. 1 ml of LB medium was added, and the bacteria incubated for 1 hour at 37°C with constant shaking. 100 µl of the culture was then streaked out on a LB-agar plate containing

selective antibiotic and incubated overnight at 37°C. Ampicilin was routinely used at 100 μg/ml and kanamycin at 50 μg/ml.

**Preparation of plasmid DNA**

A single colony of transformed bacteria was picked and inoculated into 5 ml of LB medium containing selective antibiotic. The culture was grown overnight at 37°C with constant shaking. The following evening, 1 ml was used to inoculate 250 ml of fresh LB medium containing selective antibiotic, and the culture grown for a further 12 hours. Plasmid DNA was purified using Qiagen maxiprep columns and eluted in TE buffer (10 mM Tris-HCl (pH8), 1mM EDTA) according to the manufacturers instructions. DNA was examined by agarose gel electrophoresis and the concentration determined by reading the absorbance at 260 nm using a spectrophotometer.

**Preparation of glycerol stocks of transformed bacteria**

Glycerol stocks of transformed *E. coli* were made by adding 100 μl of 80% glycerol to 900 μl of bacterial culture that had been grown to stationary phase overnight. Vials were inverted to mix the glycerol, frozen in a dry ice/ethanol bath, and stored at –70°C. To recover the bacteria, a sterile inoculating loop was used to scrape the surface of the frozen culture. Bacteria were streaked out on a LB-agar plate containing selective antibiotic and grown overnight at 37°C. A single colony was picked and plasmid DNA prepared as described.

**Agarose gel electrophoresis**

1 to 2% agarose gels were prepared in TAE buffer (40 mM Tris, 320 mM acetic acid, 1mM EDTA, pH 7.2). Plasmid DNA, PCR products and restriction digests were separated at 80 V for 2 hours, stained with ethidium bromide and visualised under ultraviolet light using a transilluminator. For preparative work a 366 nm light source was

used in order to minimise photo nicking and dimerisation of DNA. DNA was recovered from agarose gel slices using a QIAquick gel extraction kit (Qiagen) according to the manufacturers instructions.

**Restriction digest of DNA**

Single and double digests of PCR products and vectors were performed in a volume of 20 µl using 1 unit of restriction enzyme in an appropriate buffer, chosen to give maximum activity of the enzyme(s). All restriction enzymes and buffers were from Promega. Digestions were performed at 37°C for 3 hours and enzymes heat inactivated at 65°C for 10 minutes where appropriate. DNA was fractionated by agarose gel electrophoresis and the required bands cut from the gel with a clean scalpel. DNA was recovered by gel extraction as described.

**Ligation of DNA**

PCR products were ligated into linearised vector with cohesive ends using T4 DNA ligase (Promega). A molar ratio of 1:3 to 1:5 vectors:insert was normally used. The mass of insert required for each ligation was calculated using the formula below:

$$\frac{\text{100 ng of vector x kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio of } \frac{\text{insert}}{\text{vector}} = \text{ng of insert}$$

100 ng of vector and the appropriate mass of insert were added to an Eppendorf tube and made up to a volume of 8 µl with distilled water. Samples were heated to 45°C for 5 minutes then chilled on ice. 1 µl of 10 x T4 DNA ligase buffer (300 mM Tris-HCl (pH 7.8), 100 mM $MgCl_2$, 100 mM DTT, 100 mM ATP) and 1 µl of enzyme were then added, to give a final volume of 10 µl, and the reaction allowed to proceed for 2 hours at 22°C. Control reactions were set up with either vector alone or insert alone. 1 µl of the ligation

mixture was then used to transform competent DH5α *E. coli*, and transformants selected on LB-agar plates containing selective antibiotic as described. Single colonies of transformants were picked and inoculated into 5ml of LB medium containing antibiotic and grown overnight at 37°C with constant shaking. Bacteria were then harvested by centrifugation at 5,000 xg for 5 minutes and plasmid DNA prepared using Qiagen miniprep columns according to the manufacturers instructions. Restriction digests were performed to confirm the presence of DNA inserts and plasmids sequenced to ensure that the inserts did not contain any mutations. Sequencing reactions were carried out by Miss Nancy Nelson in the genomics and proteomics group at the MRC human reproductive sciences unit.

**Cell culture**

HEK 293 cells stably transfected with the rat GnRH receptor (designated SCL60 cells) were maintained in DMEM supplemented with 10% fetal calf serum, penicillin (50 IU/ml), streptomycin (50 mg/ml), G418 sulphate (0.5 mg/ml) and L-glutamine (4 mM) at 37°C in a humidified atmosphere of 5% carbon dioxide and 95% air. LβT2 gonadotroph cells were grown on Matrigel (BD Biosciences) coated plasticware in DMEM supplemented with 10% fetal calf serum, penicillin (50 IU/ml), streptomycin (50 mg/ml), and L-glutamine (4 mM) at 37°C in a humidified atmosphere of 5% carbon dioxide and 95% air.

Cell lines were routinely passaged twice weekly by enzymatic dispersal with trypsin-EDTA. Briefly, the medium was removed from confluent cultures and cells washed three times with 10 ml of PBS. 2 ml of 0.5 x trypsin-EDTA was then added to each 162 $cm^2$ flask (Corning Costar), and the flasks returned to the incubator for 2 – 5 minutes. After the cells had been dispersed, 8 ml of growth medium was added to quench the trypsin. SCL60 cells were routinely split 1:3, while LβT2 cells were split 1:2.

Dispersed cells were diluted 1:10 in DMEM and counted using a Nebauer haemocytometer. Cells were counted in all four counting areas of the haemocytometer and an average taken to give the cell number x $10^5$ per ml. They were then seeded into 100 mm dishes or 12 well plates (Corning Costar) for experiments, as required.

**Cryopreservation and resucitation of cell lines**

Stocks of each cell line were stored at –196°C under liquid nitrogen in cryoprotectant (10% DMSO (v/v) in fetal calf serum). Cells were recovered from the liquid nitrogen store and rapidly warmed to 37°C in a water bath. They were then gently resuspended in 10 ml of growth medium and seeded into flasks. Frozen stocks were preserved by banking cells for the first few passages after they were resuscitated. Confluent cultures were passaged as described, and cells collected by centrifugation at 500 xg for 5 minutes. The medium was decanted and the cell pellet gently resuspended in cryoprotectant, aliquoted into vials, and frozen at –70°C in a cryo 1°C freezing container (Nalgene). Vials were then transferred to liquid nitrogen for long term storage.

**Transient transfection by $Ca^{2+}$-phosphate precipitation**

SCL60 cells were transfected by the calcium phosphate precipitation method (Wigler *et al.*, 1979). Briefly, 3x$10^6$ cells were seeded into 100 mm dishes and allowed to attach overnight. 3 hours prior to beginning the transfection, the medium was removed and replaced with fresh growth medium. 10 µg of plasmid DNA was diluted to 438 µl in sterile deionised water and 62 µl of 2 M $CaCl_2$ added, to give a volume of 500 µl. The DNA/$CaCl_2$ solution was added drop wise to 500 µl of 2 x HEPES-buffered saline (50 mM HEPES, 280 mM NaCl, 1.5 mM $Na_2HPO_4$, pH 7.1) whilst gently vortexing, and the mixture incubated at room temperature for 2 minutes to allow the calcium phosphate and DNA to from a co-precipitate. The transfection solution was then added drop wise to the

cells, and cultures incubated overnight. After 16 hours the medium was removed and replaced with fresh growth medium. Cells were harvested for experiments 48 hours later.

**Transient transfection with Superfect reagent**

L□T2 cells were transfected with Superfect transfection reagent (Qiagen) using 5 μl of Superfect per μg of plasmid DNA. 3 x $10^6$ cells were seeded into 100 mm dishes and allowed to attach overnight. The following day 10 μg of plasmid DNA was diluted to a volume of 300 μl with Optimem serum free medium (Gibco) in a sterile Eppendorf tube. 50 μl of Superfect was added, and the mixture incubated at room temperature for 10 minutes to allow the DNA to complex with the Superfect reagent. The transfection solution was then added to the cells in 3 ml of growth medium for 5 hours. Cells were harvested for experiments 48 hours later.

**Preparation of pharmacological inhibitors and ligands**

Stock solutions of AG1478, BAPTA-AM, cytochalasin-D, GF109203X, herbimycin A, latrunculin-B, PD 98059, PP2, Ro-318220, wortmannin, and U73122 (all from Calbiochem) were prepared in dimethyl sulphoxide and stored at –20°C. RGDS and RGES were dissolved in sterile water and stored at –20°C. *C. difficile* Toxin B (Calbiochem), EGF and GnRH were dissolved in sterile water and stored at 4°C.

**Inositol phosphate formation assay**

SCL60 cells were seeded into 12 well plates at a density of 3 x $10^5$ cells per well and allowed to attach overnight. The following day, the medium was removed and monolayers washed with 1 ml of PBS. Cells were then incubated for 48 hours in 1 ml of inositol-free DMEM (Gibco) containing 1% dialysed fetal calf serum, penicillin (50 IU/ml), streptomycin (50 mg/ml), L-glutamine (4 mM) and 1 μCi/ml myo-[$^3$H]inositol

(Amersham Pharmacia Biotech). After 48 hours, the medium was removed and monolayers washed with 1 ml of pre-warmed assay buffer (140 mM NaCl, 20 mM HEPES (pH 7.2), 8 mM glucose, 4 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mg/ml fatty acid free bovine serum albumin) to remove extracellular myo-[$^3$H]inositol. Cells were then incubated at 37°C in 500 µl of assay buffer containing 10 mM LiCl for 30 to 60 minutes to block inositol phosphatases, then stimulated with GnRH as described in the figure legends. Pretreatment with chemical inhibitors was performed during the pre-incubation with LiCl.

Following stimulation with GnRH, the assay buffer was removed and cells lysed in 500 µl of 10 mM formic acid for 30 minutes at 4°C. $^3$H-inositol phosphates were purified by ion-exchange chromatography using AG1-X8 resin (Formate form, Bio-Rad Laboratories). The formic acid extract was added to 500 µl of a 50% slurry of AG1-X8 resin prepared in distilled water and mixed by vortexing. The supernatant was removed and the resin washed with 1ml of distilled water followed by 1 ml of 60 mM ammonium formate, 5 mM sodium tetraborate. Bound inositol phosphates were then eluted with 1 ml of 1 M ammonium formate, 0.1 M formic acid. 800 µl of eluate was mixed with 2.5 ml of Optiphase 3 scintillant (Perkin Elmer), and the radioactivity measured by liquid scintillation spectroscopy. Assays were performed in triplicate.

**Immunocytochemistry and confocal microscopy**

SCL60 Cells were seeded into poly-L-lysine coated 8-well chamber slides (Labtech) at a density of 7.5 x $10^4$ cells per well and allowed to attach overnight. The medium was removed, cells washed with PBS, and incubated overnight in serum free DMEM containing penicillin (50 IU/ml), streptomycin (50 mg/ml), and 10 mM HEPES (pH 7.4). Quiescent cells were then stimulated with GnRH the following morning. Chemical inhibitors were added in serum free DMEM as described in the figure legends.

After stimulation with GnRH, cells were washed with chilled PBS and fixed with ice cold methanol at –20°C for 10 minutes. The fixed cells were then blocked with 10% fetal calf serum, 1% bovine serum albumin in PBS (blocking solution) for 1 hour at room temperature. Anti- tyrosine tubulin and β-actin antibodies were added at a 1:100 dilution in blocking solution overnight at 4°C. Cells were then washed three times with PBS and FITC conjugated secondary antibody added at a 1:200 dilution in blocking solution for 1 hour at room temperature. After three washes with PBS, slides were mounted in permafluor mounting medium (Immunotech) and left to dry overnight in the dark.

Filamentous actin was stained with fluorescently labeled phalloidin. After fixing in ice cold methanol, cells were incubated for 1 hour at room temperature with a 1:50 dilution of Alexfluor-568 phalloidin (Molecular Probes) prepared in PBS. The cells were then washed three times with PBS and mounted as above. For phase contrast microscopy, cells were fixed in ice cold methanol and mounted as described.

Confocal microscopy was performed on a Zeiss LSM 510 laser scanning microscope with a 40 x 1.4 numerical aperture oil immersion lens. Images were exported to Adobe photoshop.

**Fractionating cells with non-ionic detergents**

The distinct chemical properties of non-ionic detergents allow cells to be fractionated into specific subcellular proteomes for immune precipitation and Western blotting studies. Cytoplasmic and nuclear extracts were prepared by gently lysing cells in a NP-40 (Calbiochem) based buffer. Low concentrations of this detergent do not solubilise the nuclear lamina, allowing the nuclei to be separated from the cytosol and other organelles by low speed centrifugation. In contrast, lysis in Triton X-100 based buffer allows the isolation of detergent insoluble cytoskeletons from the majority of other cellular material.

The cytoskeleton and associated proteins can then be extracted under depolymerising conditions and separated from insoluble material by centrifugation.

**Preparation of cytoplasmic and nuclear extracts**

SCL60 cells were seeded into 100mm dishes at a density of 3 x $10^6$ cells per dish and allowed to attach overnight. The medium was removed, cells washed with PBS, and incubated overnight in serum free DMEM containing penicillin (50 IU/ml), streptomycin (50 mg/ml), and 10 mM HEPES (pH 7.4). Quiescent cells were stimulated with GnRH or EGF the following morning. Chemical inhibitors were added in serum free DMEM as described in the figure legends.

Following stimulation with ligands, cells were washed with chilled PBS and lysed on ice for 10 minutes in 800 µl of NP-40 based lysis buffer (0.5% NP40, 150 mM NaCl, 5 mM HEPES (pH 7.4), 2 mM EDTA (pH 8.0), 100 µM $Na_3VO_4$, 1 mM PMSF and 10 µg/ml leupeptin). Lysates were recovered to Eppendorf tubes and nuclei sedimented by centrifugation at 1,000 xg for 3 minutes at 4°C. Cytoplasmic lysates were recovered to fresh tubes and clarified by centrifugation at 15,000 xg for 15 minutes at 4°C. The nuclear pellet was washed twice in 800 µl of NP-40 buffer to remove contaminating cytoplasmic proteins and solubilised in 50 µl of NP-40 buffer containing 0.2% (w/v) SDS. Insoluble material was sedimented by centrifugation at 15,000 xg for 15 minutes at 4°C and nuclear lysates recovered to fresh tubes.

Protein tyrosine phosphorylation was analysed by immune precipitating proteins from cytoplasmic extracts with anti-phosphotyrosine (PY20) agarose conjugated antibody, followed by Western blotting. Phosphorylation of transiently expressed epitope tagged proteins was analysed by immune precipitating proteins from cytoplasmic extracts with anti-myc or anti-HA agarose conjugated antibodies as appropriate, followed by Western blotting. ERK activation was analysed by Western blotting cytoplasmic and nuclear

extracts with a phosphospecific antibody that recognises the active phosphorylated forms of ERK 1 and 2. Protein interactions were analysed by reciprocal immune precipitation and Western blotting or by two-dimensional gel electrophoresis and MALDI-ToF mass spectrometry.

**Preparation of cytoskeletal extracts**

Cytoskeletal extracts were prepared essentially according to Payrastre *et al.* (1991). SCL60 cells were seeded into 100mm dishes at a density of 3 x $10^6$ cells per dish and allowed to attach overnight. The medium was removed, cells washed with PBS, and incubated overnight in serum free DMEM containing penicillin (50 IU/ml), streptomycin (50 mg/ml), and 10 mM HEPES (pH 7.4). Quiescent cells were stimulated with GnRH the following morning.

Following stimulation with GnRH, cells were washed with chilled PBS and lysed on ice for 15 minutes in 1 ml of Triton X-100 based lysis buffer (0.5% Triton X-100, 20 mM HEPES (pH 7.4), 50 mM NaCl, 1 mM EGTA, 100 μM $Na_3VO_4$, 1 mM PMSF and 10 μg/ml leupeptin). Lysates were recovered to Eppendorf tubes and insoluble material collected by centrifugation at 12,000 xg for 1 minute at 4°C. The pellet was washed four times in 1 ml of 20 mM HEPES (pH 7.4), 50 mM NaCl, 1 mM EGTA, 100 μM $Na_3VO_4$, 1 mM PMSF and 10 μg/ml leupeptin, to remove contaminating cytoplasmic proteins, and cytoskeletons extracted in 2 ml of 0.6 M KI, 100 mM PIPES (pH 6.5), 100 mM KCL, 100 μM $Na_3VO_4$, 1 mM PMSF and 10 μg/ml leupeptin for 20 - 30 minutes at 4°C with gentle shaking. Insoluble material was sedimented by centrifugation at 15,000 xg for 15 minutes at 4°C and the supernatant recovered to a fresh tube. The association of proteins with Triton X-100 insoluble cytoskeletons was then confirmed by immune precipitation and Western blotting.

**Determination of protein concentration**

Protein concentrations were determined according to Bradford (1976), using BSA as standard. A standard curve (0 – 2 mg/ml) was prepared by serial dilution of a 10 mg/ml stock of BSA in the appropriate buffer. 10 µl of each sample or standard was added to 1 ml of Bradford assay reagent (Bio-Rad Laboratories) in an Eppendorf tube, vortexed to mix, and incubated at room temperature for 5 minutes. The absorbance was then measured at 595 nm using a spectrophotometer. A standard curve was plotted and the concentration of protein in each sample calculated from the equation of the line.

**Immune precipitation**

Proteins were immune precipitated from 500 µg of cell extract with 20 µl of agarose conjugated antibody, or 2 µg of antiserum plus 20 µl of a 30% slurry of protein A plus protein G agarose resin (Calbiochem). Briefly, samples were tumbled overnight with antibody and protein A plus protein G agarose resin (if required) at 4°C and immune precipitates collected by centrifugation at 5,000 xg for 10 minutes at 4°C. Immune complexes were then washed twice in 1ml of NP-40 based lysis buffer to remove contaminating proteins. To prevent the non-specific binding of proteins to the agarose matrix, cell extracts were routinely pre-cleared with 20 µl of protein A plus protein G agarose resin for 1 hour prior to immune precipitating.

**SDS-PAGE**

Discontinuous Tris-glycine gels were prepared according to Westermeier (1997). 8% and 10% resolving gels were prepared in 1.5 M Tris (pH 8.8), 1% (w/v) SDS, 25% (v/v) glycerol. 5% stacking gels were prepared in 1.25 M Tris (pH 6.8), 1% (w/v) SDS. Solutions were degassed for 5 minutes and polymerisation initiated by the addition of TEMED and APS. The resolving gel was poured and then immediately overlaid with the stacking gel. The inclusion of 25% glycerol in the resolving gel buffer allows both to be

cast at the same time without them mixing together. Gels were allowed to polymerise for 1 hour at room temperature and stored at 4°C overnight before use.

Protein extracts were mixed with an equal volume of 2 x SDS-PAGE sample buffer (25 mM Tris-HCl (pH 6.8), 8% SDS, 0.9 M β-mercaptoethanol, 10% (v/v) glycerol), boiled for 5 minutes and cooled to room temperature. Immune precipitates were routinely solubilised in 25 µl of SDS-PAGE sample buffer, boiled for 5 minutes, cooled to room temperature, and agarose beads pelleted by centrifugation at maximum speed in a bench top centrifuge for 1 minute. A Hamilton syringe with a 22 gauge needle was used to load the gels to prevent agarose resin being accidentally loaded into the wells. Proteins were resolved by SDS-PAGE using a Mini Protean II electrophoresis system (Bio-Rad Laboratories). Electrophoresis was routinely performed at 200 V for 1 hour in SDS-PAGE running buffer (25 mM Tris, 192 mM glycine, 1% (w/v) SDS).

**Western blotting**

Proteins were transferred from SDS-PAGE gels to PVDF membrane (Perkin Elmer) for Western blotting using a Novablot semi-dry transfer apparatus (Amersham Pharmacia Biotech). PVDF membranes were prepared by washing with 100 % methanol for 1 minute, followed several washes with distilled water, and then equilibrated in semi-dry transfer buffer (20mM Tris, 192 mM glycine, 20% methanol, 0.1% SDS) for 10 minutes. Three thicknesses of electrode paper were soaked in semi-dry transfer buffer and placed onto the anode plate of the transfer apparatus. The PVDF membrane was then placed on top followed by the gel and then another three thicknesses of electrode paper wetted with buffer. Air bubbles were rolled out with a glass test tube, and the cathode plate carefully placed on top. Proteins were transferred at 12 V for 1 hour.

Following semi-dry transfer, membranes were rinsed in TBS-T (100 mM Tris-HCl (pH 7), 150 mM NaCl, 0.05% Tween 20, 0.05% NP-40) and blocked for 1 hour in 10 ml of blocking buffer (4% (w/v) bovine serum albumin in TBS-T) with gentle shaking on a

plate rocker. The blocking buffer was discarded and primary antibody (diluted 1:1,000 in 10 ml of blocking buffer) added for 1 hour. Membranes were washed three times for 10 minutes each in TBS-T, then alkaline-phosphatase conjugated secondary antibody (diluted 1:10,000 in 10 ml of blocking buffer) added for 1 hour. After three further 10 minute washes in TBS-T, blots were drained and developed with ECF substrate (Amersham Pharmacia Biotech) for 1 – 5 minutes according to the manufacturers instructions, and visualised using a Typhoon 9200 scanner (Amersham Pharmacia Biotech). Bands were quantified using ImageQuant software (Molecular dynamics).

After being scanned, blots were washed in 40% methanol for 30 minutes to remove precipitated fluorophore and rinsed in TBS-T. Antibodies were then stripped from the membrane by incubating in 10 ml of 25 mM Tris-HCl (pH 7), 8% (w/v) SDS, 0.72 M β-mercaptoethanol for 30 minutes at 80°C, followed by three 10 minute washes in TBS-T.

***In vitro* protein kinase assay**

The kinase activity of FAK and Src was assayed *in vitro* using rabbit muscle enolase as substrate. Cytoplasmic extracts were prepared from SCL60 cells as described, and FAK or Src immune precipitated with 2 □g of specific antiserum and 20 μl of protein A plus protein G resin for 4 hours at 4°C. Immune complexes were collected by centrifugation at 15,000 xg for 10 minutes, washed twice with 1 ml of NP-40 based lysis buffer, and once with 1ml of 150 mM NaCl, 20 mM HEPES (pH 7.4), 30 mM $MgCl_2$. They were then resuspended in 10 μl of protein kinase assay buffer (150 mM NaCl, 20 mM HEPES (pH 7.4), 30 mM $MgCl_2$, 100 μM sodium orthovanadate, 10 μCi [□-$^{32}$P]ATP (3000 Ci/mol; Amersham Pharmacia Biotech), 10 μM cold ATP, and 0.3 mg/ml rabbit muscle enolase) on ice. The reaction was started by warming samples to 30°C in a water bath, and samples incubated for 10 minutes. The reaction

was stopped by returning the samples to ice. 10 µl of SDS-PAGE sample buffer was then added to each tube, and samples boiled for 5 minutes. Proteins were resolved by SDS-PAGE as described, and the gels dried onto filter paper for 1 hour at 58°C using a vacuum gel drier (Bio-Rad Laboratories), wrapped in cellophane and exposed to X-ray film (Kodak).

**Two-dimensional gel electrophoresis of Src immune complexes**

Cytoplasmic extracts were prepared from SCL60 cells and Src immune precipitated with 20 µl of anti-Src agarose conjugated antibody as described. Immune precipitates were then washed three times with 1 ml of Tris-sorbitol buffer (40 mM Tris, 10 mM sorbitol) to remove contaminating salts, and solubilised in 50 µl of two-dimensional gel electrophoresis sample buffer (8 M urea, 4% (w/v) CHAPS, 40 mM Tris) for 1 hour at room temperature.

Proteins were separated according to their isoelectric points using an IPGphor isoelectric focusing system and immobiline drystrip gels (Amersham Pharmacia Biotech). Samples were applied to 11cm, pH 3-10 (linear) immobiline strips, in 200 µl of rehydration solution (8M urea, 2% (w/v) CHAPS and 20 mM DTT), overlayed with mineral oil to minimise evaporation and urea crystallisation, and strips rehydrated for 12 hours at 30 V. Isoelectric focusing was then performed as follows; 500 V for 1hour, 1,000 V for 1 hour and 8,000 V for 2 hours (giving a total of 17,500 V hours). Immobiline strips were then washed in SDS-PAGE equilibration buffer (50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS) containing 1% (w/v) DTT for 10 minutes, followed by a second 10 minute wash in equilibration buffer containing 4% (w/v) iodoacetamide.

The immobiline strips were then placed onto Excel gel 12-14% SDS-PAGE gels (Amersham Pharmacia Biotech) and proteins resolved by electrophoresis at 1,000 V at 20 mA for 45 minutes, followed by 1,000 V at 40 mA for 2 hours 45 min using a

Multiphor II flat bed system (Amersham Pharmacia Biotech). Proteins were visualised by staining with silver or coomassie blue.

**Silver staining SDS-PAGE gels**

Gels were stained using a Hoefer automated gel stainer with a stainless steel tray (Amersham Pharmacia Biotech). All solutions were prepared with double-distilled water immediately before use. Following electrophoresis, gels were fixed in 10% glacial acetic acid in 40% methanol for 30 minutes, washed three times for 5 minutes with distilled water, and sensitised in 0.02% (w/v) sodium thiosulphate, 200 mM sodium acetate in 30% ethanol for 30 minutes. After three 5 minute washes in distilled water, silver stain (0.25% (w/v) silver nitrate, 0.015% (w/v) formaldehyde) was added for 20 minutes. Gels were then washed twice for 1 minute in distilled water and developed with 60 mM sodium carbonate, 0.0074 % (w/v) formaldehyde for 2 to 5 minutes as required. The reaction was stopped by the removal of the developer and the addition of 10 mM EDTA to chelate free silver ions. After 10 minutes the EDTA solution was removed and gels washed three times for 5 minutes in distilled water. Gels were preserved in 87% (v/v) glycerol, wrapped in cellophane and stored at 4°C.

**Coomassie blue staining SDS-PAGE gels**

All solutions were prepared with double distilled water. Following electrophoresis, gels were fixed and stained in 0.25 % (w/v) Coomassie brilliant blue R250 in methanol/water/glacial acetic acid (45:45:10) for 1 hour. The stain was removed and gels destained in several changes of methanol/water/glacial acetic acid (45:45:10) for 4 hours. Gels were then preserved in 87% (v/v) glycerol, wrapped in cellophane and stored at 4°C.

**In-gel tryptic digests and MALDI-ToF mass spectrometry**

Protein spots of interest were excised from SDS-PAGE gels with a clean scalpel blade and recovered to Eppendorf tubes. An equal sized piece was cut from a blank area of the gel to serve as a control for keratin contamination. The gel slices were dehydrated in 200 µl of 50% acetonitrile and dried under vacuum until all traces of solvent were removed. Samples were then reduced in 15 µl of 10 mM DTT, 0.2% EDTA, 100 mM ammonium bicarbonate for 30 minutes at 56°C, cooled to room temperature and alkylated in 50 µl of 50 mM iodoacetamide, 100 mM ammonium bicarbonate for 30 minutes at room temperature in the dark. After alkylation, gel slices were washed in 200 µl of 100 mM ammonium bicarbonate for 10 minutes and dehydrated in 200 µl of acetonitrile. Samples were washed and dehydrated a further two times, then dried under vacuum to constant weight. The gel pieces were rehydrated in 5 µl of digestion buffer (50 mM ammonium bicarbonate buffer containing 12.5 ng/ml sequencing grade trypsin) at 37°C for 15 minutes, covered with 10 µl of 50 mM ammonium bicarbonate to prevent them from drying out, and incubated at 37°C overnight. Peptides were extracted in 50 µl of 5% formic acid in 50% acetonitrile for 1 hour at room temperature with constant shaking. The supernatant was recovered to a fresh Eppendorf tube, dried under vacuum, and peptides resuspended in distilled water. All steps were performed in a laminar flow hood to minimise keratin contamination. Peptides were desalted using Zip Tips (Millipore) and analysed by MALDI-ToF mass spectrometry by Dr P. Barran and Dr J. Creanor at the Department of Chemistry (University of Edinburgh), using a Voyager MALDI-ToF mass spectrometer (ABI). Proteins were identified from m/z peak lists using MS-Fit (http://prospector.ucsf.edu) to interrogate the SwissProt protein database (http://www.expasy.org). The parameters used during database searches are summarised in Table 1 below. The primary amino acid sequences of proteins identified by mass

spectrometry were analysed using scansite (http://scansite.mit.edu) to identify potential protein-interaction domains and phosphorylation sites.

| Parameter | Setting used |
|---|---|
| Digest | Trypsin |
| Maximum number of missed cleavages | 1 |
| Cysteine modification | Carboxymethylation |
| Other considered modifications | Oxidation of methionine |
| Instrument | MALDI-ToF |
| Mass ions | Mono-isotopic |
| Mass tolerance | ± 50 ppm |

**Table 1: Summary of parameters used in MS-Fit (http://prospector.ucsf.edu) while searching the SwissProt database (http://www.expasy.org).**

**Preparation of the pET42a-DGKζ**

DGKζ was cloned into pET42a (Novagen) for expression in *E. coli* as a fusion protein with an N-terminal GST and 6-His tag. The coding sequence of rat DGKζ was amplified by PCR from pEGFPBos-DGKζ using primers which contained an EcoRI or SalI restriction enzyme cut site, for directional cloning into pET42a. Primer sequences were GCGGCGGAATTCATGGAGCCGCGGGACCC (forward) and CGCCGCGTCGACCT ACACAGCTGTCTCCTGGTCCT (reverse).

PCR reactions were performed in a Hybaid thermal cycler in a volume of 30 µl containing; 1 X Thermopol buffer (New England Bioloabs, 10 mM KCl, 10 mM $(NH4)_2SO_4$, 20 mM Tris-HCl, 4 mM $MgSO_4$, 0.1% Triton X-100, pH8.8), 0.67 mM dNTPs, 100 ng of each primer, and 1 unit of Deep $Vent_R$ DNA polymerase (New England Biolabs). PCR conditions were as follows; denaturation at 94°C for 5 minutes, followed by 30 cycles of 94°C for 1 minute, 58°C for 1 minute, and 72°C for 3 minutes, with a final extension step at 72°C for 10 minutes. PCR product and pET42a vector were

digested with EcoRI and SalI, ligated together, and transformed into DH5α competent *E. coli* as described.

Single colonies of transformants were picked and inoculated into 5 ml of LB medium containing antibiotic and grown overnight at 37°C with constant shaking. Plasmid DNA was purified using Qiagen miniprep columns, and sequenced to ensure that the inserts did not contain any deleterious mutations. Once the sequence had been verified as correct, pET42a-DGKζ was transformed into BL21 competent cells (Novagen) for protein expression.

**Expression and purification of recombinant DGKζ**

A single colony of transformed BL21 cells was inoculated into 5ml of 2YT medium containing 30 μg/ml kanamycin and incubated at 37°C with constant shaking until an absorbance (600 nm) of 0.6 was reached. Cultures were stored at 4°C overnight. The following day 1 ml was inoculated into 100 ml of 2YT medium, and the incubation continued until an absorbance (600 nm) of 0.6 was reached once again. Protein expression was then induced with 1 mM IPTG for 4 hours at 37°C with constant shaking. In initial experiments, recombinant DGKζ was found to be produced as an insoluble protein and therefore had to be purified under denaturing conditions using $Ni^{2+}$-NTA resin (Qiagen). β-mercaptoethanol was included in the column wash buffer to remove contaminants that had formed disulphide bonds with the fusion protein.

Following the induction of protein expression, bacteria were harvested by centrifugation at 5,000 xg for 10 minutes and lysed in 10 ml of denaturing lysis buffer (8 M urea, 20 mM sodium phosphate (pH 7.8), 0.5 M NaCl, 1 mM PMSF) for 10 minutes at room temperature. Lysates were sonicated on ice, insoluble material sedimented by centrifugation at 15,000 xg for 15 minutes at 4°C, and the clarified lysate recovered to a fresh tube. Imidazole was added to a final concentration of 10 mM, and the cell free

extract tumbled with 2 ml of pre-equilibrated $Ni^{2+}$-NTA resin for 1 hour at 4°C. The resin was then transferred to a disposable plastic column (Bio-Rad Laboratories) and washed with 30 ml of denaturing lysis buffer containing 10 mM imidazole, followed by 30 ml of denaturing wash buffer (8 M urea, 20 mM sodium phosphate (pH 6.5), 0.5 M NaCl, 10 mM imidazole, 10 mM β-mercaptoethanol). The fusion protein was then refolded on the column by washing with 30 ml of native wash buffer (20 mM sodium phosphate (pH 7.8), 0.5 M NaCl, 20 mM imidazole) to remove the urea, and eluted with 10 ml of 250 mM imidazole in 20 mM sodium phosphate (pH 7.8), 0.5 M NaCl. 1ml fractions were collected and the elution of bound proteins monitored by Bradford assay. Fractions containing recombinant DGKζ were identified by SDS-PAGE and staining with Coomassie blue and pooled together.

Recombinant DGKζ was further purified by gel filtration on a 20 ml Sephadex G-150 column, using 20 mM sodium phosphate (pH 7.8), 150 mM NaCl, 10 mM imidazole as running buffer. 1 ml fractions were collected, and the elution of proteins monitored by Bradford assay. Fractions that contained recombinant DGKζ were identified by SDS-PAGE and staining with Coomassie blue. They were then pooled together and concentrated in dialysis tubing sprinkled with a little sephadex G-25 to absorb some of the liquid. The fusion protein was then stored at 4°C until use.

**Preparation of Sephadex G-150 gel filtration columns**

1g of Sephadex G-150 (bead size 40 – 120 μm) was swollen in 50 ml of 20 mM sodium phosphate (pH 7.8), 150 mM NaCl, for 5 hours at 80°C in a water bath, then allowed to cool to room temperature. The resin was carefully poured into a 1 x 25 cm glass column and allowed to settle under gravity. The column was then equilibrated with running buffer and the void volume calculated by running dextran blue (average molecular weight $2 \times 10^6$ Da) through it.

**Preparation of rat brain lysates**

Brains were obtained from rats, frozen in dry ice/ethanol bath, and stored at –70°C until required. Tissue was collected from animals that were being euthanised for other researchers and would otherwise have gone to waste. Brains were homogenised on ice in 1 ml of 0.5% NP40, 20 mM sodium phosphate (pH 7.8), 150 mM NaCl, 100 μM $Na_3VO_4$, 1 mM PMSF and 10 μg/ml leupeptin with a glass tissue grinder and lysates clarified by centrifugation at 15,000 xg for 10 minutes at 4°C. Lysates were recovered to fresh tubes and the protein concentration determined by Bradford assay. Imidazole was added to a final concentration of 10 mM before affinity chromatography was performed.

**DGKζ affinity chromatography**

250 μg of recombinant DGKζ was bound to 50 μl of $Ni^{2+}$-NTA resin for 1 hour at 4°C and then washed three times with 1ml of wash buffer (0.5% NP-40, 20 mM sodium phosphate (pH 7.8), 150 mM NaCl, 10 mM imidazole). Immobilised DGKζ was then incubated for 4 hours at 4°C with 500 μg of rat brain lysate. Beads were collected by centrifugation at 800 xg for 1 minute and washed twice with 1 ml of wash buffer. Proteins were solubilised in 50 μl of SDS-PAGE sample buffer, boiled for 5 minutes and cooled to room temperature. Protein interactions were analysed by SDS-PAGE and Western blotting. To prevent non-specific binding of proteins to the $Ni^{2+}$-NTA resin during affinity chromatography, rat brain lysates were routinely pre-cleared with 50 μl of resin for 1 hour prior to use.

**Diacylglycerol kinase assay**

The enzymatic activity of immune precipitated DGKζ was assayed in vitro using 1,2-dioleoyl-*sn*-glycerol as substrate, as described by Payrastre *et al.* (1991). 1,2-dioleoyl-*sn*-glycerol and phosphatidylserine were suspended in chloroform/methanol (1:1) and stored

at –20°C under nitrogen to prevent their oxidation. The required amount of each lipid was pipetted into a glass tube and dried under vacuum. Mixed micelles were formed by adding 100 mM Tris-HCl (pH7.4), 20 mM $MgCl_2$ and sonicating.

Cytoplasmic and Triton X-100 insoluble cytoskeletal extracts were prepared as described, and DGKζ immune precipitated with 2 μg of specific antiserum and 20 μl of protein A plus protein G resin for 4 hours at 4°C. Immune complexes were collected by centrifugation at 15,000 xg for 10 minutes, then washed twice with 1 ml of 50 mM Tris-HCl (pH7.4), 10 mM $MgCl_2$. They were then resuspended in 100μl of lipid kinase buffer (50 mM Tris-HCl (pH7.4), 10 mM $MgCl_2$, 10 μCi of [$\gamma$-$^{32}$P]ATP (3000 Ci/mol; Amersham Pharmacia Biotech) 60 μM cold ATP, 50 μM 1,2-dioleoyl-*sn*-glycerol, and 100 μM phosphatidylserine) on ice. The reaction was started by warming samples to 30°C in a water bath, and samples incubated for 10 minutes. Reactions were terminated by the addition of 400 μl of chloroform/methanol (1:1). Samples were then vortexed for 10 seconds, and centrifuged at 2,000 xg for 10 minutes to separate the organic and aqueous phases. The aqueous phase was discarded and the organic phase containing the lipids recovered to a glass test tube. Solvent was evaporated at 42°C under a stream of cold air, and lipids resuspended in 20 μl of chloroform for analysis by thin layer chromatography.

**Thin-layer chromatography**

Radio-labeled lipids were spotted onto 20 cm x 20 cm silica gel coated thin-layer chromatography plates and separated using chloroform/methanol/water/ammonium hydroxide (60:48:11:1.8) as solvent. Following chromatography, plates were dried for 1 hour in a fume hood to remove all traces of solvent, wrapped in cellophane and exposed to X-ray film (Kodak). The identity of radiolabeled phosphatidic acid was confirmed by

running phosphatidic acid standard, staining plates in an iodine chamber, and comparing $R_f$ values.

**Translocation of GFP-tagged DGKζ**

Membrane translocation of GFP-tagged DGKζ was observed in LβT2 cells using a Zeiss LSM 510 laser scanning microscope with a 40 x 1.4 numerical aperture oil immersion lens. LβT2 cells were transfected with GFP-DGKζ as described. 24 hours after transfection, cells were dispersed with 0.5 x trypsin-EDTA and seeded into matrigel coated 35 mm microscope dishes (MaTek) at a density of 0.25 x $10^6$ cell per dish. The following evening the medium was removed, cells washed with PBS, and incubated overnight in serum free DMEM containing penicillin (50 IU/ml), streptomycin (50 mg/ml), and 10 mM HEPES (pH 7.4). For microscopy, cells were maintained in serum free DMEM at 37°C in a heated chamber on the microscope stage, stimulated with GnRH, and images recorded periodically. Images were exported to Adobe photoshop. All experiments were repeated at least three times.

**Statistical analyses**

Data are presented as mean values + or ± the standard error of the mean (SEM). Data were analysed by Student's T-test or by one way analysis of variance (ANOVA) using Tukey's pairwise comparison to identify groups, which were significantly different.

# Role of tyrosine kinases in ERK activation by the GnRH receptor

## Introduction

Many GPCRs initiate the activation of ERK 1 and 2 by recruiting the guanine nucleotide exchange factor Sos to the plasma membrane in association with Grb2, which binds to tyrosine phosphorylated proteins via its SH2 domain and to Sos via its SH3 domain (Egan *et al.*, 1994). The EGFR and FAK have both been proposed to act as plasma membrane scaffolds upon which this Ras activation complex may assemble, and many Gq coupled GPCRs have been shown to induce their tyrosine phosphorylation (Daub *et al.*, 1996; Della Rocca *et al.*, 1999).

The utilisation of different scaffolds for the activation of ERK 1 and 2 was originally thought to represent functional redundancy within the system. However, experimental data is now beginning to suggest that this is not the case. Activation of these enzymes by the EGFR typically results in their translocation of the nucleus (Horgan and Stork, 2003; Luttrell and Luttrell, 2003). In contrast, activation of ERK 1 and 2 by FAK may spatially constrain the proteins at the plasma membrane and thus favour the phosphorylation of proteins in the cytoplasm (Fincham *et al.*, 2000; Luttrell and Luttrell, 2003). Therefore, the mechanism of ERK activation employed by a GPCR may play an important role in determining the function of the active enzymes within the cell. In this chapter, the role of tyrosine kinases in the activation of ERK 1 and 2 by the GnRH receptor is investigated in SCL60 cells.

## Results

Immune precipitation and Western blotting studies revealed that GnRH (100 nM, 10 minutes) induced the tyrosine phosphorylation of four proteins in SCL60 cells, with molecular weights of 70, 100, 115 and 125 KDa, respectively (Figure 10 A). The major band was subsequently identified as FAK (Figure 10 B). However, the EGFR was not

found to be tyrosine phosphorylated following stimulation with GnRH (data not shown). These results are consistent with previous reports by Reiss *et al.* (1997) and Johnson *et al.* (2000) who showed that GnRH induces the tyrosine phosphorylation of a 125 KDa protein in αT3-1 cells, and suggest that FAK may function as a scaffold for the activation of ERK 1 and 2 in these cells.

Time course experiments revealed that GnRH induced a rapid and sustained increase in FAK tyrosine phosphorylation, which reached a maximum at 5 minutes and remained elevated above basal levels up to 1 hour after stimulation with GnRH (Figure 11 A). The increase in FAK phosphorylation was found to be significantly different from basal levels at each time point ($P<0.01$). The increase in tyrosine phosphorylation was also accompanied by an increase in the kinase activity of immune precipitated FAK towards exogenous substrate *in vitro* (Figure 11 B).

GnRH was also found to activate Src in SCL60 cells. Western blotting anti-phosphotyrosine immune precipitates with phospho-specific antibodies revealed that GnRH induced a rapid but transient dephosphorylation of Src at Tyr530, and a sustained increase in the autophosphorylation of Tyr419 (Figures 12 A and B). Significant decreases in Tyr530 phosphorylation were observed at the 1 and 2 minute time points compared to the unstimulated control ($P<0.01$), while Tyr419 phosphorylation was found to be significantly increased at each time point examined (compared to the control) ($P<0.01$). These events are consistent with the activation of the catalytic domain of Src, and were accompanied by an increase in the kinase activity of immune precipitated Src towards exogenous substrate *in vitro* (Figure 12 C). In all subsequent studies, auto-phosphorylation of Src at Tyr419 was used as an indicator of enzyme activation.

The activation of ERK 1 and 2 by GnRH was analysed by Western blotting cytoplasmic extracts with a phospho-specific antibody that recognises both isoforms when dually phosphorylated at Thr183 and Tyr185 (ERK2 numbering) by MEK 1 or 2. The time

course of ERK activation was similar to that observed for FAK, reaching a maximum at 5 minutes but remaining elevated above basal 1 hour after treatment (Figure 13 A). The increase in ERK phosphorylation was found to be significantly different from the unstimulated control at each time point examined ($P<0.01$). Western blotting nuclear extracts revealed that GnRH also induced the nuclear translocation of ERK in SCL60 cells, however, it was found to be much less potent than EGF in this respect (Figure 13 B).

By immune precipitating FAK and Western blotting with antibodies raised against ERK2 and Src, it was found that FAK functions to assemble these proteins together into a complex. In quiescent SCL60 cells, ERK2 was found to co-precipitate with FAK. The association between the two proteins did not appear to be altered by GnRH treatment (Figure 14). FAK was also found to associate with Src. However, this association was dynamically regulated, as Src co-precipitated with FAK only following stimulation with GnRH (Figure 14).

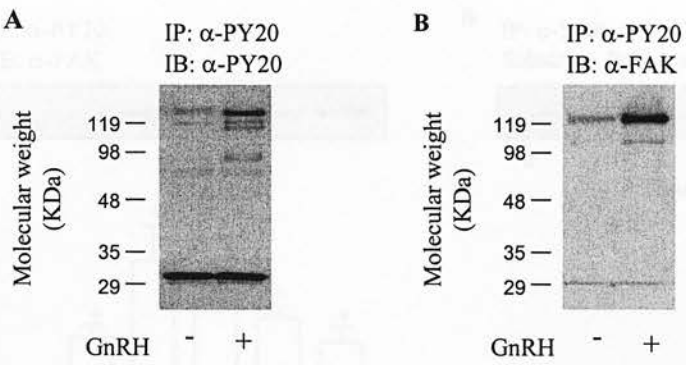


**Figure 10: Identification of proteins that are tyrosine phosphorylated in response to GnRH in SCL60 cells**. Quiescent cells were treated with vehicle or 100 nM GnRH for 10 minutes and cytoplasmic extracts prepared. Phospho-tyrosine proteins were immune precipitated and analysed by Western blotting with anti-phospho-tyrosine (A) or anti-FAK (B) antibodies. The band of approximately 30 KDa is antibody light chain. The immunoblots shown are representative of three separate experiments.

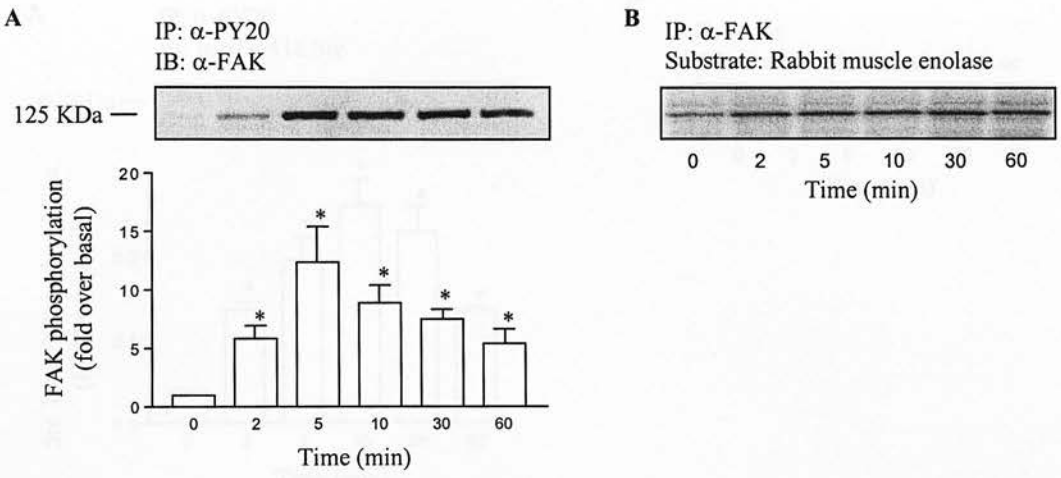


**Figure 11: GnRH induces the activation and tyrosine phosphorylation of FAK in SCL60 cells**. Quiescent cells were treated with vehicle or 100 nM GnRH and cytoplasmic extracts prepared. (A) Phospho-tyrosine proteins were immune precipitated and FAK tyrosine phosphorylation analysed by Western blotting. GnRH induced a significant increase in FAK tyrosine phosphorylation at each time point versus control (*, $P<0.01$, by ANOVA and Tukey's pairwise comparison). Data represent the mean of three separate experiments + SEM. (B) FAK was immune precipitated and kinase activity assayed *in vitro* using rabbit muscle enolase as substrate. The autoradiograph shown is representative of three separate experiments.

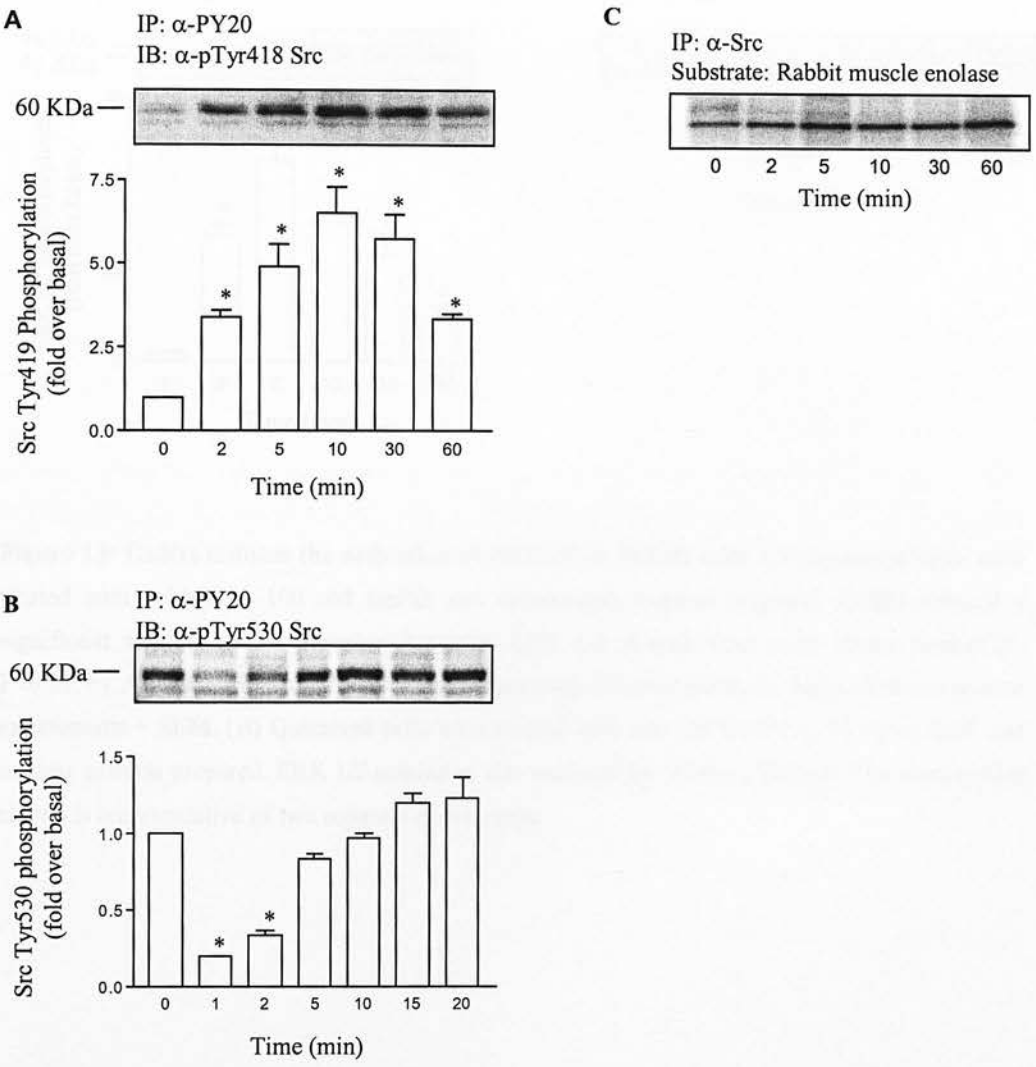


**Figure 12: GnRH induces the activation of Src in SCL60 cells**. Quiescent cells were treated with vehicle or 100 nM GnRH and cytoplasmic extracts prepared. Phospho-tyrosine proteins were immune precipitated and Src tyrosine phosphorylation analysed by Western blotting, using antibodies that recognise Src when phosphorylated at Tyr419 (A) and Tyr530 (B). GnRH induced a significant increase in Tyr419 phosphorylation at each time point and a significant decrease in Tyr530 phosphorylation at 1 and 2 minutes versus control (*, $P<0.01$, by ANOVA and Tukey's pairwise comparison). Data represent the mean of three separate experiments + SEM. Src was immune precipitated and kinase activity assayed *in vitro* using rabbit muscle enolase as substrate (C). The autoradiograph shown is representative of three separate experiments.

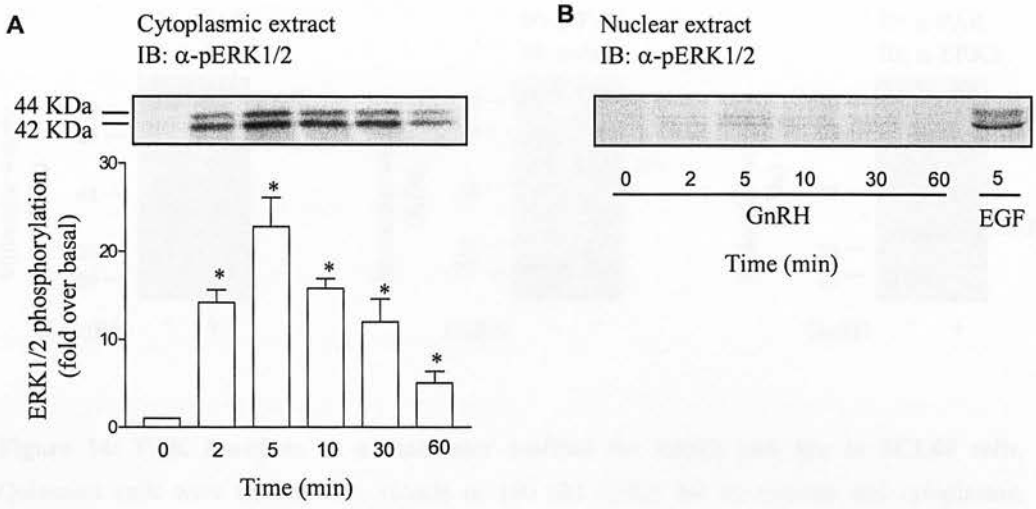


**Figure 13: GnRH induces the activation of ERK1/2 in SCL60 cells**. (A) Quiescent cells were treated with vehicle or 100 nM GnRH and cytoplasmic extracts prepared. GnRH induced a significant increase in the phosphorylation of ERK 1/2 at each time point versus control (*, $P<0.01$, by ANOVA and Tukey's pairwise comparison). Data represent the mean of three separate experiments + SEM. (B) Quiescent cells were treated with 100 nM GnRH or 10 ng/ml EGF and nuclear extracts prepared. ERK 1/2 activation was analysed by Western blotting. The immunoblot shown is representative of two separate experiments.

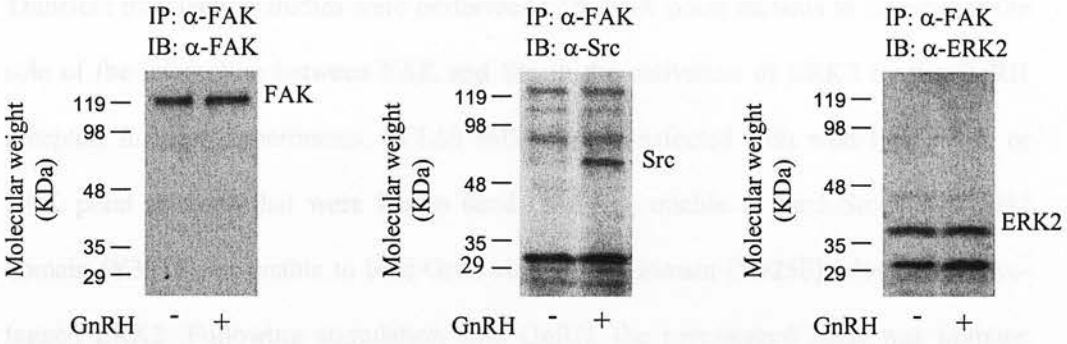


**Figure 14: FAK functions as a molecular scaffold for ERK2 and Src in SCL60 cells.** Quiescent cells were treated with vehicle or 100 nM GnRH for 10 minutes and cytoplasmic extracts prepared. FAK was immune precipitated and Src and ERK2 association analysed by Western blotting. FAK was immune precipitated with a rabbit polyclonal antibody and Western blotted with a monoclonal antibody. Src and ERK2 antibodies were rabbit polyclonal antibodies. The band of approximately 30 KDa in the Src and ERK2 blots is antibody light chain. The immunoblots shown are representative of three separate experiments.

Transient transfection studies were performed with FAK point mutants to investigate the role of the interaction between FAK and Src in the activation of ERK2 by the GnRH receptor. In these experiments, SCL60 cells were transfected with wild type FAK, or FAK point mutants that were kinase dead (K454R), unable to bind Src via its SH2 domain (Y397F), or unable to bind Grb2 via its SH2 domain (Y925F), along with myc-tagged ERK2. Following stimulation with GnRH, the myc-tagged ERK was immune precipitated from cytoplasmic extracts, and its activation analysed by Western blotting. Expression of each FAK point mutant significantly inhibited the GnRH induced phosphorylation of ERK2, compared to the wild type protein ($P<0.01$) (Figure 15). These results indicate that FAK autophosphorylation at Tyr397, association with Src, and subsequent phosphorylation at Tyr925 by Src are important events in the activation of ERK2.

A range of pharmacological inhibitors was used to investigate the proximal signals that lead to the co-ordinated activation of FAK, Src and ERK by GnRH in SCL60 cells. FAK tyrosine phosphorylation was found to be unaffected by the inhibition of PLC (U73122), PKC (GF109203X, Ro-318220), PI-3 kinase (wortmannin), MEK (PD98059), EGFR tyrosine kinase activity (AG1478), or chelation of intracellular calcium (BAPTA-AM). However, it was sensitive to the inhibition of Src family kinases with PP2 ($P<0.01$) (Figure 16). Src Tyr419 autophosphorylation displayed a similar spectrum of sensitivities, and was only significantly inhibited by pre-treatment with PP2 ($P<0.01$) (Figure 17). ERK 1 and 2 phosphorylation was also found to be unaffected by inhibition of PLC, PI-3 kinase, EGF receptor tyrosine kinase activity, or chelation of intracellular calcium. However, it was sensitive to inhibition of PKC (GF109203X, $P<0.05$; Ro-318220, $P<0.05$), MEK ($P<0.01$) and Src family kinases ($P<0.01$) (Figure 18).

As FAK and Src are known to be regulated by cell adhesion to the extracellular matrix, the role of integrins and the actin cytoskeleton in GnRH receptor signalling was

investigated. Overnight treatment of adherent cells with the fibronectin and vitronectin fragment R-G-D-S causes the displacement of integrins from the extracellular matrix, resulting in the dissolution of focal contacts, and the disassembly of the actin cytoskeleton. Treatment of SCL60 cells with the R-G-D-S peptide completely blocked FAK tyrosine phosphorylation, while the inactive control peptide R-G-E-S (that does not bind integrins) had no effect (Figure 19). Disruption of the actin cytoskeleton with cytochalasin-D or latrunculin-B, also abolished FAK tyrosine phosphorylation (Figure 19). Likewise, Src Tyr419 autophosphorylation was blocked by overnight treatment of cells with the R-G-D-S peptide, and by treatment with cytochalasin-D and latrunculin-B (Figure 20), while ERK 1 and 2 phosphorylation was significantly inhibited by treatment with R-G-D-S ($P<0.01$), cytochalasin-D ($P<0.01$) and latrunculin-B ($P<0.01$) (Figure 4.21).

The Rho family GTPases play an important role in regulating the actin cytoskeleton in eukaryotic cells. *C. difficile* Toxin B is a cell permeable bacterial toxin that monoglucosylates and inactivates RhoA, Rac1 and Cdc42. Overnight treatment with Toxin B was found to cause SCL60 cells to develop a rounded phenotype (data not shown) and significantly inhibited FAK tyrosine phosphorylation ($P<0.01$), Src Tyr419 autophosphorylation ($P<0.01$) and ERK 1 and 2 phosphorylation ($P<0.01$), following stimulation with GnRH (Figure 22).

Dominant negative cDNA constructs of RhoA (N19RhoA) and Rac1 (N17Rac1) were then used to investigate whether either of these two proteins were involved in stimulating FAK tyrosine phosphorylation by GnRH. In these experiments, SCL60 cells were transfected with HA-tagged FAK, or with HA-tagged FAK plus either N19RhoA or N17Rac1. Following agonist stimulation, HA-tagged FAK was immune precipitated from cytoplasmic extracts and its phosphorylation status analysed by Western blotting. Expression of N19RhoA did not significantly inhibit FAK phosphorylation following

stimulation with GnRH. However, it was abolished by the expression of N17Rac1 (Figure 23).

The GnRH receptor also activated PLC in SCL60 cells, leading to the hydrolysis of PtdIns(4,5)$P_2$. The accumulation of inositol phosphates in the presence of lithium chloride, which inhibits inositol monophosphatases, was found to be linear up to 1 hour following stimulation with GnRH (Figure 24 A). While the activation of the ERK signalling cascade was critically dependent on cell adhesion to the extracellular matrix and the integrity of the actin cytoskeleton, the activation of PLC was unaffected by disruption of the actin cytoskeleton with cytochalasin-D or latrunculin-B (Figure 24 B), indicating that there is a divergence between these two important pathways. The accumulation of inositol phosphates following stimulation with GnRH was however significantly inhibited by pretreating cells with the U73122 ($P<0.01$).

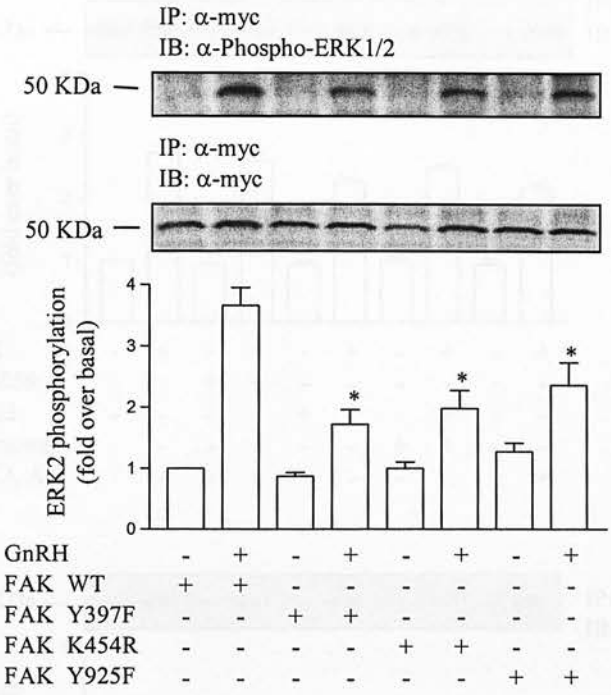


**Figure 15: The association of FAK and Src is important for the activation of ERK2 in SCL60 cells.** SCL60 cells were transfected with myc-tagged ERK2 and either wild type FAK, Y397F FAK, K454R FAK or Y925F FAK. 48 hours after transfection, quiescent cells were treated with vehicle or 100 nM GnRH for 10 minutes and cytoplasmic extracts prepared. Myc-tagged ERK2 was immune precipitated and its phosphorylation analysed by Western blotting. The expression of Y397F, K454R and Y925F FAK all significantly inhibited the GnRH induced phosphorylation of Myc-ERK compared to WT FAK (*, $P<0.01$ by ANOVA and Tukey's pairwise comparison). Data represent the mean of three separate experiments + SEM.

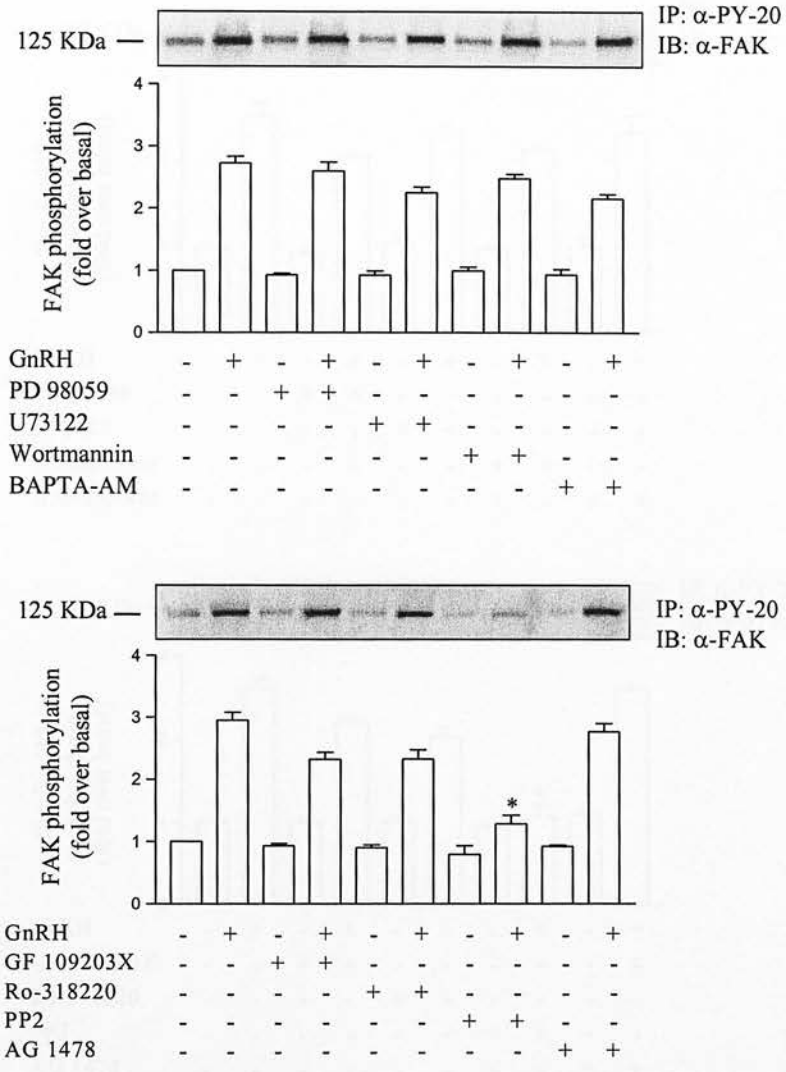


**Figure 16: Chemical sensitivities of FAK phosphorylation in SCL60 cells**. Quiescent cells were pre-treated with AG 1478 (100 nM, 30 minutes), BAPTA-AM (50 μM, 30 minutes), GF 109203X (1 μM, 30 minutes), PD 98059 (10 μM, 60 minutes), PP2 (5 μM, 30 min), Ro-318220 (100 nM, 30 min), U73122 (20 μM, 60 minutes) or wortmannin (100 nM, 30 minutes). Controls received DMSO alone. Cells were then treated with vehicle or 100 nM GnRH for 10 minutes and cytoplasmic extracts prepared. Phospho-tyrosine proteins were immune precipitated and FAK phosphorylation analysed by Western blotting. Pretreatment of cells with PP2 significantly inhibited the GnRH induced tyrosine phosphorylation of FAK (*, $P<0.01$, by ANOVA and Tukey's pairwise comparison). Data represent the mean of three separate experiments + SEM.

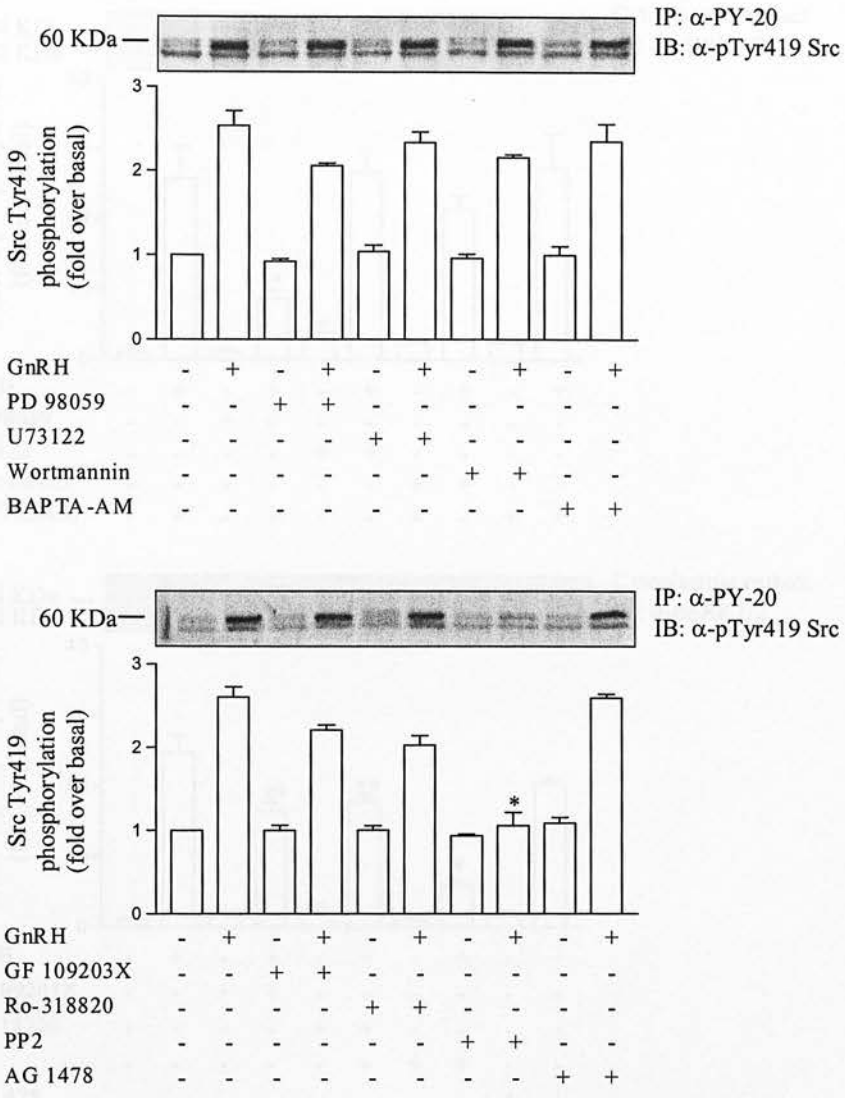


**Figure 17: Chemical sensitivities of Src activation in SCL60 cells**. Quiescent cells were pre-treated with AG 1478 (100 nM, 30 minutes), BAPTA-AM (50 μM, 30 minutes), GF 109203X (1 μM, 30 minutes), PD 98059 (10 μM, 60 minutes), PP2 (5 μM, 30 min), Ro-318220 (100 nM, 30 min), U73122 (20 μM, 60 minutes) or wortmannin (100 nM, 30 minutes). Controls received DMSO alone. Cells were then treated with vehicle or 100 nM GnRH for 10 minutes and cytoplasmic extracts prepared. Phospho-tyrosine proteins were immune precipitated and Src Tyr419 phosphorylation analysed by Western blotting. Pretreatment of cells with PP2 significantly inhibited the GnRH induced autophosphorylation of Src at Tyr419 (*, $P<0.01$, by ANOVA and Tukey's pairwise comparison). Data represent the mean of three separate experiments + SEM.

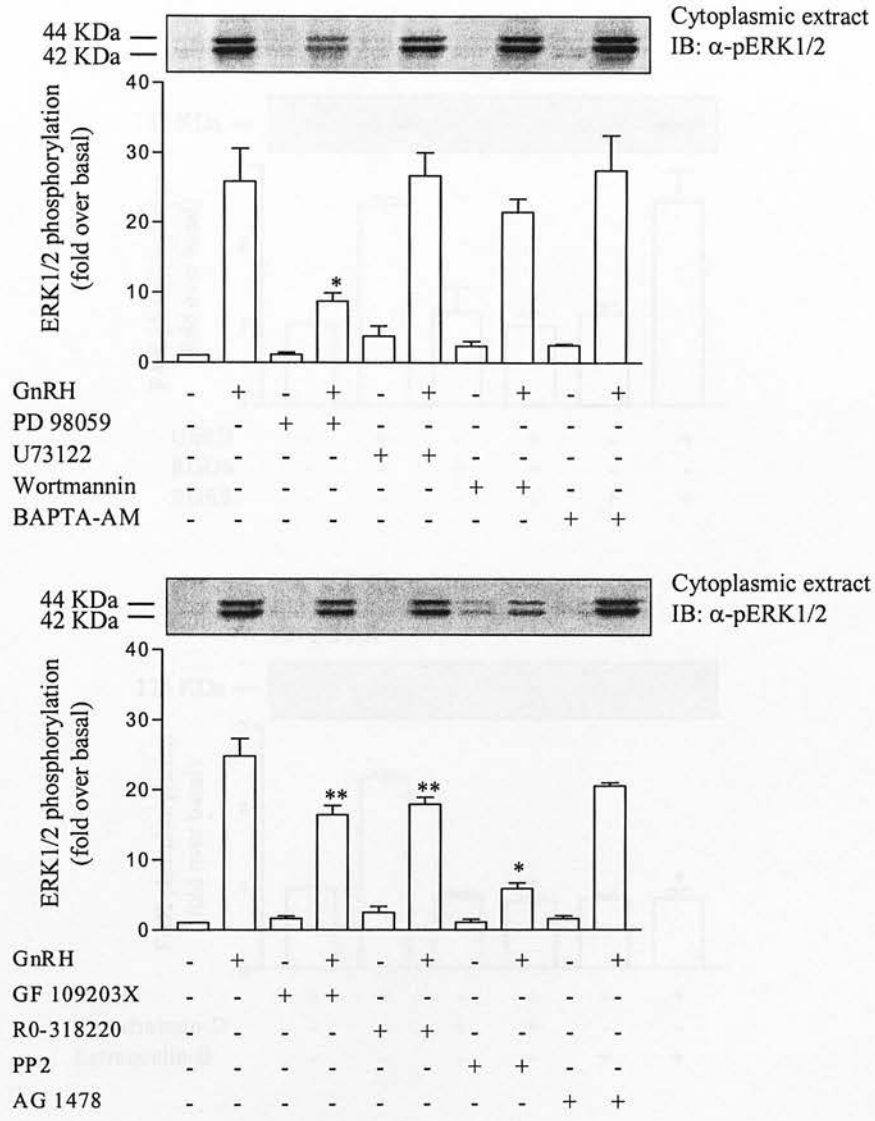


**Figure 18: Chemical sensitivities of ERK1/2 activation in SCL60 cells**. Quiescent cells were pre-treated with AG 1478 (100 nM, 30 minutes), BAPTA-AM (50 μM, 30 minutes), GF 109203X (1 μM, 30 minutes), PD 98059 (10 μM, 60 minutes), PP2 (5 μM, 30 min), Ro-318220 (100 nM, 30 min), U73122 (20 μM, 60 minutes) or wortmannin (100 nM, 30 minutes). Controls received DMSO alone. Cells were then treated with vehicle or 100 nM GnRH for 10 minutes and cytoplasmic extracts prepared. ERK1/2 phosphorylation was analysed by Western blotting. Pretreatment of cells with PD 98059, PP2, GF 109203X and R0-318220 all significantly inhibited the GnRH induced phosphorylation of ERK1/2 (*, $P<0.01$; **, $P<0.05$, by ANOVA and Tukey's pairwise comparison). Data represent the mean of three separate experiments + SEM.

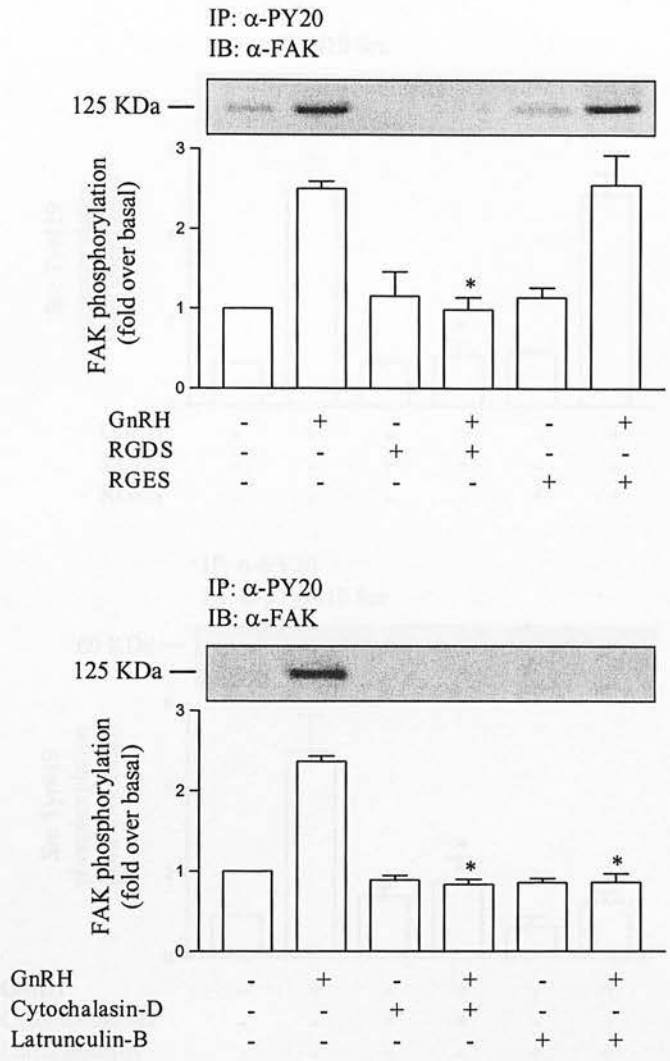


**Figure 19: Role of focal contacts and the actin cytoskeleton in FAK phosphorylation in SCL60 cells.** Quiescent cells were pre-treated with RGDS (1 mM, 16 hours), RGES (1 mM 16 hours), cytochalasin-D (1 μM, 60 minutes) or latrunculin-B (1 μM, 60 minutes). Cells were then treated with vehicle or 100 nM GnRH for 10 minutes and cytoplasmic extracts prepared. Phospho-tyrosine proteins were immune precipitated and FAK phosphorylation analysed by Western blotting. Pretreatment of cells with RGDS, cytochalasin B and latrunculin-D all significantly inhibited GnRH induced tyrosine phosphorylation of FAK (*, $P<0.01$, by ANOVA and Tukey's pairwise comparison). Data represent the mean of three separate experiments + SEM.

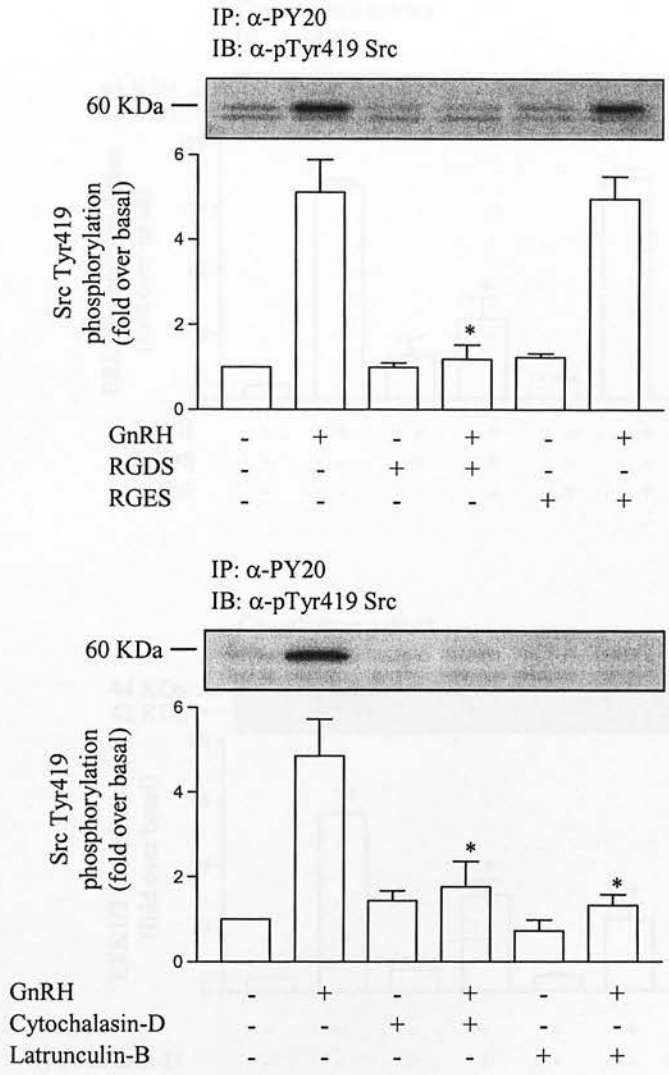


**Figure 20: Role of focal contacts and the actin cytoskeleton in Src activation in SCL60 cells.** Quiescent cells pre-treated with RGDS (1 mM, 16 hours), RGES (1 mM 16 hours), cytochalasin-D (1 μM, 60 minutes) or latrunculin-B (1 μM, 60 minutes). Cells were then treated with vehicle or 100 nM GnRH for 10 minutes and cytoplasmic extracts prepared. Phosphotyrosine proteins were immune precipitated and Src Tyr419 phosphorylation analysed by Western blotting. Pretreatment of cells with RGDS, cytochalasin B and latrunculin-D all significantly inhibited GnRH induced Src Tyr419 autophosphorylation (*, $P<0.01$, by ANOVA and Tukey's pairwise comparison). Data represent the mean of three separate experiments + SEM.

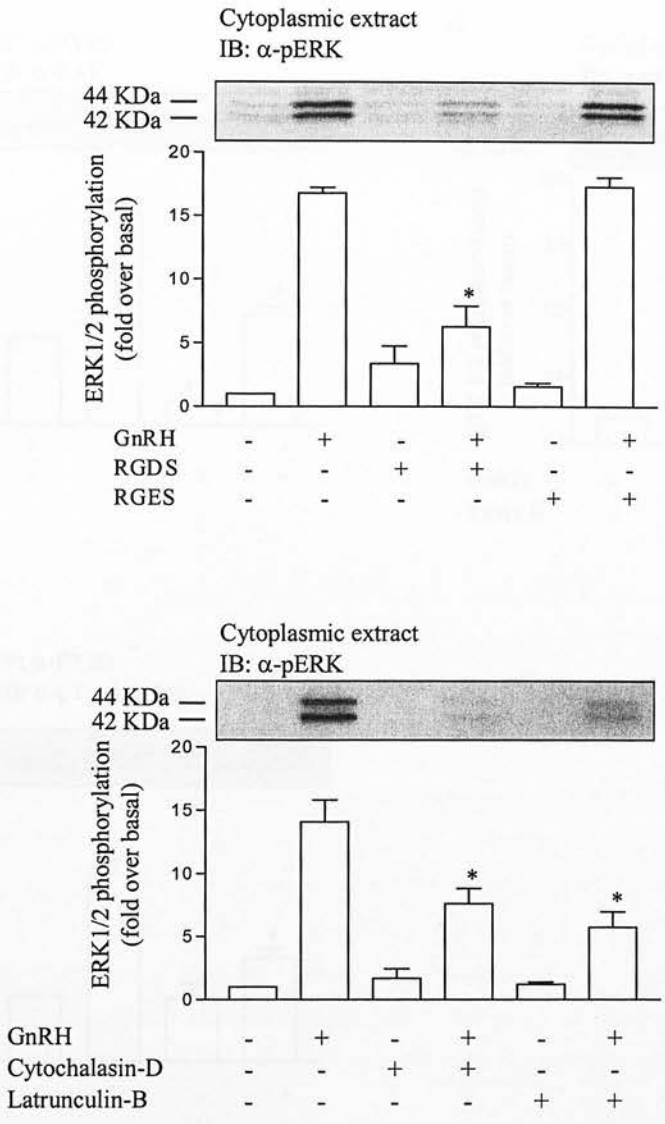


**Figure 21: Role of focal contacts and the actin cytoskeleton in ERK1/2 activation in SCL60 cells**. Quiescent cells were pre-treated with RGDS (1 mM, 16 hours), RGES (1 mM 16 hours), cytochalasin-D (1 μM, 60 minutes) or latrunculin-B (1 μM, 60 minutes). Cells were then treated with vehicle or 100 nM GnRH for 10 minutes, and cytoplasmic extracts prepared. ERK1/2 phosphorylation was analysed by Western blotting. Pretreatment of cells with RGDS, cytochalasin B and latrunculin-D all significantly inhibited GnRH induced ERK1/2 phosphorylation (*, $P<0.01$, by ANOVA and Tukey's pairwise comparison). Data represent the mean of three separate experiments + SEM.

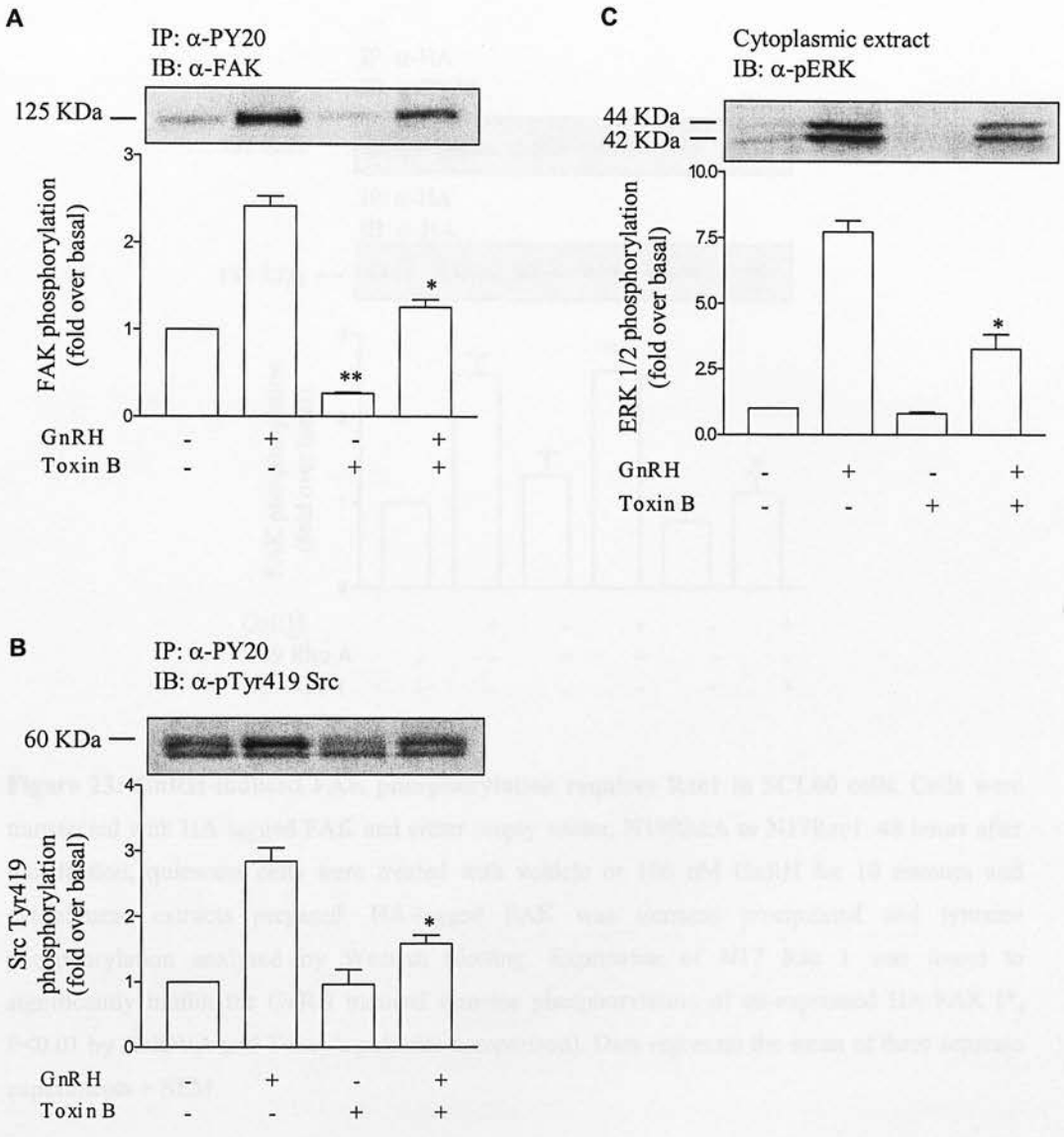


**Figure 22: Role of Rho family GTPases in the activation of FAK, Src and ERK1/2 in SCL60 cells**. Quiescent cells were pre-treated with Toxin B (5 ng/ml, 16 hours). They were then treated with vehicle or 100 nm GnRH for 10 minutes and cytoplasmic extracts prepared. Phospho-tyrosine proteins were immune precipitated and FAK phosphorylation (A) and Src Tyr419 phosphorylation (B) analysed by Western blotting. ERK phosphorylation was analysed by Western blotting cytoplasmic extracts (C). Pretreatment of cells with Toxin B significantly inhibited GnRH induced FAK, Src Tyr419 and ERK1/2 phosphorylation (*, $P<0.01$ by ANOVA and Tukey's pairwise comparison). Toxin B was also found to significantly reduce basal levels of FAK tyrosine phosphorylation (**, $P<0.05$). Data represent the mean of three separate experiments + SEM.

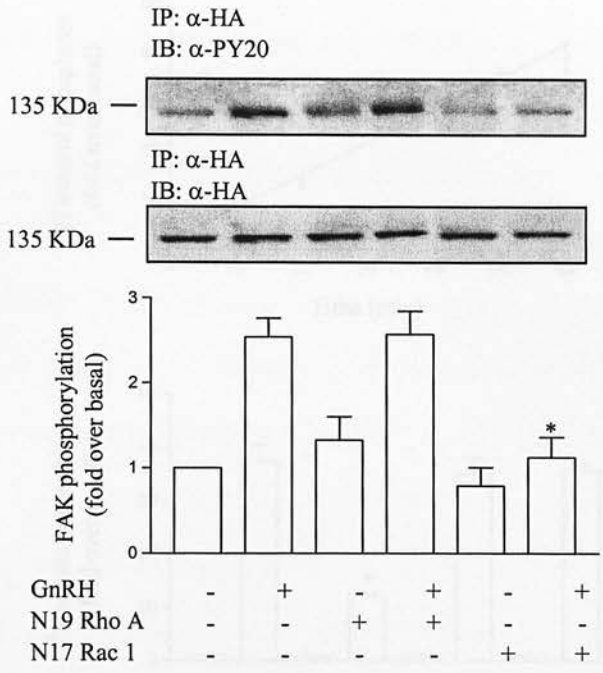


**Figure 23: GnRH-induced FAK phosphorylation requires Rac1 in SCL60 cells**. Cells were transfected with HA-tagged FAK and either empty vector, N19RhoA or N17Rac1. 48 hours after transfection, quiescent cells were treated with vehicle or 100 nM GnRH for 10 minutes and cytoplasmic extracts prepared. HA-tagged FAK was immune precipitated and tyrosine phosphorylation analysed by Western blotting. Expression of N17 Rac 1 was found to significantly inhibit the GnRH induced tyrosine phosphorylation of co-expressed HA-FAK (*, $P<0.01$ by ANOVA and Tukey's pairwise comparison). Data represent the mean of three separate experiments + SEM.

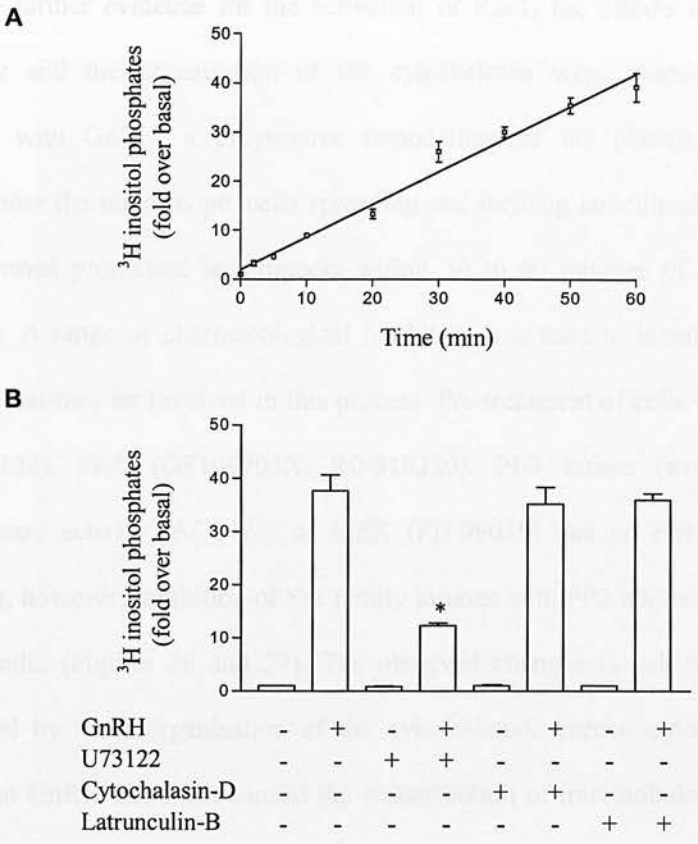


**Figure 24: The activation of PLC by GnRH is not dependent upon the actin cytoskeleton in SCL60 cells.** Cells were labeled with myo-[$^3$H]inositol for 48 hours. (A) Cells were then treated with vehicle or 100 nm GnRH for 10 to 60 minutes. Inositol phosphates were extracted and the radioactivity counted. Data represent the mean of three separate experiments ± SEM. (B) Cells were pretreated with U73122 (20 μM, 60 minutes), cytochalasin-D (1 μM, 60 minutes) or latrunculin-B (1 μM, 60 minutes). Controls received DMSO alone. They were then treated with vehicle or 100 nm GnRH for 60 minutes. Inositol phosphates were extracted and the radioactivity counted. Pretreatment of cells with U73122 significantly inhibited GnRH induced inositol phosphate accumulation (*, $P<0.01$ by ANOVA and Tukey's pairwise comparison). Data represent the mean of three separate experiments + SEM.

To provide further evidence for the activation of Rac1, the effects of GnRH on cell morphology and the organisation of the cytoskeleton were examined. Following stimulation with GnRH, a progressive remodelling of the plasma membrane was observed under the microscope, cells spreading and forming lamellipodia. Almost every cell had formed prominent lamellipodia within 30 to 60 minutes of GnRH treatment (Figure 25). A range of pharmacological inhibitors was used to identify the signalling pathway(s) that may be involved in this process. Pre-treatment of cells with inhibitors of PLC. (U73122), PKC (GF109203X, R0-318220), PI-3 kinase (wotmannin) EGFR tyrosine kinase activity (AG1478) or MEK (PD 98059) had no effect on membrane remodelling, however, inhibition of Src family kinases with PP2 abolished the formation of lamellipodia (Figures 26 and 27). The observed changes in cell morphology were accompanied by the reorganisation of the cytoskeleton. Immunocytochemical studies revealed that GnRH treatment caused the redistribution of microtubules within the cell, and stimulated actin polymerisation at the cell cortex (Figure 28). The formation of filamentous actin networks at the cell cortex was found to be restricted to sites of active plasma membrane remodelling i.e. within lamellipodia (Figure 29). In accordance with the observed effects of PP2 on GnRH induced changes in cell morphology, actin polymerisation was also found to be sensitive to the pharmacological inhibition of Src family kinases (Figure 29). Western blotting revealed that Src associates with the Triton-X100 insoluble cytoskeleton in SCL60 cells. Furthermore, GnRH stimulation lead to a significant increase in the autophosphorylation of Src (P,0.01) associated with the cytoskeleton, where it is positioned to participate in the regulation of cortical actin polymerisation (Figure 30).

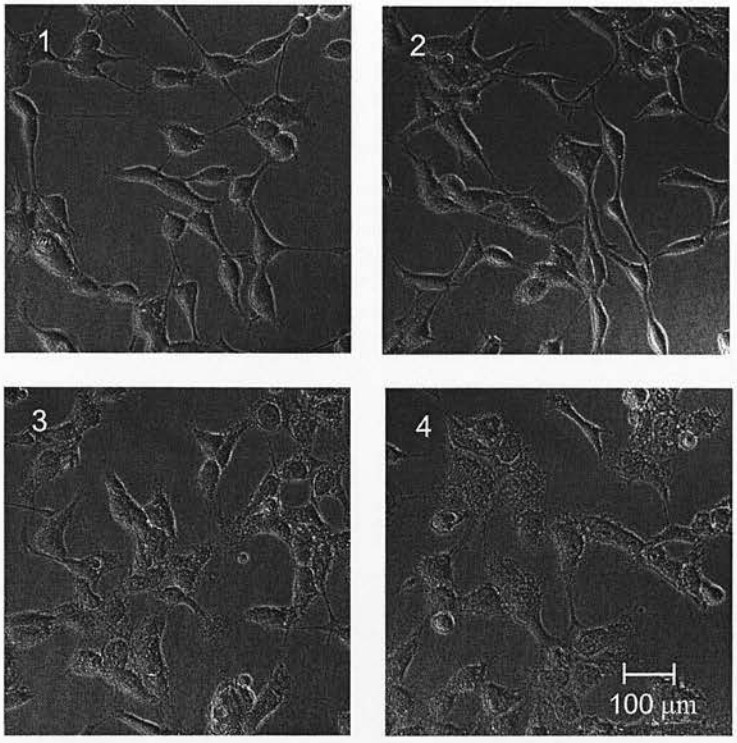


**Figure 25: GnRH induces cell spreading and the formation of lammelipodia in SCL60 cells.** Quiescent cells were treated with vehicle (1) or with 100 nM GnRH for 10 minutes (2), 30 minutes (3) or 60 minutes (4). They were then fixed in methanol, and phase contrast images taken using a Zeiss LSM 510 scanning laser microscope. Images shown are representative of at least three separate experiments.

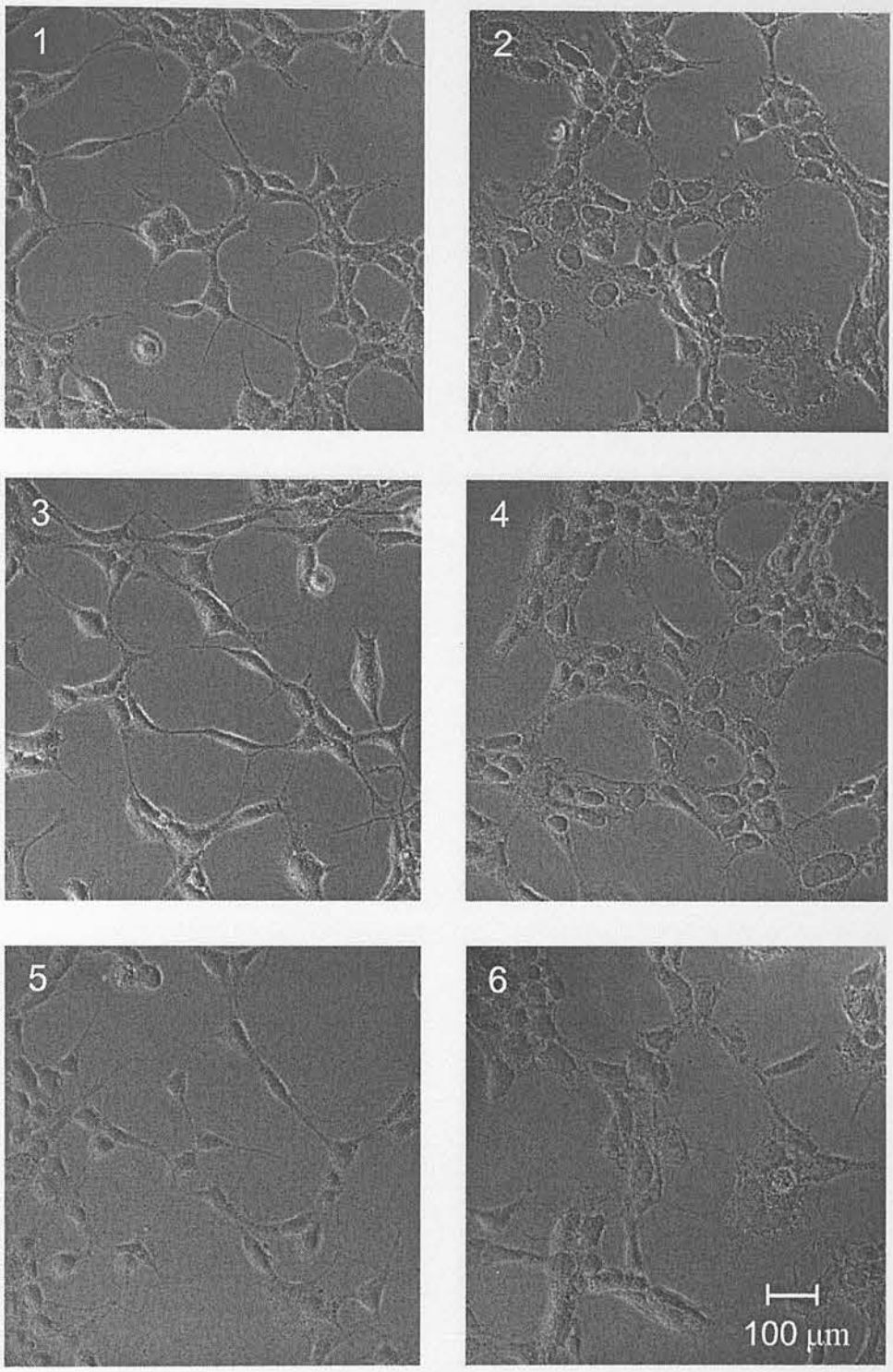


**Figure 26: Chemical sensitivities of GnRH induced cell spreading in SCL60 cells**. Quiescent cells were pretreated with 20 μM U73122 for 60 minutes (1 and 2), 100 nM Ro-318220 for 30 minutes (3 and 4), or 1 μM GF109203X for 30 minutes (5 and 6). Cells were then treated with vehicle (1,3 and 5) or 100 nM GnRH (2,4 and 6) for 30 minutes, fixed in methanol and phase contrast images taken using a Zeiss LSM 510 scanning laser microscope. Images shown are representative of at least three separate experiments.

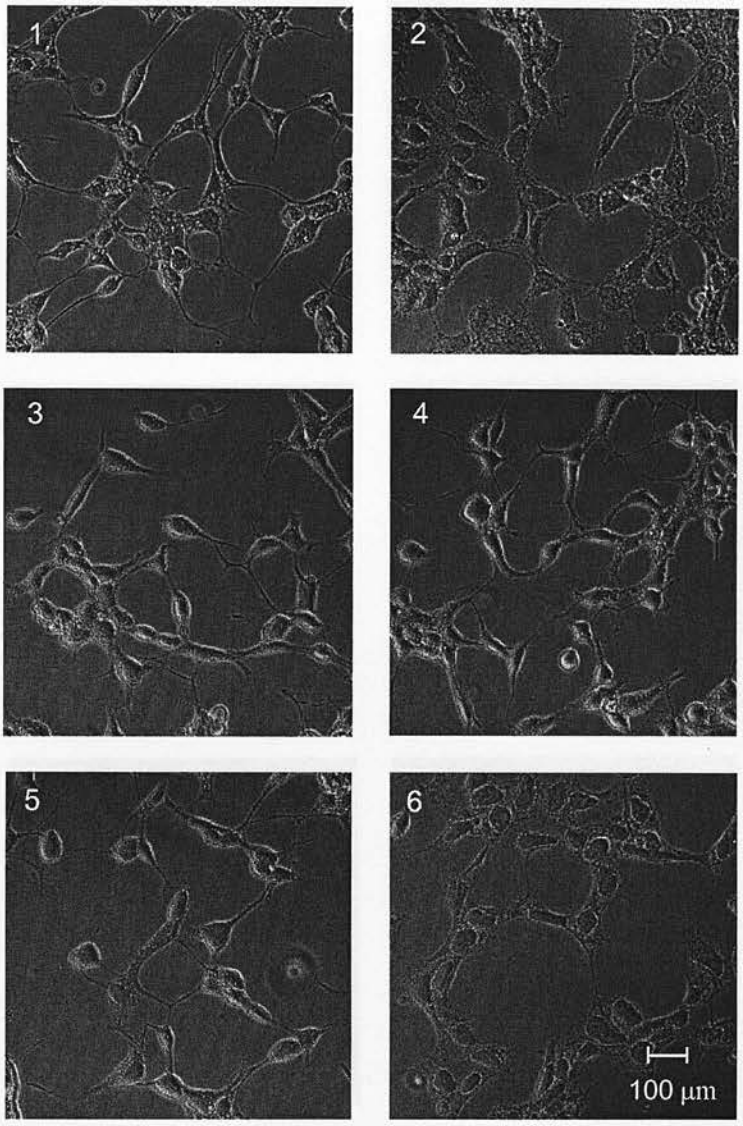


**Figure 27: Chemical sensitivities of GnRH induced cell spreading in SCL60 cells.** Quiescent cells were pretreated with 100 nM wortmannin for 30 minutes (1 and 2), 5 µM PP2 for 30 minutes (3 and 4), or 10 µM PD 98059 for 60 minutes (5 and 6). Cells were then treated with vehicle (1,3 and 5) or 100 nM GnRH (2,4 and 6) for 30 minutes, fixed in methanol and phase contrast images taken using a Zeiss LSM 510 scanning laser microscope. Images shown are representative of three separate experiments.

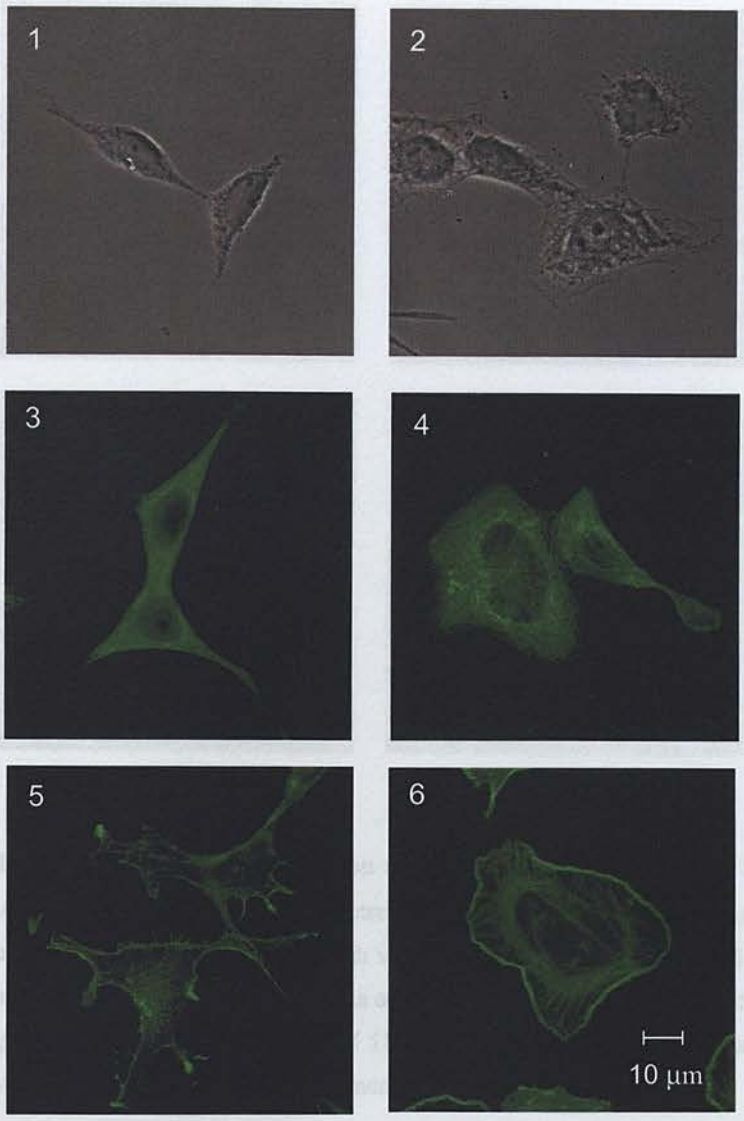


**Figure 28: GnRH induced alterations in the organisation of the cytoskeleton in SCL60 cells.** Quiescent cells were treated with vehicle (1,3 and 5) or 100 nM GnRH for 30 minutes (2, 4 and 6). Cells were then fixed in methanol and stained with antibodies to tyrosine tubulin (3 and 4) or β-actin (5 and 6) and FITC conjugated secondary antibodies. Panels 1 and 2 show phase contrast images. Images were taken using a Zeiss LSM 510 scanning laser microscope. Images shown are representative of at least three separate experiments.

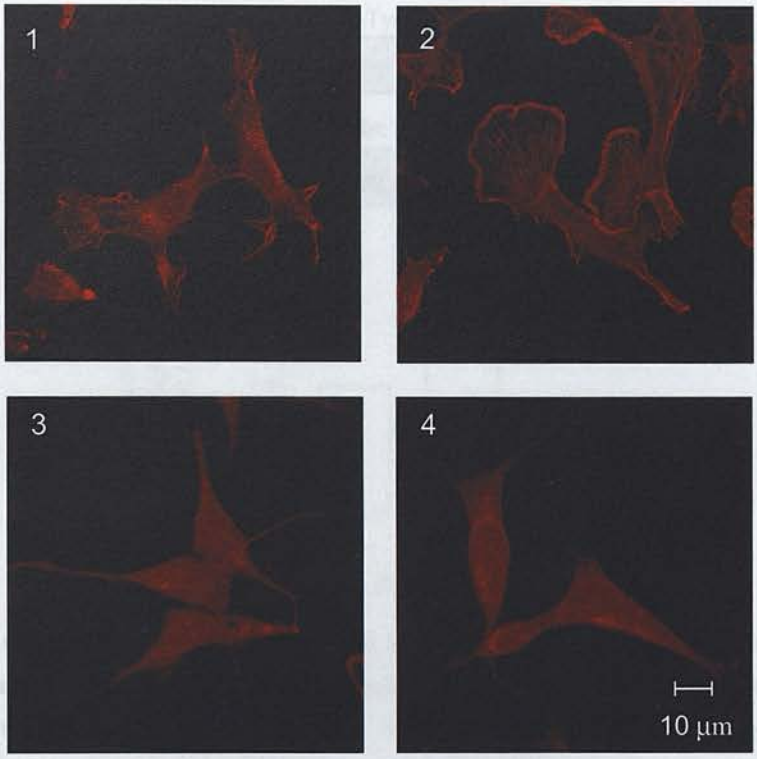


**Figure 29: GnRH induced actin polymerisation is sensitive to the inhibition of Src family kinases in SCL60 cells**. Quiescent cells were pretreated with DMSO (1 and 2) or 5 µM PP2 for 30 minutes (3 and 4). They were then treated with vehicle (1 and 3) or 100 nM GnRH (2 and 4) for 30 minutes and fixed in methanol. Filamentous actin was stained with Alexafluor-568 labeled phalloidin. Images were taken using a Zeiss LSM 510 scanning laser microscope. Images shown are representative of at least three separate experiments.

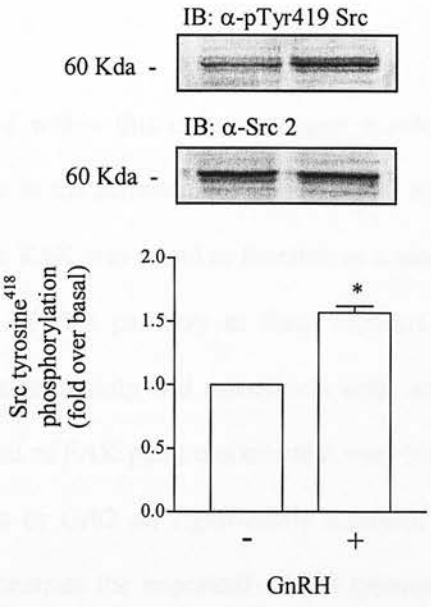


**Figure 30: GnRH induces the activation of Src, associated with Triton X-100 insoluble cytoskeletons in SCL60 cells**. Quiescent cells were treated with vehicle or 100 nM GnRH for 10 minutes. Triton X-100 insoluble cytoskeletons were prepared and Src Tyr419 phosphorylation analysed by Western blotting. GnRH induced a significant increase in Src Tyr419 phosphorylation compared to the unstimulated control (*, $P<0.01$ by Student's T-test). Data represent the mean of three separate experiments + SEM.

**Discussion**

The results presented within this chapter suggest a role for the non-receptor tyrosine kinases FAK and Src in the activation of ERK 1 and 2 by the GnRH receptor in SCL60 cells. In this cell line, FAK was found to function as a molecular scaffold for recruitment of key components of this pathway at focal contacts. This function was critically dependent on its kinase activity and association with, and tyrosine phosphorylation by Src as over expression of FAK point mutants that were kinase dead or unable to bind the SH2 domains of Src or Grb2 all significantly inhibited ERK2 activation (Figure 15). These findings demonstrate the important role of tyrosine phosphorylation and protein-protein interactions mediated by SH2 domains in this process.

While FAK has previously been shown to form a complex with Src (Eide *et al.*, 1995; Thomas *et al.*, 1998), this is the first report of an interaction between FAK and ERK2 *in vivo*. This interaction may be physiologically important as it provides a mechanism of targeting ERK2 to focal contacts where it may be activated, and may also tether the active protein at the plasma membrane, thereby inhibiting nuclear translocation. Thus, the interaction of FAK and ERK2 may provide a potential explanation for why GnRH is less potent at stimulating ERK nuclear translocation compared to EGF (Figure 13). Alternatively, when ERK 1 and 2 are activated by FAK at focal contacts, they may be spatially constrained in the cytoplasm through interactions with additional proteins. Recently, PEA-15 has been shown to regulate ERK 1 and 2 by sequestering them in the cytoplasm, thereby preventing nuclear translocation and phosphorylation of nuclear substrates (Formstecher *et al.*, 2001). In contrast, PEA-15 does not appear to inhibit the phosphorylation of substrates in the cytoplasm, including the serine/threonine kinase RSK (Formstecher *et al.*, 2001), which is an important downstream regulator of ERK dependent signalling (Frödin and Gammeltoft, 1999). As PEA-15 has previously been

shown to regulate integrin-dependent signalling (Ramos *et al.*, 1998), such an interaction seems plausible.

Though ERK 1 and 2 do not contain any modular protein-interaction domains, they interact with their substrates and regulatory molecules in a highly specific manner. Several short peptide motifs have recently been identified in these proteins that mediate these interactions. The ERK binding motifs present in MEK 1 and 2 consists of a cluster of basic amino acids surrounded by hydrophobic amino acids (Tanoue and Nishida, 2003). The docking site for this motif has recently been identified in ERK2 and features two aspartate residues that are thought to make electrostatic interactions with the basic amino acids (Tanoue *et al*, 2000). The crystal structure of ERK2 (Canagarajah *et al.*, 1997) shows that these aspartate residues are situated on the surface of the molecule and thus are available to form intermolecular interactions. The ERK binding motifs present in KSR and the transcription factors Elk-1 and SAP-1 consist of a conserved F-X-F-P motif, where X represent any amino acid (Jacobs *et al.*, 1999a; Fantz *et al.*, 2001). The phenylalanine residues within this motif are thought to mediate hydrophobic interactions with ERK. Although the binding site for this motif has not been identified in ERK, it is tought to comprise a hydrophobic patch on the surface of the molecule (Tanoue and Nishida, 2003). In *C. elegans*, gain of function mutants of lin-1, a transcription factor that is negatively regulated by the nematode homologue of ERK, have been identified in which the F-X-F-P motif is mutated, indicating that it plays a physiologically important role in kinase-substrate interactions (Jacobs *et al.*, 1999b). Recently, a third ERK binding motif has been identified in the transcription factors Ets-1 and Ets-2. This motif consists of a cluster of basic residues followed by a conserved L-X-L motif and a hydrophobic triplet (Seidel and Graves, 2002). Examination of the amino acid sequence of FAK identified a similar sequence D-R-K-G-M-L-Q-L-K-I-A-G-A-P-E, consisting of two basic amino acids, an LXL motif, and a stretch of hydrophobic residues. This putative

ERK binding site may therefore represent the site of interaction between FAK and ERK2, however, this remains to be investigated. A number of experimental approaches could be used to test whether this sequence represents the site of interaction between these two proteins, including the yeast two-hybrid system, which has proven to be a very powerful and sensitive tool for exploring protein-protein interactions *in vivo*. Because the crystal structure of FAK has not been solved, it is currently unknown whether this motif is situated on the surface of the molecule, where it could participate in molecular interactions.

By using a range of pharmacological inhibitors FAK tyrosine phosphorylation was found to be independent of PLC, PKC, and intracellular $Ca^{2+}$, but required an intact actin cytoskeleton and cell adhesion to the extracellular matrix (Figures 16 and 19). These findings are in accordance with previous studies of FAK activation by bombesin, which has been shown to be mediated by members of the Rho family GTPases (Sinnet-Smith *et al.*, 1993; Rozengurt, 1998). In SCL60 cells, Rac1 was found to be required for FAK activation by GnRH (Figure 22). Furthermore, Toxin B (which inactivates RhoA, Rac1 and Cdc42) was found to inhibit GnRH induced FAK phosphorylation (Figure 22). Toxin B was also found to significantly reduce basal levels of FAK phosphorylation in these cells, consistent with the role of the Rho family GTPases in the control of the actin cytoskeleton and the formation and turnover of focal contacts (Nobes and Hall 1995; Hotchin and Hall, 1995).

The first evidence that the Rho family GTPases were involved in the activation of FAK by Gq coupled receptors was provided by Rankin *et al.* (1994), who showed that the microinjection of Swiss 3T3 cells with C3 exoenzyme, which ADP-ribosylates and inactivates RhoA, could inhibit FAK tyrosine phosphorylation by bombesin. Subsequent studies have shown that constitutively active mutant forms of Gαq and Gα11 can activate RhoA and induce the formation of stress fibres in Swiss 3T3 cells and mouse embryo

fibroblasts (Dutt *et al.*, 2002; Vogt *et al.*, 2003), thereby providing a direct link between Gq/11 coupled receptors and the regulation of Rho activity. Gαq has been found to activate the Rho specific guanine nucleotide exchange factors p115RhoGEF, PDZ-RhoGEF and LARG (Chikumi *et al.*, 2002), which contain an RGS domain that mediates their association with G-proteins. In mouse embryo fibroblasts, expression of a dominant negative mutant of LARG inhibits RhoA activation and the formation of stress fibres by several Gq coupled receptors (Vogt *et al.*, 2003). These studies therefore provide a mechanism whereby RhoA can be activated by Gαq. However, constitutively active mutants of Gαq have not been reported to stimulate Rac1 activation or the formation of lamellipodia.

Rac1 activation by several receptor tyrosine kinases, including the PDGF and insulin receptors, is mediated by PI-3 kinase (Nobes *et al.*, 1995). However, Rac1 activation by GnRH was not blocked by the PI-3 kinase inhibitor wortmannin in SCL60 cells (Figure 27). Similarly, Rac1 activation by bombesin is insensitive to wortmannin (Nobes *et al.*, 1995), indicating that Gq coupled receptors may use a PI-3 kinase independent mechanism to activate Rac1. Data presented within this chapter demonstrate that Src plays a critical role in this pathway (Figures 27 and 29). Furthermore, Src was found to be present in Triton X-100 insoluble cytoskeleton extracts, where it is positioned to participate in the regulation of the cortical actin cytoskeleton (Figure 30).

Recent studies have shown that Src can regulate Rac activity by phosphorylating the guanine nucleotide exchange factors Ras-GRF1, Vav1, Vav2 and Tiam1. Ras-GRF1 was originally identified as a guanine nucleotide exchange factor for Ras that is activated by $Ca^{2+}$ influx (Farnsworth *et al.*, 1995). In addition, to possessing a Cdc25 homology domain that is responsible for Ras GDP/GTP exchange activity, Ras-GRF1 contains a double homology (DH) domain that is responsible for catalysing the exchange of GDP for GTP by the Rho family GTPases (Kiyono *et al.*, 1999). *In vitro* and *in vivo* studies

have shown that Ras-GRF1 is specific for Rac1, and that its Rac GDP/GTP exchange activity requires tyrosine phosphorylation by Src family kinases (Kiyono *et al.*, 1999; Kiyono *et al.*, 2000). In contrast, the Ras GDP/GTP exchange activity of the protein is unaffected by tyrosine phosphorylation (Kiyono *et al.*, 1999). However, as Ras-GRF1 expression is restricted to the brain it is unlikely to be the guanine nucleotide exchange factor that is activated by GnRH in SCL60 cells.

The Vav proteins comprise a small family of Rac specific guanine nucleotide exchange factors that share a modular structure consisting of a calponin homology (CH) domain that binds actin, a DH domain that is responsible for guanine nucleotide exchange activity, a PH domain, two SH3 domains and an SH2 domain. While Vav1 is expressed exclusively in haematopoietic cells, Vav2 is ubiquitously expressed (Adams *et al.*, 1992; Schuebel *et al.*, 1996). In quiescent cells Vav1 and Vav2 maintained in an inactive conformation through intramolecular interactions involving the DH and PH domains. Tyrosine phosphorylation of these proteins by Src family kinases disrupts this inhibitory intramolecular interaction and allows the DH domain to bind and activate Rac1 (Crespo *et al.*, 1997; Han *et al.*, 1997).

Tiam1 is a Rac specific guanine nucleotide exchange factor that was originally identified in a retroviral insertional mutagenesis screen for genes that conferred an invasive phenotype to T-lymphoma cells (Habets *et al.*, 1994). It is a widely expressed protein with a modular structure consisting of two PEST domains, two PH domains, a coiled-coil region, a PDZ domain, and a DH domain. In quiescent cells, Tiam1 is maintained in an inactive conformation by intramolecular interactions involving the N-terminus of the protein (Mertens *et al.*, 2003). Like Vav1 and Vav2, tyrosine phosphorylation of Tiam1 by Src disrupts this inhibitory intramolecular interaction, leading to an increase in its nucleotide exchange activity (Servitja *et al.*, 2003).

As Vav2 and Tiam1 are widely expressed proteins and are found in lysates from many cell types, including HEK293 cells (Servitja *et al.*, 2003), they represents good potential candidates for the Rac1 specific guanine nucleotide exchange factor regulated by GnRH in SCL60 cells. To investigate this, immune precipitation and Western blotting studies could be performed to determine if Vav2 or Tiam1 are tyrosine phosphorylated by Src in SCL60 cells following stimulation with GnRH. Because specific pharmacological inhibitors are not commercially available for these proteins, RNAi could then be used to study their role in GnRH receptor signalling.

Further evidence for a role of Src in the activation of Rac1 has been provided by studies of cells transformed with the oncogene v-Src. When cells are transformed with this oncogene they become able to grow independently of anchorage in soft agar. The colonies that are formed are morphologically very similar to those seen when cells are transformed with activated mutants of the Rho family GTPases or their guanine nucleotide exchange factors (Servitja *et al.*, 2003). Furthermore, when cells are transformed with v-Src and a dominant negative form of Rac1 (N17Rac1), the number of colonies formed is significantly reduced (Servitja et al., 2003). These results therefore suggest that Rac1 is an integral component of the signalling pathway used by v-Src and by inference, its cellular homologue.

The studies of Src activation using phosphospecific antibodies shown within this chapter suggest that Src may be initially activated by the dephosphorylation of pTyr530 (Figure 12), indicating that GnRH activates a protein tyrosine phosphatase in SCL60 cells. Perhaps the best candidate molecule for this GnRH regulated phosphatase is PTPα, which is widely expressed, localises to focal contacts and co-immune precipitates with Src and Fyn *in vivo* (Harder *et al.*, 1998; Bhandari *et al.*, 1998). However, the mechanism of activation of PTPα by extracellular signalling molecules such as GnRH remains poorly understood. Though protein kinases are known to be important molecules in GnRH

receptor signalling, protein phosphatases have received much less attention. Nevertheless, these experiments indicate that a protein tyrosine phosphatase plays a key role in the activation of Src by GnRH. This could be further investigated by using protein tyrosine phosphatase inhibitors such as pervanadate or hydrogen peroxide. Because isoform-specific pharmacological inhibitors are not currently available for these proteins, RNA interference (RNAi) could also provide a useful tool for studying the role of individual phosphatases in GnRH receptor signalling.

Though pTyr530 is only transiently dephosphorylated following stimulation of SCL60 cells with GnRH, Tyr419 autophosphorylation and Src kinase activity remained elevated above basal levels for 1 hour (Figure 12). These findings are in accordance with a previous study by Boerner *et al.* (1996), who showed that Src retains significant enzyme activity when doubly phosphorylated at Tyr530 and Tyr419. Examination of the crystal structures of the active and inactive forms of the enzyme provides clues to how this may occur. In the inactive state, the activation loop adopts an ordered helical structure and Tyr419 is sequestered in a hydrophobic pocket where it helps stabilise the closed (inactive) conformation of the catalytic domain (Xu *et al.*, 1999). However, when it is phosphorylated, Tyr419 cannot be accommodated in this hydrophobic pocket and therefore cannot help fix the N- and C-terminal lobes of the catalytic domain in the closed conformation (Xu *et al.*, 1999), even when the SH2 domain binds pTyr529. Thus the catalytic domain can remain active. Therefore the inactivation of Src requires both the phosphorylation of Tyr530 by Csk, and the dephosphorylation of Tyr419 by an unknown protein tyrosine phosphatase. Src may also be active when doubly phosphorylated at Tyr530 and Tyr419 when bound to FAK via its SH2 and SH3 domains, which relieves the intramolecular repression of the catalytic domain. Both these situations may occur in SCL60 cells, accounting for the prolonged activation of Src.

These observations also suggest that Src activation in SCL60 cells may reflect two distinct events that are temporally and mechanistically distinct. Src may be initially activated by the transient dephosphorylation of pTyr530 by a protein tyrosine phosphatase, and subsequently by interactions with FAK. Thus, Src activation may occur upstream (protein tyrosine phosphatase dependent) and downstream (FAK dependent) of Rac1 activation. Therefore, it would be expected that the initial activation of Src by GnRH would occur independently of Rho family GTPases, while at later time points it would be predicted to be sensitive to the inhibition of Rho family GTPases (which are required for the activation of FAK). Indeed, Src Tyr419 autophosphorylation was found to be inhibited by *C. difficile* toxin B at 10 minutes (Figure 22), however, it is unknown what effect this compound has on Src pTyr529 dephosphorylation at earlier time points.

Based on these observations, a model of ERK activation may be proposed in which GnRH activates a protein tyrosine phosphatase, which dephosphorylates the regulatory tyrosine residue in the C-terminal tail of Src leading to its activation. Src then phosphorylates a Rac1 specific guanine nucleotide exchange factor, perhaps Vav2 or Tiam1, leading to the activation of Rac1, Scar and the Arp2/3 complex, which initiates actin polymerisation at the cell cortex. Once nucleated, actin filaments grow rapidly towards the plasma membrane, providing the protrusive force that is required for the extension of lamellipodia. Integrins become aggregated in lamellipodia, forming focal complexes, which provide anchor points for the cell as it moves. Integrin aggregation in turn leads to the oligomerisation and activation of FAK, which *trans*-autophosphorylates at Tyr397 to create a binding site for Src. Thus, Src is recruited into a complex with FAK, which stabilises it in an active conformation. Src then phosphorylates FAK on a number of tyrosine residues, including Tyr925, which creates a binding site for the SH2 domain of Grb2. Grb2 then recruits Sos to the plasma membrane where it activates Ras to initiate the activation of the ERK cascade.

In accordance with this model, ERK activation was found to be dependent on cell adhesion to the extracellular matrix, required an intact actin cytoskeleton and FAK, Src and Rac1 activity (Figures 18, 21 and 22). In addition, ERK activation was also found to be sensitive to inhibitors of PKC (Figure 18), indicating that multiple signals converge to provide precise regulation of this pathway. Although the results presented within this chapter indicate that PKC plays only a minor role in ERK activation, this may reflect the fact that low concentrations of both PKC inhibitors were used in these studies, which may not have been sufficient to completely block cellular PKC. Therefore, the role of PKC in the activation of the ERK signalling cascade may have been under represented in these cells. However, as ERK activation was not inhibited by BAPTA-AM, which chelates intracellular $Ca^{2+}$, conventional PKC isozymes do not appear to be involved. These findings are in accordance with a study in LβT2 cells, which showed ERK activation by GnRH to require PKC but to be independent of $Ca^{2+}$ (Liu *et al.*, 2002). Because inhibition of PLC did not significantly inhibit ERK activation by GnRH (Figure 18), it may be speculated that an atypical PKC may be involved in this pathway, as they do not require diacylglycerol or $Ca^{2+}$ for their activation (Newton, 1997). However, activation of a novel PKC isozyme cannot be discounted as PLC activity was not completely inhibited by U73122 in SCL60, even when used at concentrations up to 20 μM (Figure 24). Therefore, sufficient diacylglycerol may have been generated by PLC mediated hydrolysis of PtdIns(4,5)$P_2$ in the presence of U73122 to activate these kinases. By using RNAi to knockout the expression of specific PKC isozymes, it may be possible to identify those isozymes involved in the activation of ERK in SCL60 cells, however, this was beyond the scope of this thesis.

**Future work**

To gain a better understanding of the role played by tyrosine kinases such as FAK and Src in GnRH receptor signalling, it would be desirable to identify the three other proteins

that were tyrosine phosphorylated following stimulation of SCL60 cells with GnRH (Figure 10). Potential candidate molecules for these phosphoproteins include Vav2 and paxillin, which have molecular weights of 101 KDa and 68 KDa, respectively. Paxillin is the prototypic member of a family of scaffolding proteins, which also includes hic-5 and leupaxin (Schaller, 2001). These proteins contain a number of modular protein-interaction domains, which control their subcellular localisation and interaction with other proteins. The C-terminus of paxillin contains four Lin-11, Isl-1, Mec-3 (LIM) domains (Schmiechel and Beckerle, 1994), which are responsible for targeting it to focal contacts (Brown *et al.* 1996), while the N-terminus contains multiple copies of another interaction domain called the LD motif, which features the consensus sequence L-D-X-L-L-X-X-L, where X represents any amino acid (Brown *et al.*, 1996). The LD motif is predicted to form a α-helix with the leucine residues lying along one side, presenting a hydrophobic protein-binding surface (Turner, 2000). The individual LD motifs of paxillin have been shown to bind a number of different proteins and have discrete binding preferences. LD1, LD2 and LD4 bind the structural protein vinculin, while LD2 and LD4 bind FAK (Brown *et al.*, 1996; Turner, 2000).

The N-terminus of paxillin also contains two important tyrosine residues, Tyr31 and Tyr118, which are phosphorylated by Src and FAK (Schaller and Parsons, 1995). Phosphorylation of these residues creates binding sites for the SH2 domains of the adapter protein Crk (Schaller and Parsons, 1995), which is involved in the control of integrin mediated cell motility, and Csk (Sabe *et al.*, 1994) which plays an important role in controlling Src activity by phosphorylating Tyr530 as described (Nada *et al.*, 1991). The structure of Csk is very similar to the Src family kinases, consisting of a conserved catalytic domain, an SH2 domain and an SH3 domain (Ogawa *et al.*, 2002). However, unlike the majority of other protein kinases, Csk has a high basal level of enzyme activity, and is not regulated by phosphorylation of residues within its activation loop

(Lamers et al., 1999). Rather, Csk appears to be regulated through the control of its subcellular localisation and thus access to potential substrates. Therefore, recruitment of Csk into focal contacts through its association with tyrosine phosphorylated paxillin may bring it into proximity to Src, allowing it to phosphorylate Tyr530. Thus, paxillin tyrosine phosphorylation may play a role in the control of GnRH receptor signalling by recruiting proteins to the plasma membrane that are involved in the regulation of Src activity, and the organisation of the actin cytoskeleton. Thus, identification of the remaining phosphoproteins may help provide a more complete understanding of the role of protein tyrosine phosphorylation in the regulation of GnRH receptor signalling, and may provide new avenues for future research.

# Identification of Src binding proteins involved in the regulation of GnRH receptor signalling

## Introduction

Many signalling pathways in eukaryotic cells are regulated by the reversible assembly of multi-protein complexes that integrate and transmit signals from cell surface receptors to the cytoplasm and nucleus. The assembly of these complexes is regulated by a variety of protein- and lipid-interaction domains that target proteins to specific subcellular localisations and mediate molecular interactions (Yaffe and Elia, 2001; Pawson and Nash 2003). Proteomic techniques offer a new, unbiased approach for studying the assembly of these protein complexes during signal transduction.

The combination of two-dimensional gel electrophoresis and MALDI-ToF mass spectrometry is one of the most commonly used methods in proteomics, and has become the analytical tool of choice for many researchers. These techniques allow rapid separation and identification of many cellular proteins and are applicable to high throughput screening. Protein identification has been greatly facilitated by the sequencing of the human and mouse genomes, and by the provision of detailed protein databases on the worldwide web that can be searched using user friendly software such as MS-Fit (http://prospcetor.ucsf.edu) to generate peptide mass fingerprints.

Having established a role for Src in the activation of the ERK cascade, a proteomics study was undertaken to identify novel Src binding proteins that may be involved in the regulation of GnRH receptor signalling. Src is an ideal molecule for such a study because it contains SH2 and SH3 domains that mediate interactions with its substrates, and specific antibodies are available for immune precipitation, which allows simple purification of Src complexes. In this chapter, the identification of DGKζ as a Src binding protein is described using proteomic techniques and the role of this interaction investigated.

**Results**

Two-dimensional gel electrophoresis and silver staining revealed that Src associates with many proteins in quiescent SCL60 cells. However, 20 different proteins were found to co-precipitate with Src only after stimulation with GnRH (Figure 31). These proteins were cut from the gel, digested with trypsin, and analysed by MALDI-ToF mass spectrometry. In two separate experiments, good data sets were obtained for protein number three, which had an apparent molecular weight of 100 KDa and an isoelectric point of pH 8.5 (Figure 31). One of the mass spectra obtained for this protein is shown in Figure 32. Peptide mass fingerprinting identified this protein as the lipid kinase DGKζ (Figure 33), with molecular weight search (MOWSE) scores of 6.45 $x10^6$ and 6.172 $x10^6$ being reported. A numer of keratins were also found to be present in the sample, namely cytokeratin 1 (MOWSE score 1.129 x $10^6$), cytokeratin 2e (MOWSE score 1.082 x $10^6$), cytokeratin 5 (MOWSE score 3.467 x $10^5$) and cytokeratin 9 (MOWSE score 4.609 x $10^6$). As these proteins have molecular weights in the region of 60 – 70 KDa they probably do not represent proteins co-precipitating with Src but rather contaminants from skin when handling the gel.

The other proteins analysed by MALDI-ToF mass spectrometry could not be identified with any confidence. The low success rate of protein identification may reflect the low abundance of proteins in the sample, which approached the limit of detection by silver staining and the mass spectrometer, and was further hampered by keratin contamination of the gel, which proved to be a major problem.

In the human, alternative splicing of the DGKζ gene gives rise to two isoforms of the protein with predicted molecular weights of 104 KDa (Accession number: Q13374-2) and 124 KD (Accession number: Q13374-1), respectively. The mass of the protein estimated from the two-dimensional gel would suggest that it is the 104KDa isoform that

co-precipitates with Src in these cells. Analysis of the amino acid sequence of DGKζ using scansite (http://scansite.mit.edu) identified a potential Src phosphorylation site at Tyr558 (Y*-L-E-V) and two proline rich motifs that may be ligands for the SH3 domain of Src. The sequences of these proline rich regions are L-A-P-P-P-P-T-P-G-A-P (amino acids 65 - 75) and D-L-P-T-P-T-S-P-L-P-T (amino acids 762 - 772). All three motifs are conserved in rat and mouse DGKζ, and may therefore represent the site(s) of interaction between the two proteins.

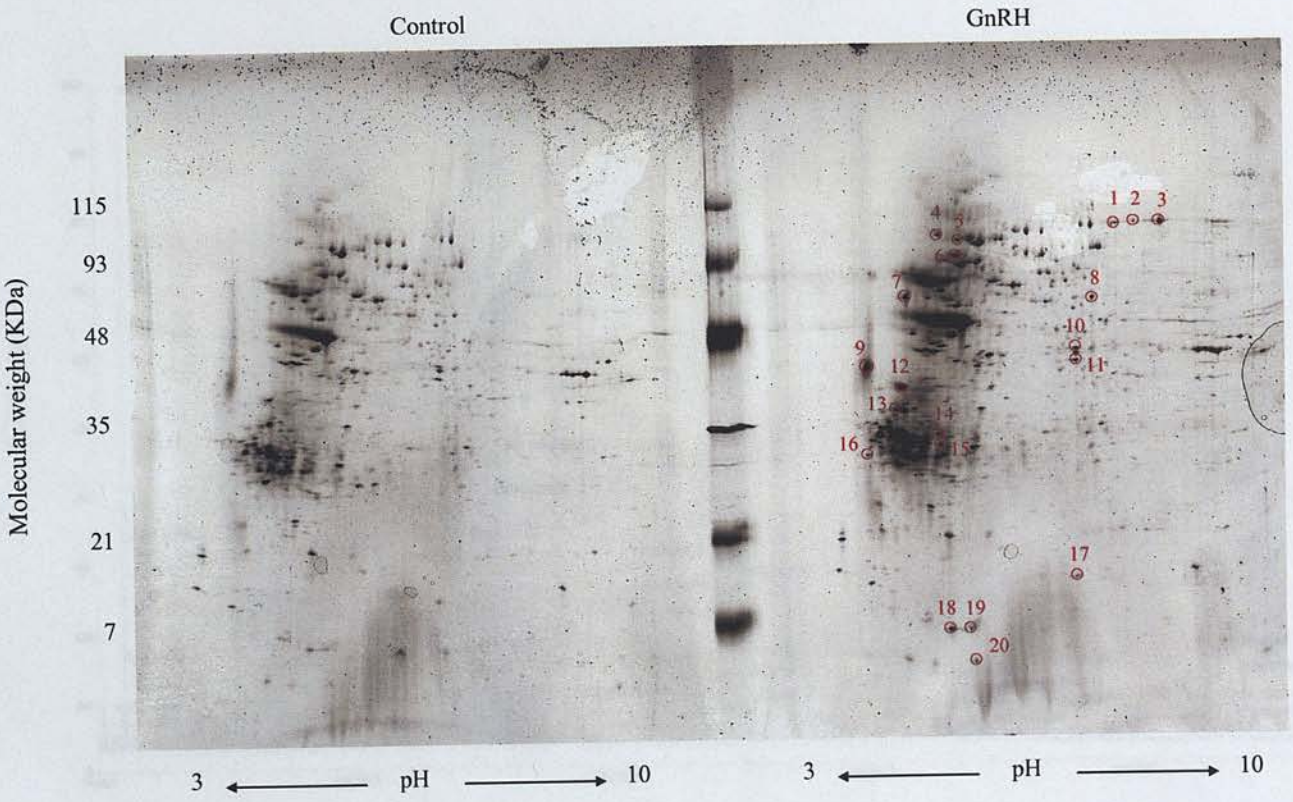


**Figure 31: Two-dimensional gel electrophoresis of Src immune precipitated from SCL60 cells**. Quiescent cells were treated with vehicle or 1 μM GnRH for 10 minutes and cytoplasmic extracts prepared. Src was immune precipitated and subjected to two-dimensional gel electrophoresis. Gels were stained with silver. The 20 proteins found to associate with Src following stimulation with GnRH are highlighted in red. The two-dimensional gel shown is representative of two separate experiments.

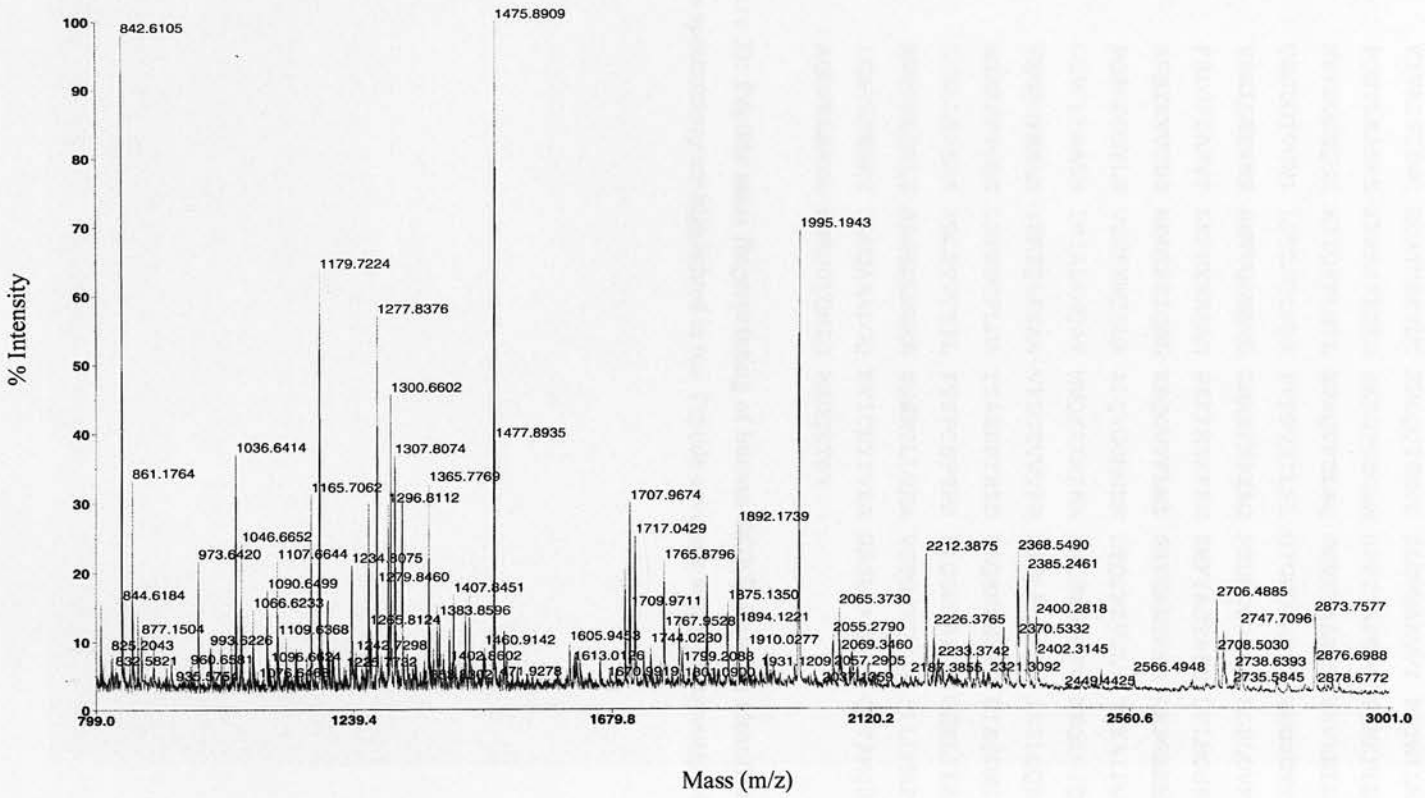


**Figure 32: MALDI-ToF mass spectrum**. The mass spectrum obtained for protein number 3, which had an apparent molecular weight of approximately 100 KDa and an isoelectric point of pH 8.5.

MEPRDGSPEA RSSDSESASA SSSGSERDAG PEPDKAPRRL NKRRFPGLRL
FGHRKAITKS GLQHLAPPPP TPGAPCSESE RQIRSTVDWS ESATYGEHIW
FETNVSGDFC YVGEQYCVAR MLKSVSRRKC AACKIVVHTP CIEQLEKINF
RCKPSFRESG SRNVREPTFV RHHWVHRRRQ **DGKCRHCGKG FQQ**KFTFHSK
EIVAISCSWC KQAYHSKVSC FMLQQIEEPC SLGVHAAVVI PPTWILRARR
PQNTLKASKK KKRASFKRKS SKKGPEEGRW RPFIIRPTPS PLMKPLLVFV
NPKSGGNQGA KIIQSFLWYL **NPRQVFDLSQ GGPKEALEMY R**KVHNLRILA
CGGDGTVGWI LSTLDQLRLK PPPPVAILPL GTGNDLA**RTL NWGGGYTDEP**
**VSKILSHVEE GNVVQLDR**WD LHAEPNPEAG PEDRDEGATD RLPLDVFNNY
FSLGFDAHVT LEFHESREAN PEKFNSRFRN KMFYAGTAFS DFLMGSSKDL
AK**HIRVVCDG MDLTPKIQDL KPQCVVFLNI PRYCAGTMPW GHPGEHHDFE**
**PQR**HDDGYLE VIGFTMTSLA ALQVGGHGER LTQCREVVLT TSKAIPVQVD
GEPCKLAASR IRIALRNQAT MVQKAKRR**SA APLHSDQQPV PEQLR**IQVSR
**VSMHDYEALH YD**KEQLKEAS VPLGTVVVPG DSDLELCRAH IERLQQEPDG
AGAKSPTCQK LSPKWCFLDA TTASRFYRID RAQEHLNYVT EIAQDEIYIL
DPELLGASAR PDLPTPTSPL PTSPCSPTPR SLQGDAAPPQ GEELIEAAKR
**NDFCKLQELH RAGGDLMHRD EQSR**TLLHHA VSTGSKDVVR YLLDHAPPEI
LDAVEENGET CLHQAAALGQ RTICHYIVEA GASLMKTDQQ GDTPRQRAEK
**AQDTELAAYL ENR**QHYQMIQ REDQETAV

**Figure 33: Peptide mass fingerprinting of human DGKζ.** Peptides identified by MALDI-ToF mass spectrometry are highlighted in red. Peptide coverage was approximately 14%.

The expression of DGKζ in SCL60 cells was confirmed by immune precipitation and Western blotting using a polyclonal antibody that was specific for the 104 KDa isoform of the enzyme. A protein of approximately 100 KDa was identified in cytoplasmic extracts, that was not detected when immune precipitations were performed with pre-immune serum (Figure 34 A). The interaction between Src and DGKζ was also confirmed by immune precipitation and Western blotting. Src was found to co-precipitate with DGKζ only following stimulation with GnRH (Figure 34 B). Src was also found to bind to DGKζ *in vitro* by affinity chromatography, using immobilised recombinant rat DGKζ as ligand (Figure 35). Note the recombinant protein has a molecular weight of approximately 130 KDa, due to the presence of GST and 6 His tags at the N-terminus.

The association between Src and DGKζ was found to be dependent on Src enzyme activity, as it could be significantly inhibited by the pre-treatment of cells with the general tyrosine kinase inhibitor herbimycin A or the Src family kinase inhibitor PP2 ($P<0.01$) (Figure 34 B). Although Src enzyme activity was appeared to be required for the association of the two proteins, DGKζ was not found to be tyrosine phosphorylated following stimulation of SCL60 cells with GnRH, as determined by Western blotting DGKζ immune precipitates with an anti-phosphotyrosine antibody (Figure 36).

*In vitro* kinases assays, performed with mixed micelles containing phosphatidylserine and dioleoyl-*sn*-glycerol as substrate, revealed that GnRH stimulated the enzymatic activity of DGKζ, that was immune precipitated from SCL60 cells (Figure 37). The activation of DGKζ by GnRH appeared to require Src enzyme activity, as it could be inhibited by pre-treating cells with PP2 (Figure 37). However, PP2 had no direct effect on DGKζ kinase activity *in vitro* when added to immune precipitates for 30 minutes prior to performing the kinase assay. Thus, these effects were not due to the direct inhibition of the catalytic domain of DGKζ by this compound (data not shown). In many cells, the cytoskeleton functions as a scaffold for enzymes involved in lipid metabolism. Immune precipitation

and Western blotting revealed that DGKζ was associated with Triton X-100 insoluble cytoskeletons in SCL60 cells (Figure 38 A), and *in vitro* kinases assays revealed that GnRH activated the DGKζ that was associated with the cytoskeleton (Figure 38 B).

DGKζ was also found to be expressed in LβT2 gonadotroph cells (Figure 39). As the enzyme activity of the DGKs has been shown to be controlled in part by regulating their access to substrate in the plasma membrane, the effects of GnRH on the subcellular localisation of GFP-tagged DGKζ, was investigated in these cells by confocal microscopy. In quiescent LβT2 cells, GFP-DGKζ was found to be present in the nucleus and cytoplasm. However, following stimulation with GnRH, it was observed to translocate to the plasma membrane (Figure 40). Membrane translocation was rapid, occurring within 10 minutes of agonist treatment, and was found to be sensitive to the inhibition of Src enzyme activity with PP2 (Figure 41). Membrane translocation also required an intact actin cytoskeleton, as it could be abolished by pre-treatment of cells with cytochlasin-D (Figure 42) or latrunculin-B (Figure 43), and required PKC activity (Figure 44), as previously reported (Santos *et al.*, 2002).

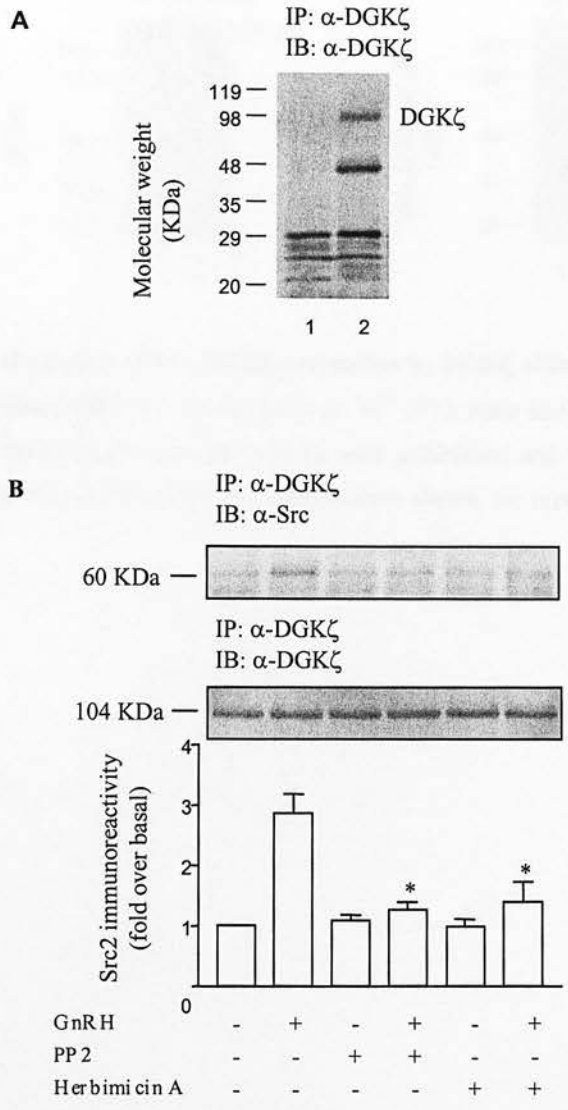


**Figure 34: Confirmation of DGKζ expression and association with Src in SCL60 cells.** (A) Cytoplasmic extracts were prepared and proteins immune precipitated with pre-immune serum (lane 1) or anti-DGKζ (lane 2). Proteins were resolved by SDS-PAGE and DGKζ expression analysed by Western blotting. (B) Quiescent cells were pre-treated with PP2 (5 μM, 30 minutes), herbimicin A (100 nM 30 minutes) or DMSO. They were then treated with vehicle or 100 nM GnRH for 10 minutes and cytoplasmic extracts prepared. DGKζ was immune precipitated and Src association analysed by SDS-PAGE and Western blotting. Pretreatment of cells with herbimycin A or PP2 significantly inhibited the GnRH induced association of Src with DGKζ (*, $P<0.01$ by ANOVA and Tukey's pairwise comparison). Data represent the mean of three separate experiments + SEM.

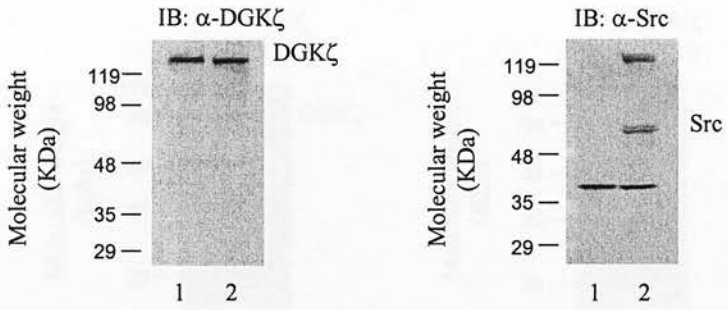


**Figure 35: Confirmation of Src-DGKζ association by DGKζ affinity chromatography**. 6 His tagged recombinant DGKζ was immobilised on $Ni^{2+}$-NTA resin and incubated with buffer alone (lane 1) or rat brain lysate (lane 2). Proteins were solubilised and Src association analysed by SDS-PAGE and Western blotting. The immunoblots shown are representative of three separate experiments.

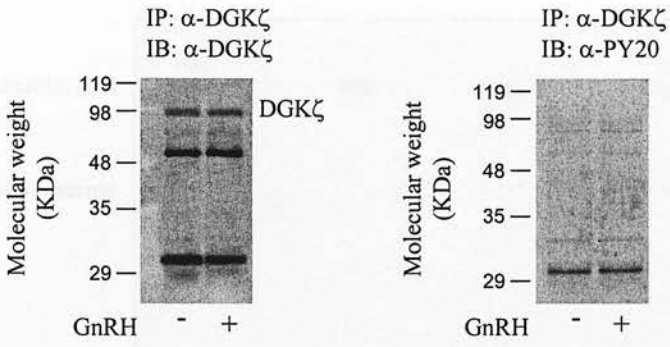


**Figure 36: DGKζ is not tyrosine phosphorylated in SCL60 cells following stimulation with GnRH**. Quiescent cells were treated with vehicle or 100 nM GnRH for 10 minutes and cytoplasmic extracts prepared. DGKζ was immune precipitated, and its tyrosine phosphorylation status analysed by SDS-PAGE and Western blotting with an anti-phopshotyrosine antibody. The immunoblots shown are representative of three separate experiments.

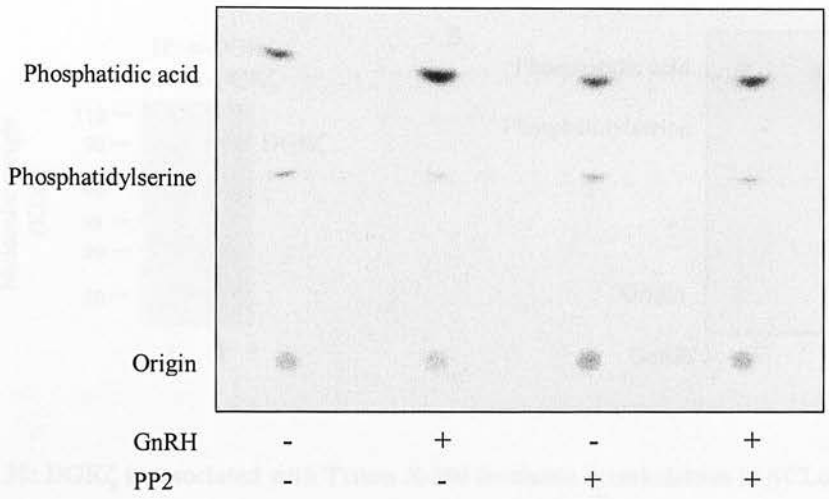


**Figure 37: Src mediated activation of DGKζ by GnRH in SCL60 cells**. Quiescent cells were treated with DMSO or PP2 (5 μM, 30 min). They were then treated with vehicle or 100 nM GnRH for 10 minutes and cytoplasmic extracts prepared. DGKζ was immune precipitated and kinase activity assayed *in vitro* using [γ-$^{32}$P]ATP and 1,2-dioleoyl-*sn*-glycerol as substrate. Radiolabelled lipids were extracted, resolved by thin-layer chromatography, and visualised by autoradiography. The autoradiograph shown is representative of three separate experiments.

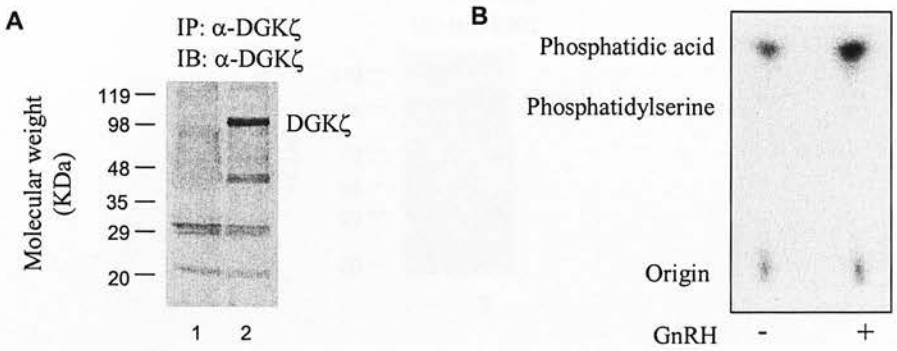


**Figure 38: DGKζ is associated with Triton X-100 insoluble cytoskeletons in SCL60 cells.** (A) Proteins were immune precipitated from solubilised cytoskeletons with pre-immune serum (lane 1) or anti-DGKζ antibody (lane 2). Proteins were resolved by SDS-PAGE and the association of DGKζ with the cytoskeleton analysed by Western blotting. The immunoblot shown is representative of three separate experiments. (B) Quiescent cells were treated with vehicle or 100 nM GnRH for 10 minutes and cytoskeletal extracts prepared. DGKζ was immune precipitated and kinase activity assayed *in vitro* using [$\gamma$-$^{32}$P]ATP and 1,2-dioleoyl-sn-glycerol as substrate. Radiolabelled lipids were extracted, resolved by thin-layer chromatography and visualised by autoradiography. The autoradiograph shown is representative of three separate experiments.

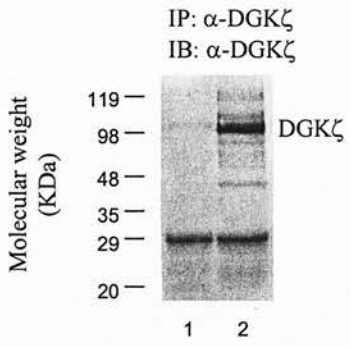


**Figure 39: Expression of DGKζ in LβT2 cells**. Cytoplasmic extracts were prepared from LβT2 cells and proteins immune precipitated with pre-immune serum (lane 1) or anti-DGKζ antibody (lane 2). Proteins were resolved by SDS-PAGE and DGKζ expression analysed by Western blotting. The immunoblot shown is representative of three separate experiments.

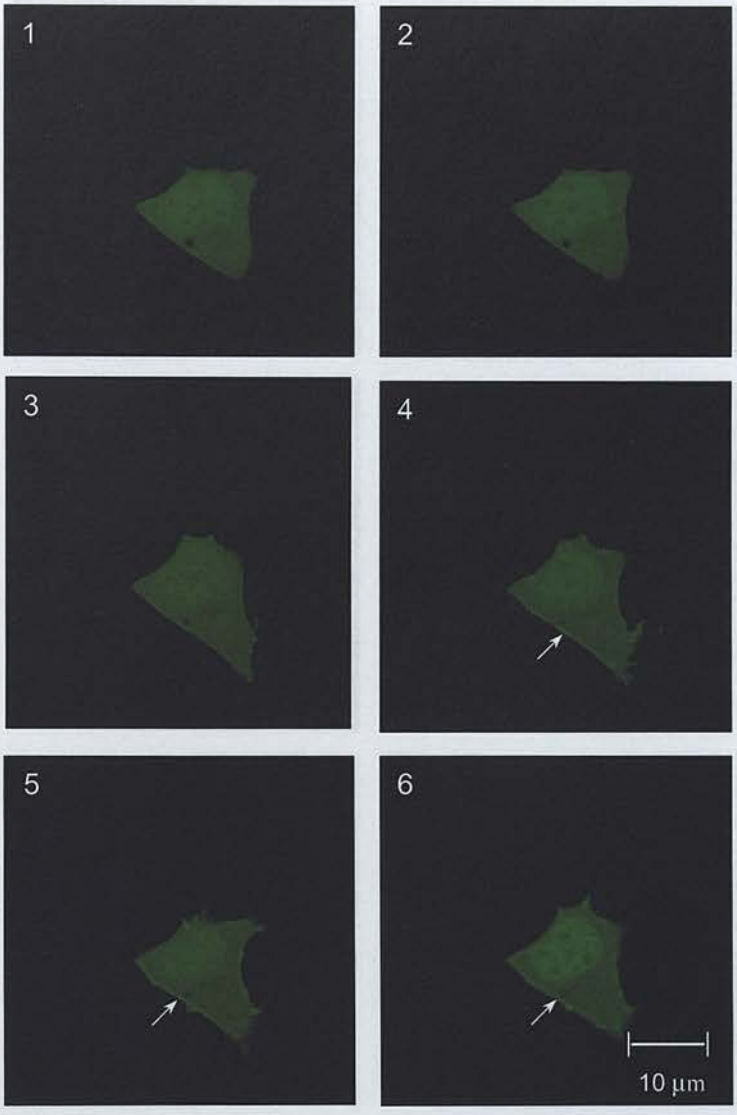


**Figure 40: GnRH stimulates the membrane translocation of GFP-DGKζ in LβT2 cells.** 48 hours after transfection quiescent cells were stimulated with 100 nM GnRH. Images were before treatment, (1) and at 2 minutes (2), 5 minutes (3), 10 minutes (4), 15 minutes (5) and 20 minutes (6) after stimulation with GnRH, using a Zeiss LSM 510 laser scanning microscope. Images shown are representative of five separate experiments.

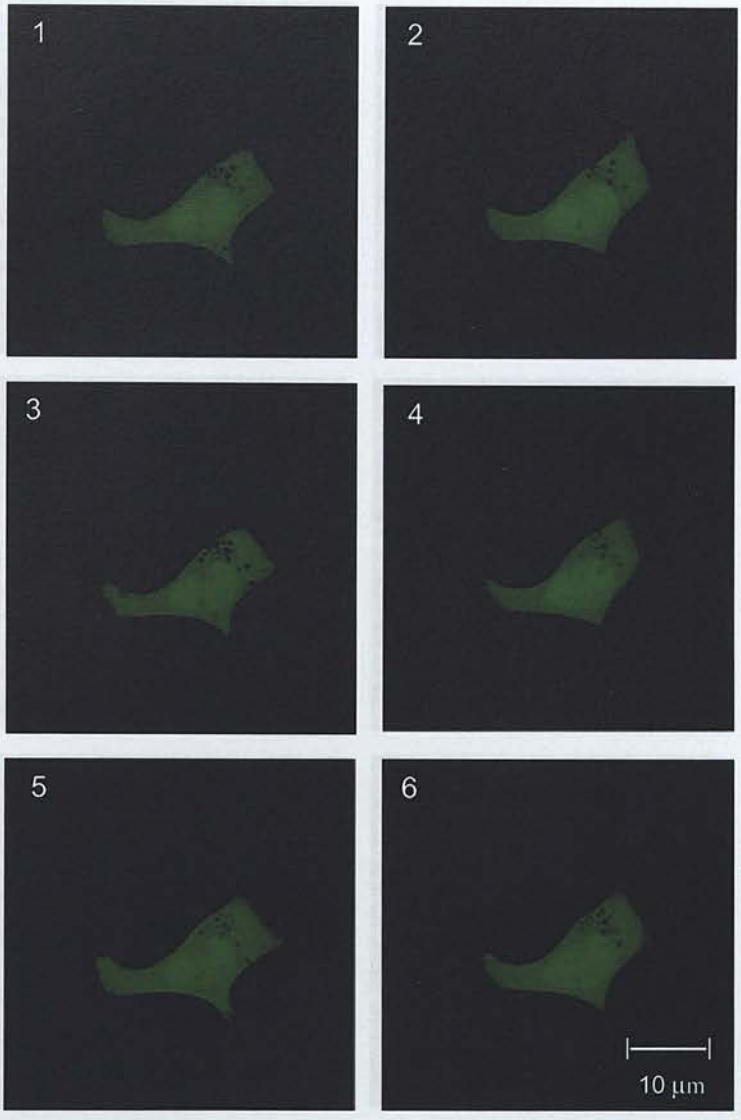


**Figure 41: Membrane translocation of GFP-DGKζ is sensitive to the inhibition of Src family kinases in LβT2 cells**. 48 hours after transfection quiescent cells were pretreated with PP2 (5 µM, 30 minutes) then stimulated with 100 nM GnRH. Images were taken before treatment (1) and at 2 minutes (2), 5 minutes (3), 10 minutes (4), 15 minutes (5) and 20 minutes (6) after stimulation with GnRH, using a Zeiss LSM 510 laser scanning microscope. Images shown are representative of five separate experiments.

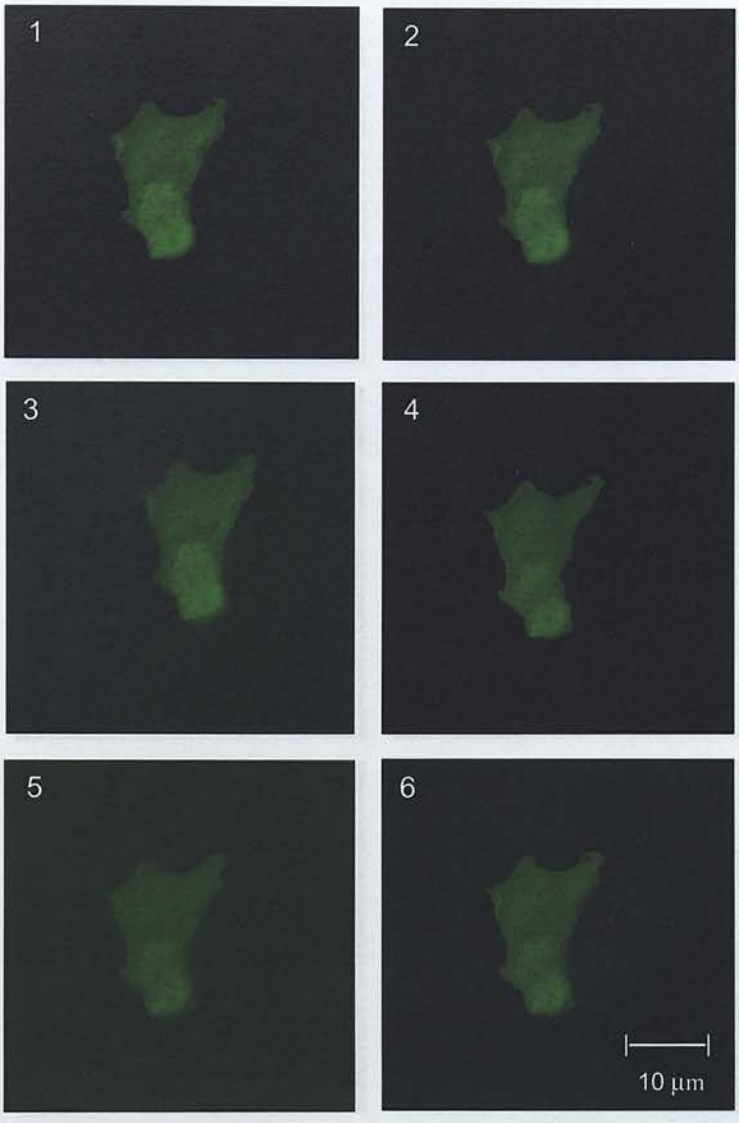


**Figure 42: Membrane translocation of GFP-DGKζ is sensitive to actin filament disruption with cytochalasin-D in LβT2 cells.** 48 hours after transfection quiescent cells were pretreated with cytochalasin-D (1 µM, 30 minutes) then stimulated with 100 nM GnRH. Images were taken before treatment (1) and at 2 minutes (2), 5 minutes (3), 10 minutes (4), 15 minutes (5) and 20 minutes (6) after stimulation with GnRH, using a Zeiss LSM 510 laser scanning microscope. Images shown are representative of five separate experiments.

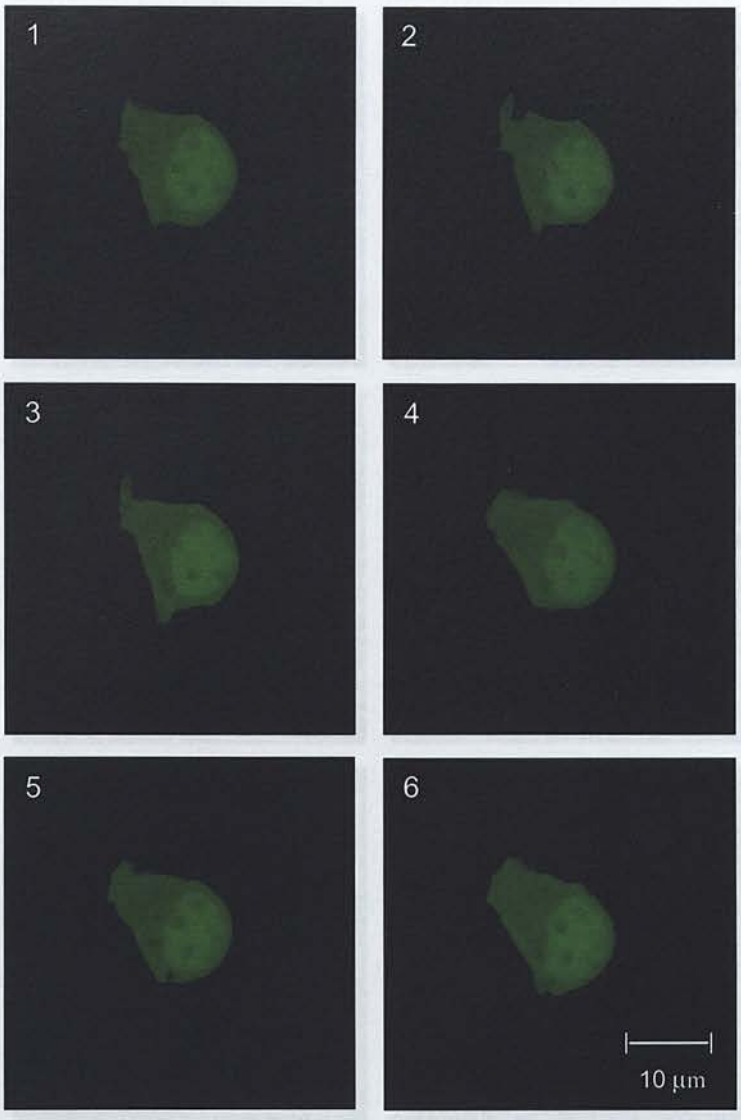


**Figure 43: Membrane translocation of GFP-DGKζ is sensitive to actin filament disruption with latrunculin-B in LβT2 cells**. 48 hours after transfection quiescent cells were pretreated with latrunculin-B (1 µM, 30 minutes) then stimulated with 100 nM GnRH. Images were taken before treatment (1) and at 2 minutes (2), 5 minutes (3), 10 minutes (4), 15 minutes (5) and 20 minutes (6) after stimulation with GnRH, using a Zeiss LSM 510 laser scanning microscope. Images shown are representative of five separate experiments.

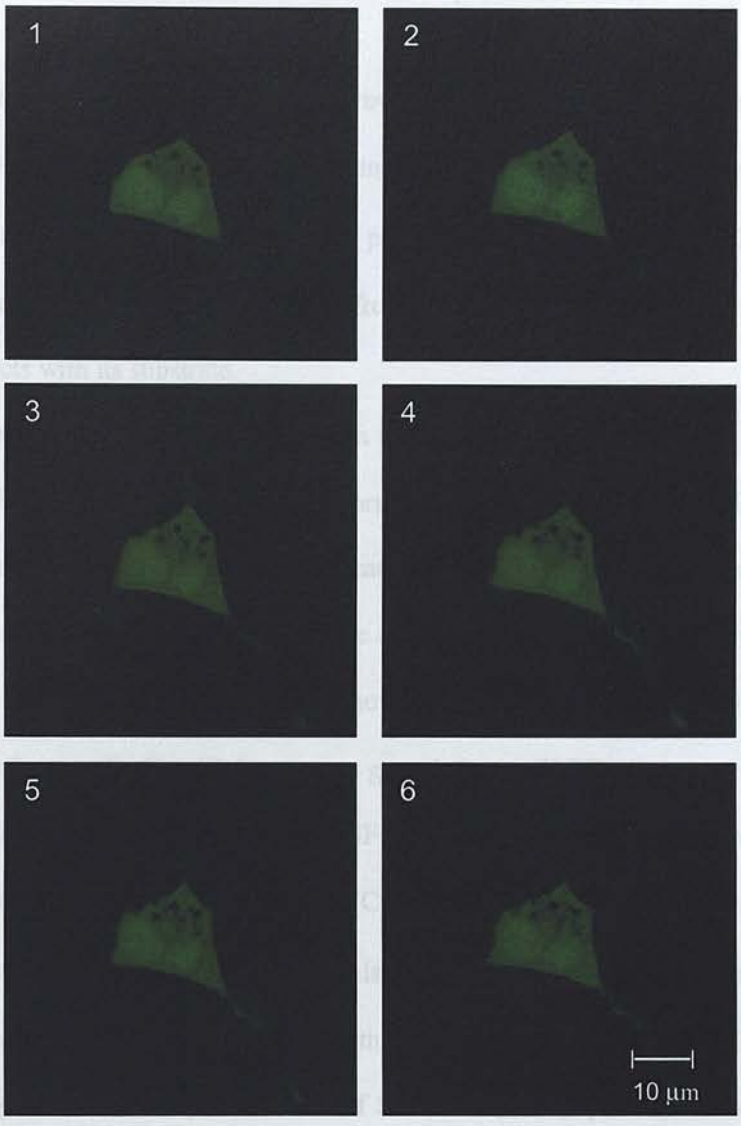


**Figure 44: Membrane translocation of GFP-DGKζ is sensitive to inhibition of PKC in LβT2 cells**. 48 hours after transfection quiescent cells were pretreated with Ro-318220 (100 nM, 30 minutes) then stimulated with 100 nM GnRH. Images were taken before treatment (1) and at 2 minutes (2), 5 minutes (3), 10 minutes (4), 15 minutes (5) and 20 minutes (6) after stimulation with GnRH, using a Zeiss LSM 510 laser scanning microscope. Images shown are representative of five separate experiments.

**Discussion**

The results presented within this chapter provide evidence for the first time that Src associates with the lipid kinase DGKζ following stimulation of SCL60 cells with GnRH. Furthermore, this interaction was found to play a role in regulating DGKζ enzyme activity and was required for it to translocate from the cytoplasm to the plasma membrane where it interacts with its substrate.

The first evidence that Src may be involved in the regulation of diacylglycerol signalling was provided by studies of fibroblasts transformed with the oncogene v-Src. Expression of this oncogene was found to cause a significant increase in the cellular concentration of phosphatidic acid, and a diacylglycerol kinase activity co-purified with v-Src (Sugimoto *et al.*, 1984). Subsequently, it has been shown that Src can associate with DGKα following stimulation of cells with hepatocyte growth factor (HGF) (Cutrupi *et al.*, 2000) and that this interaction is required for HGF and vascular endothelial growth factor (VEGF) to stimulate DGKα enzyme activity (Cutrupi *et al.*, 2000; Baldanzi *et al.*, 2004). The association between Src and DGKα was also reported require Src kinase activity as it could be abolished by pretreating cells with the Src family kinase inhibitor PP1, or by over expressing a kinase dead point mutant of Src (Cutrupi *et al.*, 2000). These findings suggest that Src may regulate the activity of DGKα and DGKζ through a conserved mechanism.

While DGKα contains three conserved tyrosine residues that are putative Src phosphorylation sites, tyrosine phosphorylation could not be detected with endogenous levels of protein without inhibiting protein tyrosine phosphatases with pervanadate (Cutrupi *et al.*, 2000). Phosphorylation of DGKα by Src appears to be transient and occur at low stoichiometry, nevertheless, the tyrosine phosphorylated protein has high levels of enzyme activity, indicating that tyrosine phosphorylation may still be an important event

in its activation (Cutrupi *et al.*, 2000). Therefore, Src mediated phosphorylation of DGKζ may also be a transient event, which may explain why it could not be detected by immune precipitation and Western blotting. Furthermore, if Src phosphorylates DGKζ at low stoichiometry, a more sensitive means of detection may be required. Alternatively, protein tyrosine phosphatases may need to be inhibited if tyrosine phosphorylation is to be detected. By mutating the putative Src phosphorylation site in DGKζ (Tyr558) to alanine it may be possible to determine if Src mediated tyrosine phosphorylation of this residue is required for its DGKζ by GnRH. Conversely, substitution of this residue with the phosphomimetic amino acid aspartic acid may lead to constitutive enzyme activity.

Activation of the DGKs also requires that they translocate from the cytoplasm to the plasma membrane where they interact with their substrate. A recent study by Santos *et al.* (2002) has provided many details of the mechanism whereby DGKζ is recruited to the plasma membrane following the activation of Gq coupled receptors. GFP-tagged DGKζ rapidly translocates to the membrane following the activation of the type I muscarinic receptor with carbachol, when expressed in Jurkat cells. Membrane recruitment was found to be independent of DGKζ enzyme activity, but required serine phosphorylation by PKC (Santos *et al.*, 2002). DGKζ contains an18 amino acid motif (K-A-S*-K-K-K-K-R-A-S*-F-K-R-K-S*-S*-K-K) that is very similar to the PKC phosphorylation motif of the myristoylated alanine rich C kinase substrate (MARCKS) protein (Bunting *et al.*, 1996). Pharmacological inhibition of PKC, or mutation of the serine residues within this MARCKS homology domain to alanine prevented enzyme activation and membrane translocation of DGKζ in response to carbachol. When the serine residues within this motif were substituted with aspartic acid, DGKζ retained the ability to translocate to the plasma membrane. However, it was not found to be constitutively associated with the membrane, indicating that phosphorylation of the

MARCKS homology domain by PKC is necessary but not sufficient for membrane recruitment (Santos *et al.*, 2002).

MARCKS homology domains are found in a number of proteins that shuttle between the cytoplasm and the plasma membrane. In the MARCKS protein, the basic residues in this motif mediate electrostatic interactions with acidic phospholipids in the membrane (Arbuzova *et al.*, 1997; Bahr *et al.*, 1998). Phosphorylation of the serine residues dramatically alters the electrostatic potential of the domain, reducing its affinity for membranes, which causes the protein to become cytoplasmic (Kim *et al.*, 1994a; Kim *et al.*, 1994b). However, phosphorylation of the serine residues within the MARCKS homology domain of DGKζ appears to have the opposite effect as it is required for membrane translocation. It has been proposed that phosphorylation of these residues may facilitate membrane recruitment of DGKζ by causing a conformational change in the molecule that alters its association with regulatory proteins (Santos *et al.*, 2002). Alternatively, the MARCKS homology domain may make electrostatic contacts with an acidic patch on DGKζ that maintains it in an inactive conformation and prevents interactions with substrate or regulatory molecules until it is phosphorylated by PKC. Santos *et al.* (2002) also demonstrated that both C1 domains are required for enzyme activity and membrane recruitment of DGKζ. Mutation of the two conserved histidine residues required for the co-ordination of $Zn^{2+}$ in either of the C1 domains to glycine abolished enzyme activity and membrane recruitment in response to carbachol (Santos *et al.*, 2002).

The results presented within this chapter provide further details of the mechanism of DGKζ membrane recruitment following the activation of Gq coupled receptors. In addition to phosphorylation of serine residues within the MARCKS homology domain, and the C1 domains, which are thought to bind diacylglycerol, membrane translocation appears to require an intact actin cytoskeleton and Src activity. The role of Src-mediated

tyrosine phosphorylation in this process could be investigated by mutating the putative Src phosphorylation site (Tyr558) to phenylalanine and aspartic acid. If tyrosine phosphorylation of this residue by Src is a prerequisite for membrane recruitment substitution with phenylalanine would be predicted to abolish it, while substitution with aspartic acid should facilitate it. Alternatively, DGKζ may be recruited to the plasma membrane in association with an additional protein that is activated by Src, or by binding to the SH3 domain of Src, which is itself targeted to the plasma membrane via N-terminal myristoylation. This latter point could tested by creating deletion mutants of DGKζ lacking the proline rich regions that were identified as potential ligands for the SH3 domain of Src.

By phosphorylating diacylglycerol to produce phosphatidic acid, the diacylglycerol kinases may play an important role in regulating the activity of the conventional and novel PKC isozymes. Thus the results presented in this chapter provide a mechanism for the feedback regulation of PKC activation by GnRH. In addition, phosphatidic acid plays a number of roles in the cell. It is an important intermediate in the synthesis of phosphatidylinositol, and also has second messenger functions. Perhaps the best evidence for the essential role of diacylglycerol kinases in phosphatidylinositol biosynthesis has been provided by studies of retinal degeneration mutants in *D. melanogaster*. In the rdgA mutant, photoreceptor cells differentiate normally but degenerate rapidly after eclosion (Hotta and Benzer, 1970). Their degeneration starts with the breakdown of the subrhabdomeric cisternae, which is the specialised smooth endoplasmic reticulum that forms immediately beneath the rhabdomeric microvilli in the compound eye (Matsumoto *et al.*, 1988; Matsumoto-Suzuki *et al.*, 1989; Masai *et al.*, 1997). Biochemically, the rdgA mutant lacks an eye specific diaclyglycerol kinase in a gene dosage-dependent manner (Inoue *et al.*, 1989). Cloning of the rdgA gene showed that it encoded a diacyglycerol kinase, designated DGK2, that is expressed exclusively in the compound eye (Masai *et*

*al.*, 1993; Massai *et al.*, 1997). The *Drosophila* DGK2 contains a catalytic domain that is conserved with the mammalian diacylglycerol kinases, two C1 domains and four tandem ankyrin repeats (Masai et al., 1993), and therefore is very similar in structure to the mammalian type IV DGKs, which includes DGKζ (Figure 9). In the retina, DGK2 is found almost exclusively in association with the membranes of the subrhabdomeric cisternae, where diacylglycerol is converted to phosphatidic acid. In the rdgA mutant, it is thought that the lack of phosphatidic acid in the subrhabdomeric cisternae leads to its breakdown and results in an insufficient supply of phosphatydlinositol to the photoreceptive membrane, which is essential for the maintenance of the photoreceptors as well as for phototransduction (Masai *et al.*, 1997).

Phosphatidic acid is also recognised to play a role in the regulation of the ERK signalling cascade (Andresen *et al.*, 2002), and has been shown to bind Raf-1 *in vitro* (Ghosh *et al.*, 1996). Mutational analysis has determined that the phosphatidic acid binding site in Raf-1 is contained within a 35 amino acid polybasic motif that is adjacent to the catalytic domain (Ghosh *et al.*, 1996). Mutation of a single arginine residue (Arg398) within this polybasic motif to alanine is sufficient to reduce the affinity of the mutant protein for phosphatidic acid, to impair membrane binding, and inhibit ERK activation by extracellular signalling molecules (Rizzo *et al.*, 1999). It is now thought that phosphatidic acid binding rather than Ras binding drives the plasma membrane translocation of Raf-1 (Rizzo *et al.*, 1999). A putative polybasic phosphatidic acid binding motif has also been identified in the ERK scaffolding protein KSR (Figure 45), indicating that phosphatidic acid may also recruit this protein to the plasma membrane to facilitate the assembly of the kinases of the ERK cascade into a cohesive signalling module. Thus, activation of DGKζ by GnRH may also contribute to the activation of ERK 1 and 2. Because the currently available diacylglycerol kinase inhibitors do not affect DGKζ activity, RNAi could be used to investigate its role in this pathway.

Raf-1 FRNEVAVRKTRHVNILLFMGYMTKDNLAIVTQWCEG

KSR FKKEVMNYRQTRHENVVLFMGACMNPPHLAIITSFCKG

**Figure 45: The phosphatidic acid binding domains of Raf-1 (amino acids 390 – 426) and KSR (amino acids 497 – 533).**

Phosphatidic acid has also been shown to stimulate the activity of PIP-5 kinase, which phosphorylates PtdIns(4)P to produce PtdIns(4,5)$P_2$ (Ishihara *et al.*, 1996). Although PtdIns(4,5)$P_2$ represents less than 1% of total membrane phospholipids it has a pivotal role in numerous signalling events (Toker, 1998). It is hydrolysed by PLC to generate diacylglycerol and Ins(1,4,5)$P_3$ as described previously, and is phosphorylated by PI-3 kinase to produce PtdIns(3,4,5)$P_3$. In addition PtdIns(4,5)$P_2$ regulates the activity of many proteins involved in the regulation of the actin cytoskeleton (Sechi and Wehland, 2000). Recently, PIP5Iα has been shown to bind to DGKζ, and both proteins co-localise in lamellipodia in migrating cells (Lou *et al.*, 2004b), where they are positioned to participate in the regulation of the cortical actin cytoskeleton. Thus, DGKζ may also be involved in the regulation of the actin cytoskeleton in SCL60 cells by activating PIP-5Iα kinase.

**Future work**

Although the data presented within this chapter show that Src interacts with DGKζ following stimulation of SCL60 cells with GnRH, many of the molecular details regarding this interaction remain unknown. By using techniques such as the yeast-two hybrid screen, it may be possible identify which domains in Src are important for this interaction. However, it is also possible that the interaction between these proteins is not direct but mediated by an additional protein in a putative complex with Src. Improved proteomic techniques such as tandem affinity purification may therefore help to solve this

problem by reducing the number of non-specific proteins co-precipitating with Src during immune precipitation and allowing a more detailed analysis of Src complexes.

By using purified proteins, it may be possible to show Src mediated phosphorylation of DGKζ *in vitro* and allow the phosphorylation sites to be mapped. Such studies would undoubtedly shed light on the mechanism of DGKζ activation by Src.

# Conclusion

Protein phosphorylation on serine, threonine and tyrosine residues plays an important role in GnRH receptor signalling, regulating the activity of protein and lipid kinases and creating binding sites for protein-interaction domains to nucleate the formation of signalling complexes. The non-receptor tyrosine kinase Src appears to play a central role in GnRH receptor signalling and is intimately involved in numerous processes, including the regulation of the actin cytoskeleton, diacylglycerol signalling and the activation of the ERK signalling cascade. The data presented within this thesis indicate that these events may not be separate, but represent a co-ordinated cellular response to receptor activation. Activation of Src stimulates the formation of lamellipodia, which in turn leads to the activation of FAK. Src is then recruited into a complex with FAK and phosphorylates it on tyrosine residues to allow the binding of Grb2/Sos, which initiates the activation of ERK (Schlaepfer *et al.*, 1994).

Src was also found to be involved in the activation of DGKζ, which phosphorylates diacylglycerol to produce phosphatidic acid. This provides a potential mechanism for the feedback regulation of conventional and novel PKC isozymes, which are allosterically activated by diacylglycerol. In addition, Raf-1 has been shown to be recruited to the plasma membrane by binding to phosphatidic acid (Ghosh *et al.*, 1996). Thus, DGKζ may also participate in the activation of ERK 1 and 2.

Phosphatidic acid is also known to be an important intermediate in the biosynthesis of phosphatydlinositol and activates PIP-5 kinase, which phosphorylates PtdIns(4)P to produce PtdIns(4,5)$P_2$ (Toker, 1998). As well as serving as serving as a substrate for PLC, PtdIns(4,5)$P_2$ regulates the activity of many proteins involved in the regulation of the actin cytoskeleton (Sechi and Wehland, 2000). Therefore, DGKζ may also be involved in the regulation of the cytoskeleton in SCL60 cells.

Overall these studies demonstrate the integrative nature of signal transduction in eukaryotic cells, and that several pathways may interact to give a co-ordinated response to a given signal.

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# Appendix A: Antibodies

| Antibody | Supplier | Species |
|---|---|---|
| Anti-goat AP conjugate | Sigma (A4187) | Rabbit |
| Anti-mouse AP conjugate | Sigma (A1293) | Goat |
| Anti-mouse FITC conjugate | Sigma (F0257) | Goat |
| Anti-rabbit AP conjugate | Sigma (A3687) | Goat |
| 12CA5 | Boehringer Mannheim (583816) | Mouse |
| β-actin | Sigma (A1978) | Mouse |
| DGKζ | Santa Cruz Biotech (SC-8721) | Goat |
| ERK2 | Santa Cruz Biotech (SC-154) | Rabbit |
| ERK 1/2 (pThr83, pTyr185) | New England Biolabs (9101S) | Rabbit |
| EGFR | Santa Cruz Biotech (SC-03) | Rabbit |
| FAK | Santa Cruz Biotech (SC-558) | Rabbit |
| FAK | Transduction Laboratories (F15020) | Mouse |
| HA-agarose conjugate | Santa Cruz Biotech (SC-7392) | Mouse |
| Myc | Santa Cruz Biotech (SC-40-AC) | Mouse |
| PY20 | Santa Cruz Biotech (SC-508) | Mouse |
| PY20-agarose conjugate | Santa Cruz Biotech (SC-508-AC) | Mouse |
| Src | Santa Cruz Biotech (SC-18) | Rabbit |
| Src-agarose conjugate | Santa Cruz Biotech (SC-18-AC) | Rabbit |
| Src pTyr419 | Bioscource international (44-662) | Rabbit |
| Src pTyr530 | Bioscource international (44-662) | Rabbit |
| Tyrosine tubulin | Sigma (T9028) | Mouse |

# Appendix B: cDNA constructs

| cDNA construct | Vector | Supplier |
|---|---|---|
| HA-FAK | pCDNA 3.1 | Dr D Schlaepfer |
| HA-Y397F FAK | pCDNA 3.1 | Salk Institute, California, USA |
| HA-R454K FAK | pCDNA 3.1 | |
| HA-Y925F FAK | pCDNA 3.1 | |
| Myc-ERK2 | pCMV5 | Dr E Nishida |
| | | University of Tokyo, Japan |
| N19RhoA | pCDNA 3 | Dr A Hall |
| N17Rac1 | pCDNA 3 | University College London, UK |
| GFP-DGKζ | pEGFPBos | Dr I Merida |
| | | University of Madrid, Spain |