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The Significance of FRS2 Phosphorylation in EGF- and FGF-Induced Signaling

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A journey of a thousand miles begins with a single step.

- Lao Zi (6th century B.C.)

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1. INTRODUCTION

Cells continuously monitor changes in their extracellular environments and elicit appropriate adaptive responses. Nearly every aspect of cell life is controlled by signal transduction processes, by which extracellular perturbations are perceived, converted into intracellular signals, and conveyed to effectors whose activities are necessary to generate cellular responses. Many of these intracellular processes are controlled by signal transduction pathways that regulate protein phosphorylation. It has been estimated that more than a third of all proteins in a mammalian cell can be modified by phosphorylation and that up to 2% of the genes in a vertebrate genome encode either protein kinases or phosphatases (Manning *et al.*, 2002). It has becoming apparent that phosphorylation is the most common type of protein modification, and the major mechanism for reversible regulation of protein activity and function. In particular, reversible tyrosine phosphorylation is one of the fundamental mechanisms for controlling cell proliferation, differentiation as well as development. Though the overall level of phosphotyrosine (ca. 0.5%) in proteins in normal vertebrate cells is very low compared to the levels of phosphoserine (ca. 90%) and phosphothreonine (ca 10%) (Hunter and Sefton, 1980; Hunter, 2000), after oncogenic transformation or growth factor stimulation, the level of tyrosine phosphorylation increases to 1-2 % of total protein phosphorylation in the cells (Zhang, 1998; Zhang, 2002). The extent of tyrosine phosphorylation is determined by the opposing actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). Thus, PTKs and PTPs are important regulators of various cellular processes whose activities are normally tightly controlled. When dysregulated, both PTKs and PTPs can become potent oncoproteins causing cellular transformation.

1.1 Receptor Tyrosine Kinases (RTKs)

One of the fundamental mechanisms by which cells of multicellular organisms communicate is the binding of ligands to cell surface transmembrane receptors that possess tyrosine kinase activity. After binding of ligands, the receptors become activated, the incoming signal is transmitted through the cell membrane and distributed to intracellular signal molecules. The surface receptors can be classified in a variety of different receptor families, such as cytokine receptors, G-protein-coupled receptors (GPCR), receptor serine/threonine kinase (RS/TK) or receptor tyrosine kinases (RTKs). Although each receptor family uses individual cellular signaling mechanisms, they do not simply form linear pathways but are part of a complex and interdependent cellular network (Hackel *et al.*, 1999; Hubbard and Till, 2000; Hunter, 2000).

Receptor tyrosine kinases form a major family of signal transducing surface receptors that use their intrinsic tyrosine kinase activity to phosphorylate other proteins (van der Geer *et al.*, 1994). All RTKs possess a glycosylated extracellular ligand-binding domain, a single hydrophobic transmembrane domain and a cytoplasmic region containing the catalytic domain. With the exception of the insulin receptor (IR) subfamily members that form heterotetramers, RTKs are single polypeptide chains in their inactivated states.

The extracellular domains contain distinctive patterns of cysteine-rich regions or characteristic arrays of structural motifs, such as immunoglobulin (Ig)-like structures (van der Geer *et al.*, 1994; Hunter, 2000). Based on these structural properties of their extracellular domains, the RTKs can be classified into subfamilies (Figure 1). The ligand-binding domain is the most distinctive feature in RTKs and reflects the individual requirements for the binding of a specific ligand.

The transmembrane domain consists of a stretch of hydrophobic amino acids. Although the transmembrane domains function primarily as passive lipid anchors, they can also influence receptor function. For example, the oncogenic form of receptor HER2 from the epidermal growth factor receptor (EGFR) family is constitutively activated due to a point mutation in its transmembrane domain (Bargmann *et al.*, 1986). Mutations in the transmembrane region of the fibroblast growth factor receptor (FGFR) are associated with clinical disease (Klint and Claesson-Welsh, 1999; Powers *et al.*, 2000; Ornitz and Marie, 2002).

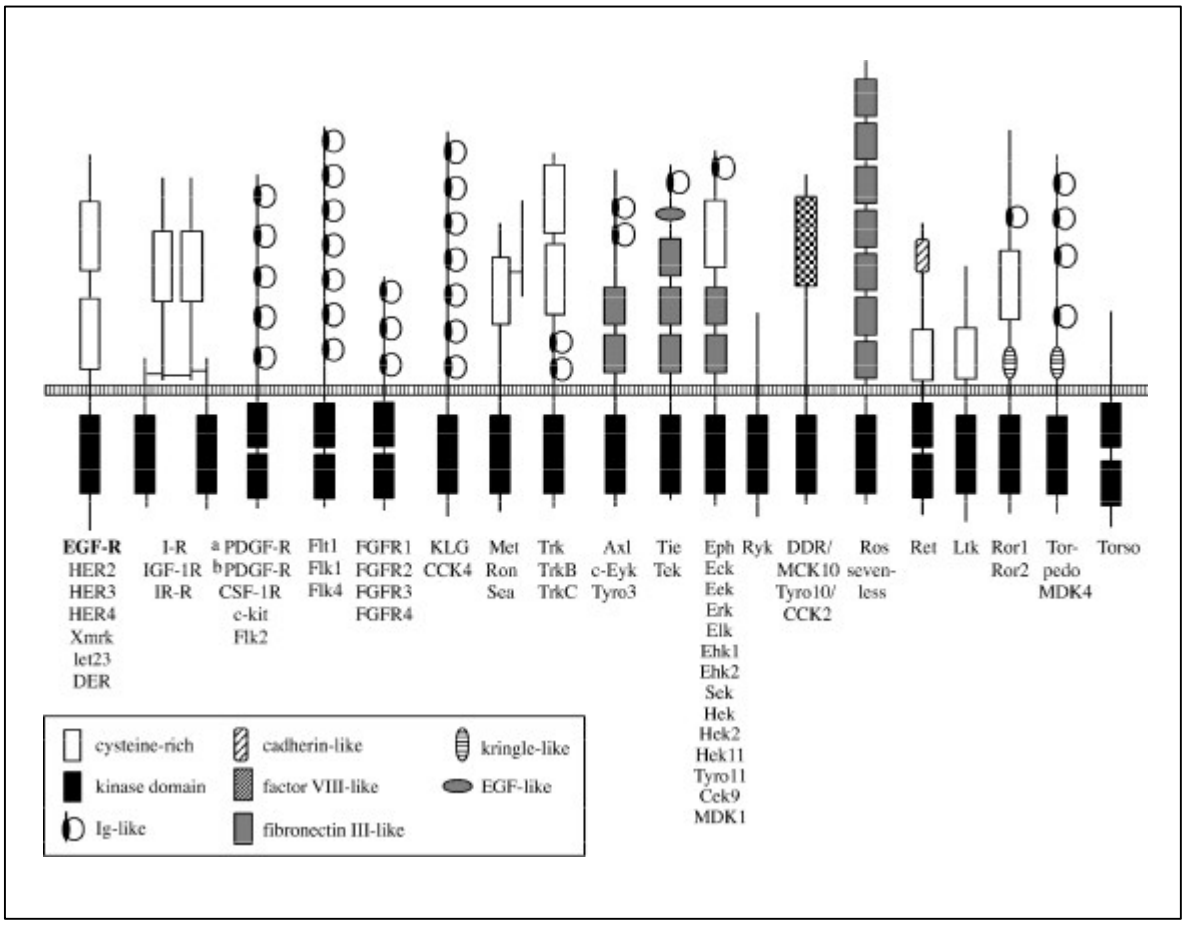


Figure 1: The receptor tyrosine kinases subfamilies.
The figure shows the division of the receptor tyrosine kinase family into 19 subfamilies. The classification is based on the structural differences of the extracellular domains. (Adopted from Wallasch, 1996.)

The intracellular domain can be subdivided into a juxtamembrane domain followed by a tyrosine kinase catalytic domain and a carboxyl-terminal regulatory tail (Ullrich and Schlessinger, 1990). The catalytic domain, which distinguishes RTKs from other receptors, consists of 250-300 residues and is related to that of cytoplasmic protein tyrosine kinases as well as serine/threonine kinases. Three separate roles can be ascribed to the catalytic domain: first, binding of the adenosine triphosphate (ATP); second, binding and orientation of the protein substrate; and third, transfer of the γ-phosphate from ATP to the acceptor hydroxyl residue (tyrosine, serine or threonine) of the protein substrate (Hunter, 1998). In some subfamilies, the kinase domain is divided into two halves by a kinase insert, which participates in the recruitment of cytoplasmic

molecules to receptor-based signaling complexes. Within the carboxyl-terminal sequence there are autophosphorylation sites that serve as binding sites for substrates and effectors (Ullrich and Schlessinger, 1990).

Ligand binding to the extracellular portion of these receptors results in receptor dimerization, which facilitates *trans*-autophosphorylation of specific tyrosine residues in the cytoplasmic portion. These phosphotyrosine residues enhance receptor catalytic activity and/or provide docking sites for downstream signaling proteins. Because of the critical roles played by RTKs in cellular signaling processes, their catalytic activity is normally under tight control by intrinsic regulatory mechanisms as well as by protein tyrosine phosphatases.

1.1.2 Epidermal Growth Factor Receptor Signaling

The epidermal growth factor receptor (EGFR) family consists of four closely related receptors: EGFR (also known as ErbB1), HER2 (or ErbB2/neu), HER3 (ErbB3) and HER4 (ErbB4). An interesting feature of HER3 is the exchange of several amino acids, which are conserved in all other protein tyrosine kinases (Guy *et al.*, 1994). As a consequence, HER3 has no intrinsic kinase activity. However, it is transphosphorylated by HER2 and thus participates in signal transduction of the EGFR family (Wallasch *et al.*, 1995). For all EGFR family members, except for the orphan receptor HER2, a number of different ligands have been described. Seven epidermal growth factor (EGF)-like mammalian gene products are known to directly activate the EGFR: EGF, transforming growth factor alpha (TGF-α), heparin-binding EGF (HB-EGF), heregulin (also known as the neu differentiation factor, NDF), amphiregulin, betacellulin and epiregulin (Yarden and Sliwkowski, 2001). Each of these molecules is synthesized as a transmembrane precursor and subjected to proteolytic cleavage of its ectodomain to produce the soluble form of the growth factor. The integrated biological responses to EGFR signaling are pleiotropic, including mitogenesis, apoptosis, cell mobility, differentiation, as well as tumor progression such as invasion and metastasis.

Binding of EGF to its receptor at the cell surface results in activation of the intrinsic tyrosine kinase of the receptor and tyrosine phosphorylation of the C-terminus of the EGFR. The amino acid sequences containing phosphorylated tyrosine residues

serve as binding sites for signal transduction molecules that contain Src homology 2 (SH2) and phosphotyrosine-binding (PTB) domains. Some of these proteins possess catalytic activity, whereas others serve as adaptors that couple receptors and downstream enzymes involved in signal transduction. Therefore, interactions of the receptor with these signal molecules are the key initial steps of signaling pathways activated by growth factors. For example, binding of Shc and Grb2 (growth-factor-receptor-bound protein 2) adaptor proteins to phosphorylated EGFR is essential for activation of Ras GTPase and mitogen-activated protein kinase (MAPK).

The SH2 domain of Grb2 binds to the activated EGFR (Lowenstein *et al.*, 1992), while its SH3 domains constitutively bind to son-of-sevenless (SOS), the Ras guanidine-nucleotide-exchange factor (Li *et al.*, 1993; Rozakis-Adcock *et al.*, 1993). Binding of Grb2 to the EGFR recruits the Grb2-SOS complex to the membrane and to the membrane-anchored Ras, thus linking EGFR to the Ras/MAPK signaling cascade. Grb2 can bind to EGFR directly through its main binding site on EGFR, pY-1068, or indirectly through three phosphotyrosines of Shc (Lowenstein *et al.*, 1992; Batzer *et al.*, 1994; Okabayashi *et al.*, 1994). Shc binds to pY-1148 and pY-1173 of the EGFR through its PTB and SH2 domains, respectively, and is phosphorylated efficiently by the EGFR kinase (Soler *et al.*, 1994b; Batzer *et al.*, 1995). Membrane translocation and phosphorylation of Shc are sufficient to recruit the Grb2-SOS complex to Ras (Soler *et al.*, 1994a). Other signal molecules directly involved in EGFR signaling include phospholipase Cγ (PLCγ) and its downstream calcium- and protein kinase C (PKC)-mediated cascades; phosphatidylinositol-3-OH kinase (PI3K) and its downstream effector Akt (or protein kinase B, PKB); ubiquitin ligase Cbl; adaptor proteins Gabl (Grb2-associated binder-1), Grb7, Nck, Crk; as well as the protein tyrosine phosphatase and adaptor protein SHP2 (Hackel *et al.*, 1999; Zwick *et al.*, 1999; Prenzel *et al.*, 2000).

1.1.3 Fibroblast Growth Factor Receptor Signaling

The fibroblast growth factors (FGFs) belong to a family of heparin-binding polypeptides with more than 20 members (Ornitz and Itoh, 2001; Moroni *et al.*, 2002). FGFs are potent mitogens for a variety of different cell types in tissue culture and in vivo, and are implicated in differentiation of endothelial and neuronal cells (Klint and Claesson-Welsh, 1999). The binding of FGFs to heparin or heparin-like glycoaminoglycan (HLGAG) may serve two physiologically relevant goals: the protection of the FGFs from degradation and the creation of a local reservoir of growth factors. The local reservoir allows for a strict spatial regulation of FGF signaling. Moreover, the association of FGFs with heparin is required for effective activation of the FGF receptor (FGFR), where heparin increases the affinity and half-life of the FGF-FGFR complex (Ornitz and Itoh, 2001; Ornitz and Marie, 2002).

The FGFR family has four members: FGFR1 (also known as Flg), FGFR2 (Bek), FGFR3, and FGFR4, which share between 55% and 72% homology at the protein level. The FGFRs play important roles in embryonic development, angiogenesis, as well as wound healing (Powers *et al.*, 2000). One distinct feature of FGFRs is that they contain three extracellular Ig-like domains (IgI, IgII and IgIII), with an acidic box (eight consecutive acidic residues) inserted between the first and second Ig-like domains. Alternative mRNA splicing of the *fgfr1-3* genes leads to variants with two or three Ig-like domains, or specifies the sequence of the carboxyl-terminal half of the third Ig-like domain that resulting in either IIIa, IIIb or IIIc isoforms of FGFR. The *fgfr4* gene is unique in that there is only one possible form of its IgIII domain. The IIIa splice variant codes for a truncated, secreted protein unable to independently transduce extracellular signals. However, it may sequester released FGFs and inhibit FGF signaling. Expression of IIIb and IIIc isoforms is differentially regulated in a tissue-specific manner and determines the ligand-receptor binding specificity. The expression of FGFR2 isoforms of IgIIIb and IgIIIc is restricted to cells of epithelial and mesenchymal lineages, respectively. Since FGF-7 (also known as keratinocyte growth factor, KGF) is known to bind FGFR2/IIIb but not FGFR2/IIIc. Thus, this may explain the selectivity of FGF-7 for keratinocytes over fibroblasts as due to the expression of the different FGFR splice variants (Powers *et al.*, 2000; Ornitz and Itoh, 2001).

A single transmembrane stretch connects the FGFR extracellular part with its intracellular juxtamembrane (JM) domain. The JM domains of FGFR1 and FGFR2 contain one phosphorylatable tyrosine residue that may serve as docking site for downstream signaling molecules, while the FGFR3 and FGFR4 lack tyrosine residues in this region. The tyrosine kinase domain of FGFRs is split in two parts by insertion of about 15 amino acids, which comprises two tyrosine residues in FGFR1 and FGFR2, one in FGFR3 and none in FGFR4. The mitogenic potential of the FGFR4 appears to be lower than the other FGFRs, which in part is due to the lack of tyrosine residues in the kinase insert. The C-terminal tails of the FGFRs contain a number of conserved tyrosine residues that play critical roles in mediating the FGFR signaling (Klint and Claesson-Welsh, 1999). For example, pY-653 and pY-654 are important for kinase activity; pY-766 is the binding site of PLCγ. Unlike the EGFR, FGFR recruits the Grb2/SOS complex only indirectly through the docking protein FRS2 (FGFR substrate 2, also known as SNT) (Rabin *et al.*, 1993; Kouhara *et al.*, 1997). FRS2 associates directly with Grb2 or indirectly through SHP2, thereby linking the FGFR to the MAPK cascade (Kouhara *et al.*, 1997; Hadari *et al.*, 1998).

1.2 Protein Tyrosine Phosphatases (PTPs)

The precise and rapid propagation of signals demands both strict and flexible regulatory processes. Protein phosphorylation is one of the major posttranslational modification mechanisms that cells utilize to control various cellular regulatory processes. Addition or removal of a phosphoryl moiety from a protein can generate a recognition motif for protein-protein interactions, control protein stability, and most importantly, modulate enzyme activity. In vivo, protein phosphorylation is reversible and dynamic; the phosphorylation states are governed by the opposing activities of protein kinases and protein phosphatases. Based on substrate specificity, the family of phosphatases can be grouped into two subfamilies: the protein serine/threonine phosphatases (PPs) and the protein tyrosine phosphatases (PTPs). Amino acid sequence comparison of the catalytic domains has shown no sequence similarity between the two groups (Charbonneau *et al.*, 1988). Moreover PPs and PTPs use different chemical reaction mechanisms to catalyze the hydrolysis of phosphate monoesters suggesting a separate evolution of both families.

The PTPs include both the tyrosine-specific phosphatases that hydrolyze phosphotyrosine (pY)-containing proteins, and dual-specific phosphatases that target proteins containing pY, as well as phosphoserine (pS) and phosphothreonine (pT) as substrates. The PTPs are characterized by the presence of the active site signature motif (H/V)C(X)$_5$R(S/T) in the conserved catalytic domain (Zhang, 2002), and are traditionally further divided into receptor-like and cytoplasmic PTPs (Figure 2). The receptor-like PTPs generally have an extracellular domain, a single transmembrane region and one or two cytoplasmic catalytic domains (Tonks and Neel, 2001; Zhang, 2002). The extracellular domains of the receptor-like PTPs show high variability, presumably due to different functions and physiological ligands. The cytoplasmic PTPs contain a single catalytic domain and various amino- or carboxyl-terminal extensions, including SH2 domains, that have targeting or regulatory functions. All PTPs are characterized by their sensitivity to vanadate, ability to hydrolyze *p*-nitrophenyl phosphate, insensitivity to okadaic acid, and lack of metal ion requirement for catalysis (Tonks and Neel, 2001; Zhang, 2002).

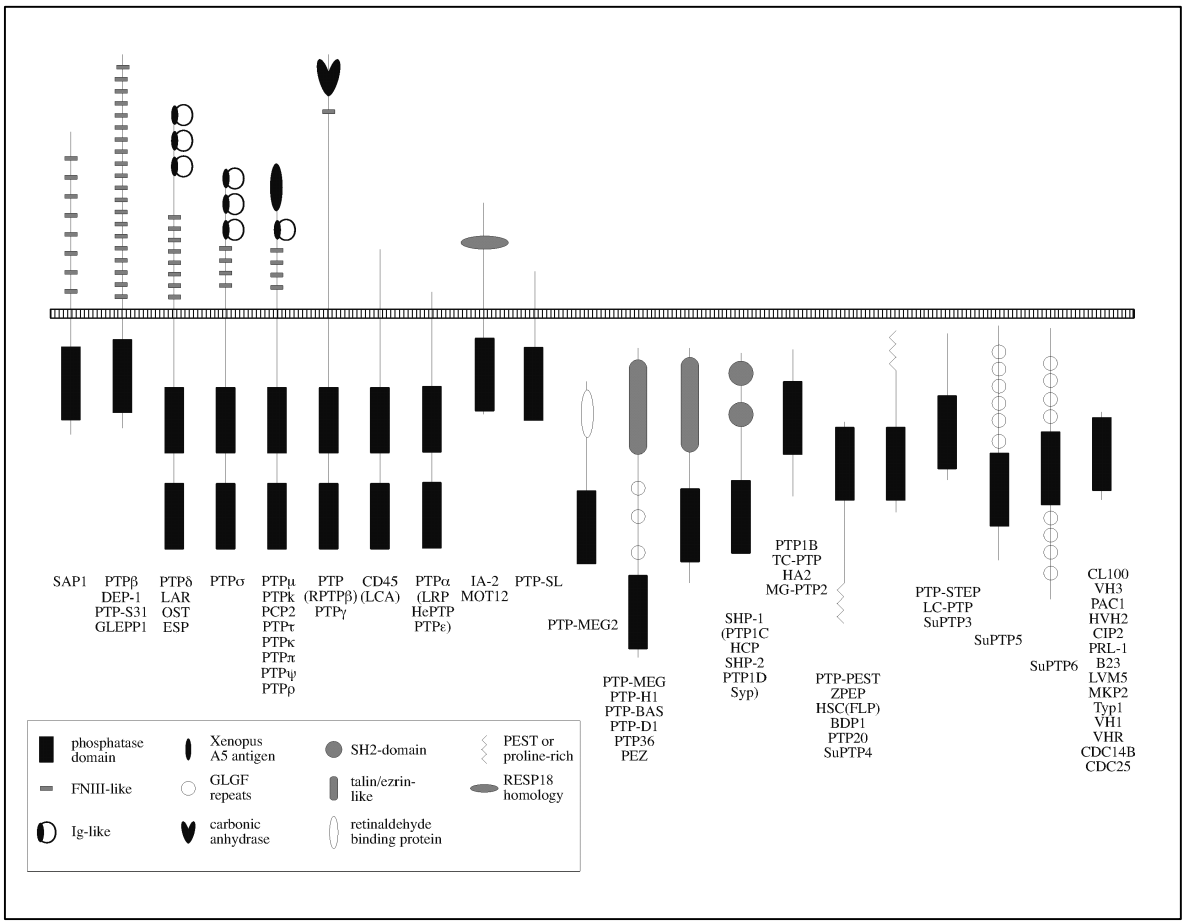


Figure 2:The family of protein tyrosine phosphatases.
The protein tyrosine phosphatases (PTPs) are divided into receptor like and cytoplasmic PTPs. The receptor-like PTPs have an extracellular domain, a single transmembrane region and one or two cytoplasmic PTP domains. The cytoplasmic PTPs contain a single catalytic domain and additional amino- or carboxyl-terminal domains. (Adopted from Stein-Gerlach, 1998a.)

The content of tyrosine phosphorylated proteins within a cell is the result of the balanced interplay between tyrosine kinase and phosphatase activity (Tonks and Charbonneau, 1989; Tonks and Neel, 2001; Tiganis, 2002). Whereas activation of RTKs generates tyrosine phosphorylation of proteins, PTPs dephosphorylate proteins and could therefore lead to termination of signals. For example, studies of PTP1B knockout mice show that this phosphatase is a major negative regulator of insulin receptor (IR) signaling in muscle and liver (Elchebly *et al.*, 1999; Klaman *et al.*, 2000). In addition to IR, PTP1B might also negatively regulate insulin-like growth factor-1 receptor (IGF-IR) and EGFR signaling (Kenner *et al.*, 1996; Flint *et al.*, 1997; Lee *et al.*, 1998). The receptor-like PTP LAR has also been suggested as a negative regulator of multiple RTKs, as reduction of LAR protein levels by an antisense approach

increased autophosphorylation of the insulin, EGF and hepatocyte growth factor (HGF) receptors (Kulas *et al.*, 1996).

1.2.1 Phosphotyrosine Phosphatase SHP2

Protein tyrosine kinase and phosphatase do not simply oppose each other's action; rather, they may work in concert to maintain a fine balance of effector activation needed for the regulation of cell growth and differentiation, as the PTP SHP2 demonstrated. SHP2, also known as SH-PTP2, SH-PTP3, PTP2C, PTP1D, and Syp, was identified independently by several groups as a cytosolic SH2 domain-containing PTP (Adachi *et al.*, 1992; Freeman *et al.*, 1992; Ahmad *et al.*, 1993; Feng *et al.*, 1993; Lechleider *et al.*, 1993a; Vogel *et al.*, 1993). It is ubiquitously expressed and contains two tandem SH2 domains at its N-terminus and one tyrosine phosphatase domain at the C-terminus.

SHP2 has been implicated in diverse signaling pathways including those initiated by platelet-derived growth factor (PDGF), EGF, IGF-1, insulin and cytokines (Feng *et al.*, 1993; Kazlauskas *et al.*, 1993; Kuhne *et al.*, 1993; Vogel *et al.*, 1993; Manes *et al.*, 1999). Within a single signaling pathway, SHP2 may act at multiple sites to participate in signal transduction. SHP2 directly binds to activated RTKs and becomes rapidly tyrosine phosphorylated. At the same time, it can interact with downstream signaling intermediates such as Grb2, p85 subunit of PI3 kinase, IRS-1, and Gab1 and 2 (Stein-Gerlach *et al.*, 1998; Feng, 1999; Qu, 2000).

The function of SHP-2 is assumed to be dephosphorylation of associated signaling molecules and to result in diminished local signals. However, the ultimate effect of SHP2 in most signaling pathways is to enhance the signals relayed from RTKs (Tang *et al.*, 1995; Bennett *et al.*, 1996; Hadari *et al.*, 1998; O'Reilly and Neel, 1998; Shi *et al.*, 1998). The enzymatic activity is required for its function since introduction of a catalytically inert SHP2 markedly inhibited activation of MAP kinase in response to insulin, EGF, PDGF, and FGF (Noguchi *et al.*, 1994; Yamauchi *et al.*, 1995; Bennett *et al.*, 1996; Maegawa *et al.*, 1999; Manes *et al.*, 1999; Inagaki *et al.*, 2000). Although the precise biochemical basis for such a positive regulation by SHP2 in the MAP kinase

pathway remains unclear, SHP2 appears to act in association with the Grb2-SOS complex (Li *et al.*, 1994; Hadari *et al.*, 1998).

On the other hand, SHP-2 does play a negative role in certain intracellular signaling processes. It inhibits signaling pathways initiated by the cytokines interferon-α and -γ , leukemia inhibitory factor, ciliary neurotrophic factor, and IL-6 (Kim *et al.*, 1998; Qu and Feng, 1998; Burdon *et al.*, 1999; You *et al.*, 1999; Ohtani *et al.*, 2000). However, signaling via the erythropoietin receptor, a member of the cytokine receptor family, was found to be enhanced by SHP2 (Tauchi *et al.*, 1995; Tauchi *et al.*, 1996). Thus, SHP2 may have dual functions both in cytokine and growth factor signal transduction.

1.3 G-protein Coupled Receptor Signaling and EGFR Transactivation

G-protein-coupled receptors (GPCRs) constitute the largest family of cell-surface molecules involved in signal transmission. They play key physiological roles in secretion from endocrine and exocrine glands, exocytosis, chemotaxis, photo- and chemoreception, neurotransmission, as well as embryogenesis, angiogenesis, tissue regeneration, and control of normal and aberrant cell growth. These receptors are activated by a wide variety of ligands, including peptide and non-peptide neurotransmitters, hormones, growth factors, odorant molecules as well as light. GPCRs owe their name to their extensively studied interaction with heterotrimeric G-proteins (composed of α, β and γ subunits). In response to receptor activation the G-proteins undergo conformational changes that lead to the exchange of GDP for GTP bound to the α-subunit. Consequently, the G_{α} and $G_{\beta\gamma}$ subunits stimulate effector molecules, thereby activating or inhibiting the production of second messengers, promoting increases in intracellular Ca^{2+} concentration and opening or closing of ion channels (Figure 3) (Gutkind, 2000; Marinissen and Gutkind, 2001; Pierce *et al.*, 2002).

Many potent mitogens such as thrombin, lysophosphatidic acid (LPA), bombesin, vasopressin, bradykinin, substance K, acetylcholine receptor agonists and angiotensin II stimulate cell proliferation by acting on their cognate GPCRs. The nature of the intracellular signaling pathways mediating these proliferative effects is still poorly understood. Whereas conventional second messenger-generating systems, such as adenylyl cyclases, ion channels and phospholipases, were the focus of the early research efforts addressing this issue, an emerging body of information indicates that additional effector pathways participate in proliferative signaling by GPCRs. In particular, GPCRs have been shown to activate members of the MAPK family, which are key components of intracellular signaling pathways that control cell proliferation.

It is well established that various stimuli which are unrelated to EGF-like ligands, can also activate the EGFR. These stimuli include GPCR agonists such as endothelin, thrombin, lysophosphatidic acid, angiotensin II, as well as cytokines, chemokines and cell adhesion elements (Pawson, 1994; Daub *et al.*, 1996; Daub *et al.*, 1997; Carpenter, 1999; Carpenter, 2000; Gschwind *et al.*, 2001). Furthermore, EGFR signaling may also be induced by environmental stress factors, such as ultraviolet and

gamma-radiation, hydrogen peroxide and heavy metal ions (Gschwind *et al.*, 2001). This so-called transactivation of the EGFR by these seemingly unrelated stimuli might occur through a mechanism common to at least a part of the agonists. In this context, stimulation of cells via GPCR or ionizing radiation results in the activation of a metalloprotease that cleaves a precursor of the EGF-like family of ligands and thereby produces a soluble growth factor which in turn, activates the EGFR (Figure 3) (Dent *et al.*, 1999; Prenzel *et al.*, 1999). Activation of the EGFR by GPCR-agonists results in the RTK tyrosine phosphorylation, association with signal adaptors, such as Shc or Grb2 and activation of the MAP kinase pathway. In addition to its role in GPCR-activated mitogenic signaling, the EGFR also serves as signal transducer of LPA-induced stress fiber formation and in the modulation of potassium channel activity (Tsai *et al.*, 1997; Gohla *et al.*, 1998). In GPCR-mediated EGFR transactivation, the intrinsic receptor kinase activity is necessary for downstream signaling (Daub *et al.*, 1996; Daub *et al.*, 1997; Prenzel *et al.*, 1999). In contrast, upon stimulation with growth hormone the EGFR is phosphorylated directly by the cytosolic tyrosine kinase Jak2 at tyrosine 1068, the major Grb2 binding site, and allows downstream signaling even by a kinase deficient EGFR mutant (Batzer *et al.*, 1994; Batzer *et al.*, 1995; Yamauchi *et al.*, 1997).

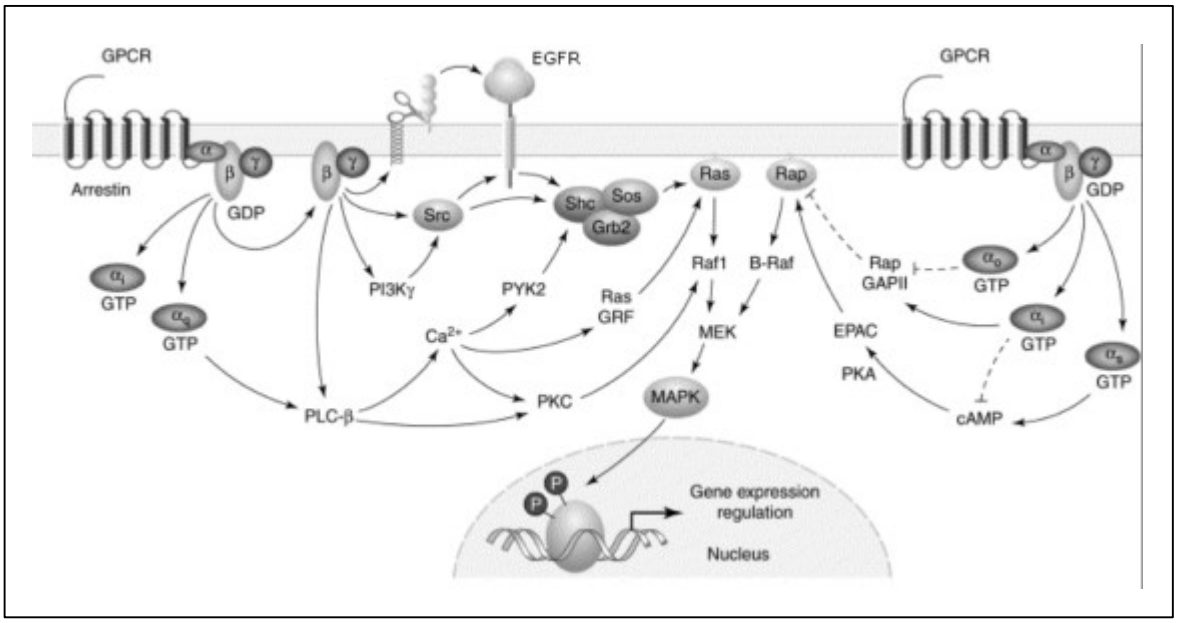


Figure 3: Multiple pathways link GPCRs to MAP kinase.
GPCR-induced metalloprotease-mediated proteolytic cleavage of EGF-like growth factor precursors leads to transactivation of the EGFR and activation of MAPK. The metalloprotease is presented as a pair of scissors. In response to receptor activation the G-proteins stimulate effector molecules, such as PKC and PLC, thereby activating the Ras/MAPK cascade. (Modified from Marinissen and Gutkind, 2001.)

1.4 Adaptor and Docking Proteins

A common feature in intracellular signaling pathways is the assembly of multi-protein complexes involving extensive protein-protein interactions. Typical examples are the interactions between RTKs and their downstream effectors. Upon activation and autophosphorylation of the RTKs, phosphotyrosine residues at their carboxyl-terminal tail serve as binding sites for downstream signal transducing molecules. These associations are usually directed by protein interaction domains, such as Src homology 2 (SH2), Src homology 3 (SH3), phosphotyrosine binding (PTB) and pleckstrin homology (PH) domains, among others (Figure 4). The SH2 domain interacts with proteins that contain a phosphotyrosine embedded in a specific sequence, in general residues at position +1 through +3 relative to the phosphotyrosine are recognized (Songyang *et al.*, 1993). The SH3 domain interacts with target proteins in a phosphorylation-independent, sequence-specific fashion, binding to short sequences containing a central PXXP sequence (Pawson, 1994; Songyang, 1999). The PTB domain, first identified in Shc and IRS-1, consists of about 150 residues and recognizes the consensus NPXpY motif. The PH domain acts as a phospholipid-dependent membrane-anchoring domain.

Docking proteins are one group of proteins containing N-terminal interaction domains and multiple C-terminal tyrosine phosphorylation sites that function as phosphorylation-dependent docking sites for other SH2 as well as PTB domain-containing proteins (Figure 4). Often these proteins have a means of membrane attachment, such as a PH domain for IRS and myristylation for FRS2 (Kouhara *et al.*, 1997; White, 1998). A number of such docking proteins have been identified, including Shc, insulin receptor substrates (IRS) 1-4, FRS2 and Gab1-2 (Grb2-associated binder) (Hunter, 2000; Schlessinger, 2000; Guy *et al.*, 2002).

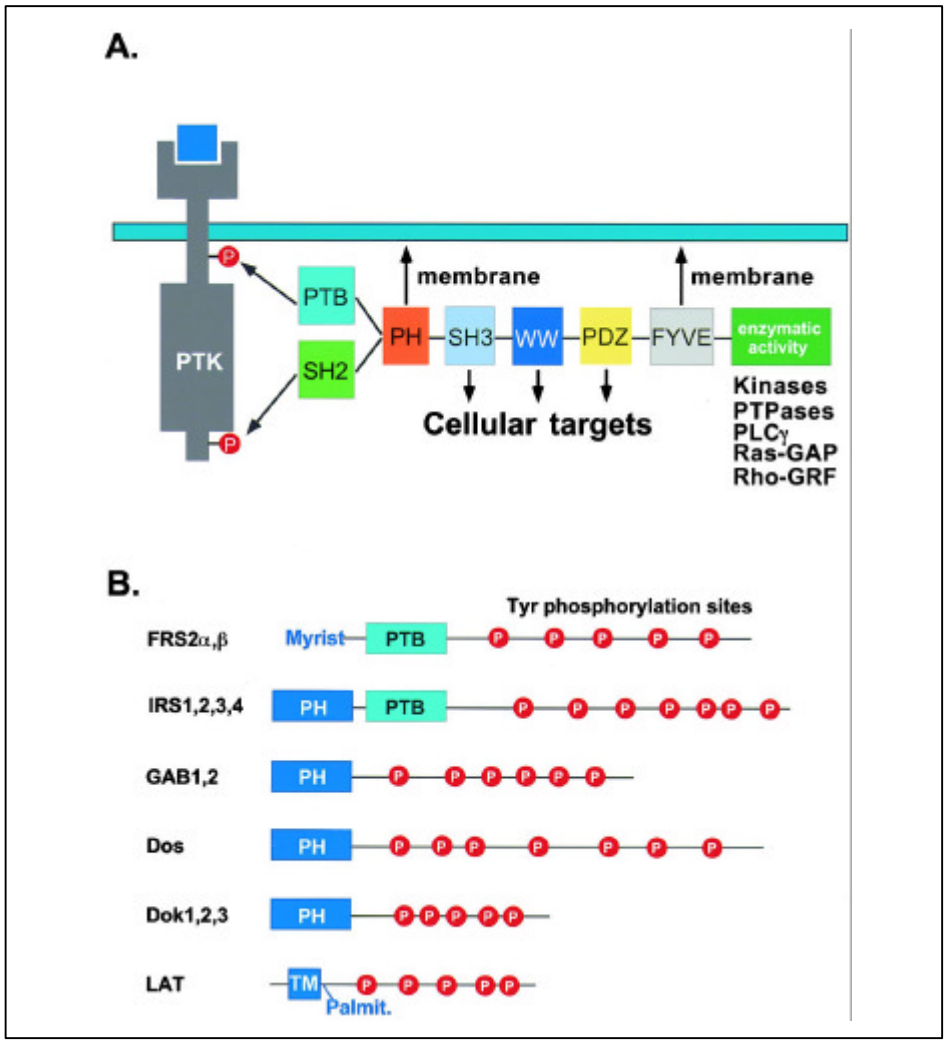


Figure 4: Protein modules and docking proteins that participate in signaling via receptor tyrosine kinases
(**A**) Protein modules implicated in the control of intracellular signaling pathways. Activated RTKs form a complex with SH2 and PTB domains of signaling proteins. SH2 domains bind to phosphotyrosine sites in activated receptors while PTB domains bind to tyrosine phosphorylated and nonphosphorylated regions in RTKs. PH domains bind to different phosphoinositides leading to membrane association. SH3 and WW domains bind to proline-rich sequences in target proteins. PDZ domains bind to hydrophobic residues at the C-termini of target proteins. FYVE domains bind specifically to PdtIns(3)P. While adaptor proteins such as Grb2 or Nck contain only SH2 and SH3 domains, other signaling proteins contain additional enzymatic activities such as protein kinases (Src, PKB), PTP (SHP2) phospholipase C (PLCγ), Ras-GAP or Rho-GRF (Vav). (**B**) Docking proteins that function as platforms for recruitment of signaling proteins. All docking proteins contain a membrane-targeting region in their N-termini as well as multiple tyrosine phosphorylation sites that function as binding sites for SH2 or PTB domains of a variety of signaling proteins. (Adopted from Schlessinger, 2000)

The ability of docking proteins to recruit diverse signaling molecules may help to expand the repertoire of signaling pathways activated by a given receptor. Mammalian Shc has one C-terminal SH2 domain and one N-terminal PTB domain.

Although both the SH2 and PTB domains recognize phosphotyrosine motifs on activated receptors, they are structurally distinct and recognize phosphopeptides in quite different fashions. The Shc PTB and SH2 domains flank a central region with two principal sites of tyrosine phosphorylation, both in Y-X-N sequences. Once phosphorylated, Shc can recruit multiple Grb2 molecules and potentially other SH2-domain proteins (Pawson *et al.*, 2001). Thus, Shc functions, in part, as an auxiliary docking subunit of activated RTKs, which can extend or amplify the binding potential of a receptor. Moreover, Shc becomes tyrosine phosphorylated upon activation of a variety of RTKs, demonstrating that a single docking protein can integrate signals from various receptors by serving as a common substrate to integrate multiple inputs.

Adaptor proteins, such as Grb2, Nck and Crk, comprise a single SH2 domain and multiple SH3 domains but lack intrinsic catalytic function. They can couple a phosphotyrosine signal, recognized by the SH2 domain, to downstream targets with proline-rich motifs that bind to the SH3 domains (Pawson *et al.*, 2001). Thus, the Grb2 SH2 domain binds to specific pY-X-N motifs on activated RTKs or cytoplasmic docking proteins, while its SH3 domains associate with SOS, the Ras GDP-GTP exchange factor.

The interaction domain-containing proteins include not only adaptors or docking proteins, but also enzymes, such as p85 subunit of PI3K, PLCγ, tyrosine kinases Src and Syk. The cytoplasmic protein tyrosine phosphatase SHP2 and the related SHP1 are unique among tyrosine phosphatases as they have two tandem SH2 domains. Besides serving to target SHP2 to various tyrosine-phosphorylated proteins, these SH2 domains also regulate the activity of SHP2 (Stein-Gerlach *et al.*, 1995; Feng, 1999). Moreover, SHP2 is also able to serve as a docking protein (Li *et al.*, 1994).

1.4.1 Regulation of Docking Proteins through Serine/Threonine Phosphorylation

The importance of tyrosine phosphorylation for generating protein binding sites and mediating protein complex formation has been realized early on (Pawson and Scott, 1997; Pawson and Nash, 2000). However, the same concept for serine/threonine

phosphorylation started to draw attention only recently (Yaffe and Cantley, 1999; Yaffe and Elia, 2001; Yaffe, 2002).

One of the best-studied docking proteins is IRS-1. Upon activation of the insulin receptor, IRS-1 provides phosphotyrosine residues as docking sites for downstream effector proteins and subsequently triggers the PI3K and MAPK pathways (Le Roith and Zick, 2001). In addition, IRS-1 also becomes phosphorylated on serine/threonine residues. These modifications may either positively or negatively modulate IRS-1 signaling (Zick, 2001). Phosphorylation of IRS-1 on serine residues by PKB upon insulin stimulation protects it from rapid dephosphorylation by PTPs, thus implicating PKB-mediated phosphorylation as a positive regulatory module (Paz *et al.*, 1999). In contrary, serine/threonine phosphorylation of IRS-1 by protein kinase $C\zeta$ ($PKC\zeta$) or c-Jun N-terminal kinase (JNK) has been shown to act as a negative-feedback control mechanism that uncouples IRS-1 from its upstream regulator or downstream effector and terminates signal transduction (Paz *et al.*, 1997; Aguirre *et al.*, 2000; Liu *et al.*, 2001; Aguirre *et al.*, 2002).

Similar effects were observed for the docking protein Gab1, which becomes tyrosine phosphorylated in cells stimulated with growth factors, cytokines, and ligands for GPCRs (Gu and Neel, 2003). The major Gab1-binding proteins detected in cells treated with extracellular stimuli include the p85 subunit of PI3K and the tyrosine phosphatase SHP2. In addition to tyrosine phosphorylation, Gab1 is phosphorylated by the MAP kinase family member ERK2 (extracellular-regulated kinase) on serine/threonine residues (Roshan *et al.*, 1999). Interestingly, EGF and HGF mediated ERK activation result in divergent effects on Gab1/PI3K signaling. HGF-stimulated ERK activation increases the Gab1/PI3K association, whereas EGF-stimulated ERK activation results in a decrease in the tyrosine phosphorylation of Gab1 and a decreased association with the PI3K. As SHP2 is shown to dephosphorylate Gab1, EGF-stimulated ERK might act through the regulation of SHP2 (Yu *et al.*, 2001; Yu *et al.*, 2002).

1.5 Mitogen-Activated Protein Kinase Signaling Pathway

The MAP kinase cascade is one of the best-characterized signaling pathways. Initiated by a wide variety of extracellular stimuli including growth factors, mitogens and cytokines, as well as environmental stress factors, through a series of protein-protein interactions and phosphorylation, a protein kinase cascade consisting of at least three kinases, MEKK (MAP kinase kinase kinase), MKK (MAP kinase kinase), and MAPK (MAP kinase), is activated. A sequential phosphorylation mechanism by which one kinase phosphorylates and activates a downstream kinase ultimately results in MAPK activation. Once activated, the MAPKs, which are serine/threonine kinases, phosphorylate different cytosolic, membrane-bound and nuclear substrates including transcription factors. Thereby MAPKs can regulate transcription of particular sets of genes. (Lewis *et al.*, 1998; Whitmarsh and Davis, 1999; Chen *et al.*, 2001; Zhan *et al.*, 2001).

The family of MAPKs can be divided into three subfamilies: extracellular-regulated kinase (ERK), c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) and p38 kinase. The members of the ERK subfamily are key downstream components of RTK mediated signal transduction pathways which control fundamental cellular processes such as cell cycle, proliferation, differentiation, survival and apoptosis. More than 50 different proteins have been reported to be substrates of ERK (Whitmarsh and Davis, 1999; Sharrocks *et al.*, 2000; Chen *et al.*, 2001). Besides transcription factors, ERK phosphorylates signal proteins that function upstream such as Syk, SOS, Raf, MEK and Gab1, as well as signal proteins that function downstream such as the ribosomal S6 kinase p90Rsk.

As all MAP kinases phosphorylate serine and threonine residues within the minimal consensus sequence PXS/TP (Songyang *et al.*, 1996), it is important that the substrate proteins provide further specificity determinants. A comparison of different MAP kinase substrates and interaction partners reveals several different arrangements of specificity-determining domains, which have been termed MAP kinase-recognition modules (Figure 5). Besides sequences surrounding the phosphoacceptor motifs, docking domains and FxF motifs may be present in these modules. The requirement for multiple determinants might reflect that each of the different components of the module

provides a unique function. For example, the docking site might serve to recruit MAP kinases to a substrate and once bound, the FxF motif might then act to stabilize this binding. Alternatively, the two motifs could act together to recruit the kinase. The presence of both the docking domain and FxF motif is likely to enhance the specificity of kinase selectivity and to improve the fidelity and efficiency of phosphorylation by directing the kinase to the phosphoacceptor motifs (Sharrocks *et al.*, 2000).

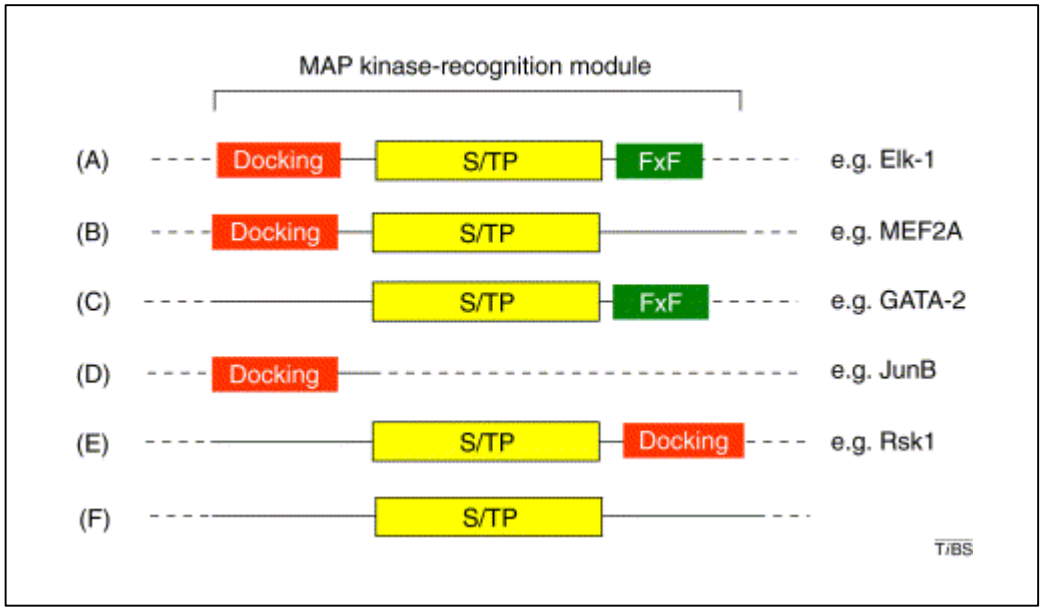


Figure 5: MAP kinase-recognition modules.
The docking domains, FxF motifs and the local context of the phosphoacceptor motifs have been identified as three determinants that constitute MAP kinase-recognition modules. The different arrangements of these determinants found are shown in (A)–(E). S/TP indicates the domain containing the phosphoacceptor motifs. Abbreviations: MEF2A, myocyte-specific-enhancer-binding factor 2A; Rsk1, ribosomal S6 kinase. (Adopted from Sharrocks, 2000.)

Studies demonstrate that specific targeting of MAP kinases to substrates via docking domains is found in all categories of its substrates, including transcription factors, protein kinases, protein phosphatases and other signaling molecules (Jacobs *et al.*, 1999; Sharrocks *et al.*, 2000; Tanoue *et al.*, 2000; Fantz *et al.*, 2001). Furthermore, docking sites for MAPK kinase MEK1 and other upstream kinases have been identified (Bardwell and Thorner, 1996; Xu *et al.*, 1999). Analysis of the docking domain in the phosphatase PTP-SL lead to identification of critical residues designed kinase

interaction motif (KIM, L-X-X-K/R-K/R-X_{1-5}-L/I-X-L) (Pulido *et al.*, 1998; Zuniga *et al.*, 1999). MAPK docking domains not directly related to substrate phosphorylation have also been described. For example, JIP-1 binds specifically to JNK MAP kinases via its docking domain and acts as a scaffold protein that nucleates the formation of a single complex containing multiple components of the JNK cascade (Whitmarsh and Davis, 1999; Davis, 2000; Enslen and Davis, 2001).

While multiple motifs on substrates cooperate to determine their specificity towards upstream kinases, multiple determinants must exist on the kinases themselves to recognize such motifs. All MAPKs possess a conserved common docking (CD) domain located in the near the C-terminal region outside the catalytic domain, that is commonly used for recognition of both their activators, regulators as well as substrates (Tanoue *et al.*, 2000).

1.6 Fibroblast Growth Factor Receptor Substrate 2 (FRS2)

The FGF receptor substrate 2 (FRS2) was identified as a major downstream mediator of FGFR signaling (Kouhara *et al.,* 1997). FRS2 is also known as SNT (suc-associated neurotrophic factor target) and is rapidly tyrosine phosphorylated upon stimulation of nerve growth factor (NGF) and FGF (Rabin *et al.*, 1993). FRS2 comprises an amino-terminal myristylation site followed by a PTB domain, and multiple tyrosine phosphorylation sites in its carboxyl-terminal region. Two isoforms of FRS2 coded by two different genes have been identified: FRS2$_\alpha$/SNT1 with 508 amino acids and FRS2$_\beta$/SNT2 with 492 residues, which share over 70% identity at the protein level. The FRS2$_\alpha$ is the most studied form and little is known about the β-form. If not indicated, the FRS2$_\alpha$ is referred to as FRS2 in this study.

As a membrane anchored docking protein, FRS2 interacts with the juxtamembrane domain of FGF receptor 1 (Xu *et al.*, 1998; Ong *et al.*, 2000). This interaction allows for FGF-induced, receptor-mediated FRS2 tyrosine phosphorylation, which in turn creates docking sites for the adaptor protein Grb2 and the tyrosine phosphatase SHP2 (Kouhara *et al.*, 1997; Ong *et al.*, 1997; Hadari *et al.*, 1998). Tyrosine phoshorylated FRS2 binds to Grb2 in association with the Ras activator SOS. This FRS2-Grb2-SOS ternary complex then activates Ras/MAP kinase signaling cascade. FRS2 also indirectly recruits Grb2 via complex formation with SHP2. In NGF signaling, FRS2 functions in a similar way. However, while the FRS2 PTB domain constitutively associates with a juxtamembrane region of FGFR1 lacking both tyrosine and asparagine residues, it recognises a canonical tyrosine phosphorylated NPXpY motif on the NGF receptor TrkA that also serves as binding site for Shc (Figure 6) (Xu *et al.*, 1998; Meakin *et al.*, 1999). In the rat pheochromocytoma PC12 cells, overexpression of FRS2 enhances and mediates sustained MAPK activity. Since neuronal differentiation correlates with sustained MAPK activation, FRS2 is believed to play an essential role in differentiation (Traverse *et al.*, 1992; Traverse *et al.*, 1994; Marshall, 1995; Kouhara *et al.*, 1997; Xu and Goldfarb, 2001).

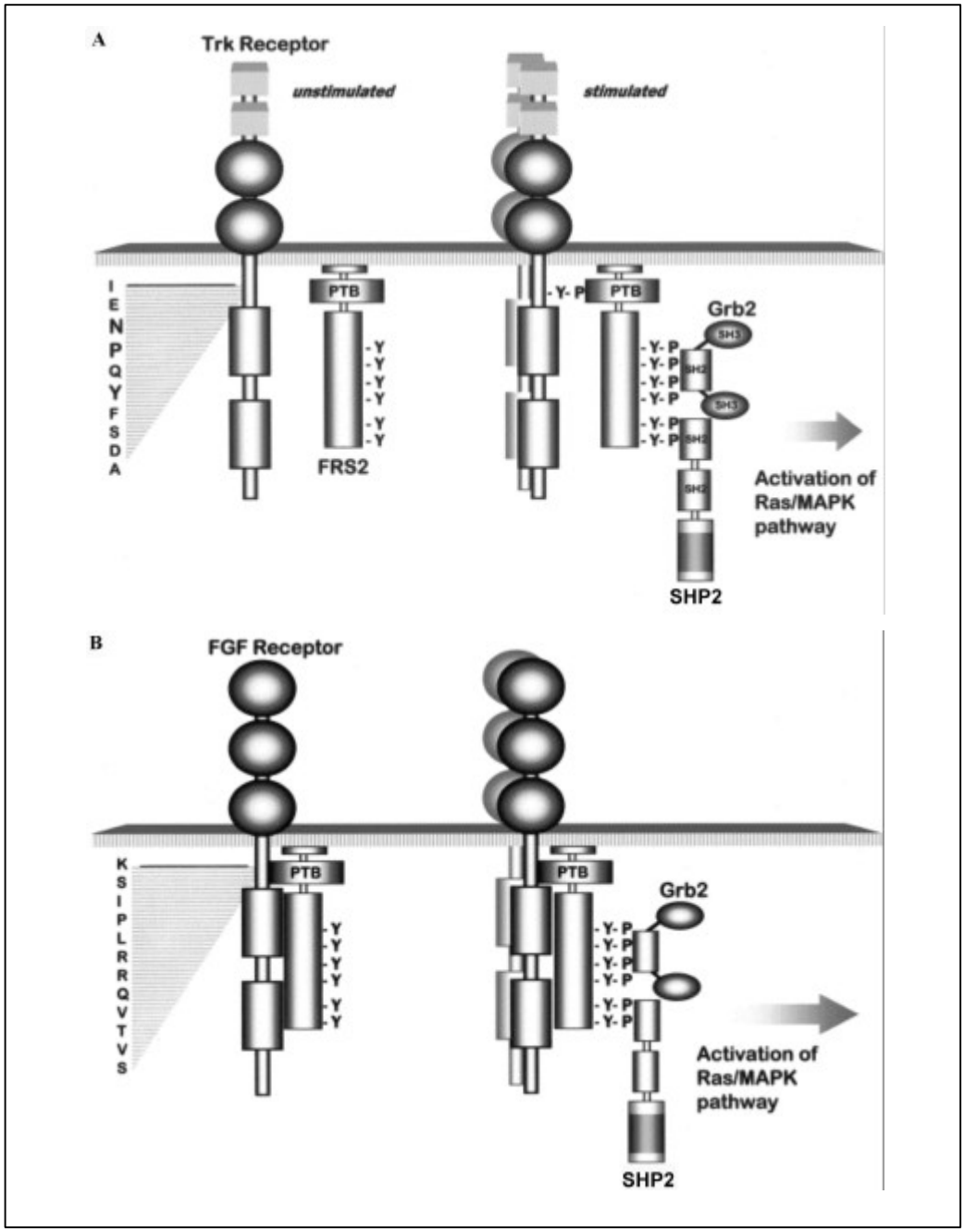


Figure 6: FRS2 in Trk and FGFR signaling.
Schematic representation of FRS2 in Trk receptor (**A**) and FGF receptor (**B**) signaling. The right and left halves represent the disposition of the proteins in unstimulated and stimulated cells, respectively. While FRS2 only binds to the activated Trk receptor, it constitutively associates with the FGF receptor. Activation of the receptors leads to phosphorylation of FRS2 on strategically located tyrosine residues that become targets for the SH2 domains on Grb2 and SHP2. FRS2 binds to Grb2 directly or indirectly via SHP2, resulting in activation of the Ras/MAPK pathway. (Modified from Guy, 2002.)

In addition to the Ras/MAPK cascade, FRS2 also plays a pivotal role in FGF-induced recruitment and activation of PI3K and its downstream effector protein PKB/Akt, a mediator of a cell survival pathway (Ong *et al.*, 2001). Tyrosine phosphorylated FRS2 binds to the SH2 domain of Grb2, which interacts primarily via its N-terminal SH3 domain with the nucleotide exchange factor SOS, and via its C-terminal SH3 domain with a proline-rich region in Gab1. Within this complex, Gab1 becomes tyrosine phosphorylated resulting in the recruitment and activation of PI3K.

Recently, involvement of FRS2 in attenuation of FGFR signaling was reported (Wong *et al.*, 2002). The product of *Cbl* protooncogene functions as an ubiquitin ligase that ubiquitinates and promotes the degradation of a variety of cell signaling proteins. FRS2-Grb2-Cbl form a ternary complex resulting in the ubiquitination and degradation of FGFR and FRS2 in response to FGF stimulation. These observations highlight the importance of FRS2 in the assembly of both positive (i.e., SOS, PI3K) and negative (i.e., Cbl) signaling proteins to mediate a balanced FGF signal transduction (Wong *et al.*, 2002).

Targeted disruption of the FRS2α gene causes severe impairment in mouse development resulting in embryonal lethality at embryonic day E7.0-E7.5, establishing that FRS2 mediates multiple FGFR-dependent signaling pathways critical for mammalian embryonic development (Hadari *et al.*, 2001). FRS2 also functions as a critical mediator of FGF signaling in *Xenopus* early embryogenesis through its association with the FGFR and the Src family kinase Laloo (Hama *et al.*, 2001; Kusakabe *et al.*, 2001; Akagi *et al.*, 2002). The mechanism of the FRS2/Laloo interaction, however, is controversial. While Kusakabe *et al*. suggest that the FRS2 N-terminal region lacking the PXXP motif and the SH4 domain of Laloo are sufficient for the association, Hama *et al.* show that both the SH3 and SH4 domains are necessary in a yeast two-hybrid system (Hama *et al.*, 2001; Kusakabe *et al.*, 2001). It is suggested that the FGFR and Laloo functionally cooperate to induce tyrosine phosphorylation of FRS2, since Laloo mediated FRS2 tyrosine phosphorylation is independent of the kinase activity of FGFR but requires the intracellular domain of the receptor (Kusakabe *et al.*, 2001). Conversely, the Laloo kinase activity is dependent on the tyrosine phosphorylation of FRS2 (Hama *et al.*, 2001). However, injection of high dose wild type xFRS2 mRNA alone caused embryonic death. Overexpression of xFRS2 in the

marginal zone appeared also to have toxic effect on the embryo (Kusakabe *et al.*, 2001). All these results indicate an important role of FRS2 in early mesoderm development.

There is evidence of FRS2 being involved in signaling induced by other growth factors, such as brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), IGF-I, as well as insulin (Easton *et al.*, 1999; Delahaye *et al.*, 2000; Kimpinski and Mearow, 2001; Kurokawa *et al.*, 2001; Melillo *et al.*, 2001). FRS2 becomes tyrosine phosphorylated by the activated receptors TrkB, Ret, IGF-IR and IR. In case of TrkB and Ret, FRS2 associates with the receptors through the known Shc binding sites. Recently, it has been reported that FRS2 also participates in vascular endothelial growth factor (VEGF) signaling through the VEGFR2 (or kinase insert domain-containing receptor, KDR) in a similar manner as in FGFR signaling. FRS2 constitutively associates with the VEGFR2 and undergoes tyrosine phosphorylation upon activation of the receptor (Stoletov *et al.*, 2002).

1.7 Objective

FRS2/SNT was identified as a specific intermediate of FGFR and NGFR signaling pathways in rodent cells (Rabin *et al.*, 1993; Kouhara *et al.*, 1997). The goal of this study was to investigate the function of the human homologue in cellular signal transduction.

Being a downstream signaling molecule of receptor tyrosine kinases (RTKs) that play important role in cellular and oncogenic signaling, the expression pattern of FRS2 in tumor cells as well as normal cells would be examined. This might give a hint about a possible involvement of FRS2 in oncogenesis.

As a membrane-anchored docking protein comprising a phosphotyrosine-binding (PTB) domain, FRS2 is very likely to target to more RTKs and function as a general mediator in various cellular signaling pathways. This work addresses this issue by testing different ligands for their effects on FRS2.

FRS2 contains multiple tyrosine phosphorylation sites that serve as docking sites for other phosphotyrosine-binding proteins. Identification of further interaction partners of FRS2 by appropriate association experiments would help to study the differential role of FRS2 in signal transduction as well as its underlying regulation mechanism.

FRS2 possesses multiple potential serine and threonine phosphorylation sites. This raised the question whether FRS2 becomes phosphorylated on these residues, and what potential function this modification could have. These subjects will be investigated by phosphoamino acid analysis and by interfering the function of different signal transduction molecules as well as by examination of truncation mutants.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Acrylamide	Serva, Heidelberg, Germany
Agarose	BRL, Eggenstein, Germany
Ampicillin	Roche Diagnostics, Mannheim, Germany
Aprotinin	Roche Diagnostics, Mannheim, Germany
APS (ammoniumperoxydisulfate)	Sigma, Taufkirchen, Germany
ATP (adenosinetriphosphate)	Amersham Biotech, Freiburg, Germany
β-mercaptoethanole	Sigma, Taufkirchen, Germany
Bisacrylamide	Roth, Karlsruhe, Germany
Bromophenolblue	Sigma, Taufkirchen, Germany
BSA (bovine serum albumine)	Sigma, Taufkirchen, Germany
Bisbenzimide	Roche Diagnostics, Mannheim, Germany
Comassie G250	Serva, Heidelberg, Germany
DAPI (4',6-diamidino-2-phenylindole)	Sigma, Taufkirchen, Germany
Desoxynucleotides	Roche Diagnostics, Mannheim, Germany
Didesoxynucleotides	Amersham Biotech, Freiburg, Germany
Dimethyldichlorsilan	Sigma, Taufkirchen, Germany
DTT (dithiothreitol)	Sigma, Taufkirchen, Germany
Ethidiumbromid	Sigma, Taufkirchen, Germany
Formamide	Merck, Darmstadt, Germany
Formaldehyde	Merck, Darmstadt, Germany
Freund's adjuvant	Sigma, Taufkirchen, Germany
Geneticin (G418)	Gibco, Eggenstein, Germany
Gelatine	Merck, Darmstadt, Germany
Glucose	Sigma, Taufkirchen, Germany
Gluthatione	Sigma, Taufkirchen, Germany
HEPES (4-(2-hydroxylethyl)-1-piperazin-ethansulfonicacid)	Serva, Heidelberg, Germany
tRNA	Sigma, Taufkirchen, Germany
IPTG (isopropyl-β-thiogalactopyranoside)	Biomol, Hamburg, Germany
Lipofectamine®	Gibco, Eggenstein, Germany
Lithium acetate	Sigma, Taufkirchen, Germany
MBP (myelin basic protein)	Sigma, Taufkirchen, Germany
MOPS (3-(N-morpholino)-propanesulfonic acid)	Serva, Heidelberg, Germany
Phenol	Roth, Karlsruhe, Germany
PMSF (phenylmethylsulfonylfluorid)	Sigma, Taufkirchen, Germany
Polbren (hexadimethrinbromide)	Sigma, Taufkirchen, Germany
Polyethylenglycol 4000	Sigma, Taufkirchen, Germany
Ponceau S	Sigma, Taufkirchen, Germany

Sodium azide	Sigma, Taufkirchen, Germany
Sodium fluoride	Sigma, Taufkirchen, Germany
Sodium Orthovanadate	Sigma, Taufkirchen, Germany
SDS (sodium dodecyl sulfate)	Roth, Karlsruhe, Germany
TEMED (N,N,N'N'-Tetraethyl-methylene diamine)	Serva, Heidelberg, Germany
Triton X-100	Serva, Heidelberg, Germany

2.1.2 Restriction, Modification Enzymes and Polymerases

Alkaline phosphatase	Roche Diagnostics, Mannheim, Germany
Lysozyme	Roche Diagnostics, Mannheim, Germany
Restriction enzymes	New England Biolabs, Schwalbach, Germany MBI Fermentas, St. Leon-Rot, Germany
RNase A	Sigma, Taufkirchen, Germany
Reverse transciptase	Roche Diagnostics, Mannheim, Germany
RNaseH	Roche Diagnostics, Mannheim, Germany
T4-DNA-ligase	MBI Fermentas, St. Leon-Rot, Germany
T7-DNA-polymerase	Amersham Biotech, Freiburg, Germany
DNA-polymerase	TaKaRa Biomedicals, Japan

2.1.3 Kinase Inhibitors

Inhibitor	Kinase	Source
AG1295	PDGFR	Calbiochem, Schwalbach, Germany
AG1478	EGFR	Calbiochem, Schwalbach, Germany
GÖ6976	PKC	Calbiochem, Schwalbach, Germany
H-89	PKA	Calbiochem, Schwalbach, Germany
KN-93	CaMK II	Calbiochem, Schwalbach, Germany
ML-7	MLCK	Calbiochem, Schwalbach, Germany
PKG inhibitor	PKG	Calbiochem, Schwalbach, Germany
PP1	Src	Calbiochem, Schwalbach, Germany
Roscovitine	Cdc2	Calbiochem, Schwalbach, Germany
SB202190	p38 kinase	Calbiochem, Schwalbach, Germany
SB600125	JNK	Calbiochem, Schwalbach, Germany
Staurosporine	PKC, PKA, PKG, MLCK, CaMK-II	Calbiochem, Schwalbach, Germany
SU5402	FGFR	Calbiochem, Schwalbach, Germany
U-0126	MEK1	Calbiochem, Schwalbach, Germany
U73122	PLC γ	Calbiochem, Schwalbach, Germany
Wortmannin	PI-3K	Calbiochem, Schwalbach, Germany

2.1.4 Ligands

EGF (mouse)	Toyoba, Japan
FGF (acid, human)	Peprotech, USA
Insulin (human)	Lilly, Giessen, Germany
IGF-I (human)	Sigma, Taufkirchen, Germany
NGF-β (human)	Sigma, Taufkirchen, Germany
PDGF B/B (human)	Roche Diagnostics, Mannheim, Germany
Carbachol (carbamylcholine chloride)	Sigma, Taufkirchen, Germany
LPA (lysophosphatidic acid)	Sigma, Taufkirchen, Germany
Thrombin	Sigma, Taufkirchen, Germany

2.1.5 Radiochemicals

[α-^{32}P]-dATP, [γ-^{32}P]-ATP	>5000 Ci/mmol
[α-^{35}S]-dATP, L-[^{35}S]-Methionine	>1000 Ci/mmol
[^{32}P]-Phosphate	10mCi/ml

All radiochemicals were purchased from Amersham Biotech (Freiburg, Germany) or NEN (Cologne, Germany) and used before the first half life.

2.1.6 Commertial Kits and Diverse Materials

QIAEX II gel extraction kit	Qiagen, Hilden, Germany
QIAquick PCR purification kit	Qiagen, Hilden, Germany
QIAGEN plasmid maxi kit	Qiagen, Hilden, Germany
Random primed DNA labeling kit	Amersham Biotech, Freiburg, Germany
Micro BCA protein assay kit	Pierce, USA
ECL kit	Amersham Biotech, Freiburg, Germany PerkinElmer Life Science, Boston, USA
Nitrocellulose membrane	Schleicher & Schuell, Germany
Polyvinylidene difluoride (PVDF) membrane	Millipore, USA
Hyperfilm	Amersham Biotech, Freiburg, Germany
CNBr-activated sepharose 4B	Amersham Biotech, Freiburg, Germany
Protein A-sepharose	Amersham Biotech, Freiburg, Germany
Protein G-sepharose	Amersham Biotech, Freiburg, Germany
Glutathione Sepharose 4B	Amersham Biotech, Freiburg, Germany
Tissue cultur plates	Nunclon, Danemark Flacon, U.K.

2.1.7 Media and Buffers

2.1.7.1 Bacterial Media

LB-medium	1.0	%	tryptone
	0.5	%	yeast extract
	1.0	%	NaCl
			pH 7.2
2 x TY-medium	1.6	%	tryptone
	1.0	%	yeast extract
	0.5	%	NaCl
			pH 7.2

The following concentrations of the antibiotics were used if required:

Ampicillin	100	µg/ml
Kanamycin	100	µg/ml
Chloramphenicol	30	µg/ml

1.5 % agar was added to the media for production of agar plates.

2.1.7.2 Cell Culture Media

All the media for the cell culture were purchased from Gibco (Eggenstein, Germany). The fetal calf serum (FCS) was purchased from Sigma (Taufkirchen, Germany).

Dulbecco's Modified Eagle Medium (DMEM) with 4.5 mg/ml glucose, 5 or 10% FCS, 2 mM glutamin, 1 mM sodium pyruvat.

Dulbecco's Modified Eagle Medium (DMEM) with 1.0 mg/ml glucose, 10% FCS, 2 mM glutamin, 1 mM sodium pyruvat.

RPMI 1640 with 10%FCS, 2 mM glutamin, 1 mM sodium pyruvat.

Leibovitz's L-15 with 10%FCS, 2 mM glutamin, 1 mM sodium pyruvat.

Methionine free media for radioactive labeling of cells:
MEM (Eagle) with Earle's salts, 2 mM glutamin, 40 µCi/ml L-[^{35}S]-methionine.

Phosphate free media for radioactive labeling of cells:
MEM (Eagle) with Earle's salts, 2 mM glutamin, 200 µCi/ml [^{32}P]-phosphate.

Media for storage of frozen cells:
90 % FCS, 10 % DMSO.

Coating medium for tissue culture plate (for PC12 cells):
0.01% collagen type I, 30% ethanol in 1xPBS.

2.1.8 Stock Solutions and Frequently Used Buffers

Denhardt (100x)	2.0	%	polyvinylpyrollidone
	2.0	%	ficoll
	2.0	%	bovine serum albumine (BSA)
DNA sample buffer (6x)	2.5	‰	bromophenolblue
	2.5	‰	xylenecyanol
	30.0	%	glycerine
	100.0	mM	EDTA pH 8.0
HNTG (4x)	200.0	mM	HEPES-NaOH pH 7.5
	600.0	mM	NaCl
	40.0	%	glycerine
	0.4	%	Triton X-100
Laemmli buffer (2x)	187.5	mM	Tris-HCl pH 6.8
	6.0	%	sodium dodecyl sulfate (SDS)
	30.0	%	glycerine
	0.01	%	bromophenolblue
	5.0	%	β-mercaptoethanol
MOPS (10x)	200.0	mM	3-(N-morpholino)-propanesulfonic acid (MOPS)
	80.0	mM	sodium acetate
	10.0	mM	EDTA pH 8.0
	pH 7.0		(NaOH)
NET (10x)	500.0	mM	Tris-HCl pH 8.0
	50.0	mM	EDTA pH 8.0
	1500.0	mM	NaCl
	0.5	%	Triton X-100
PBS	13.7	mM	NaCl
	2.7	mM	KCl
	80.9	mM	Na2HPO4
	1.5	mM	KH2PO4
	pH 7.4		(HCl)
RNA sample buffer (2x)	48.0	%	formamide
	17.0	%	formaldehyde
	11.0	%	glycerine
	0.1	%	bromophenolblue
SSC (20x)	3.0	M	NaCl
	30.0	mM	sodiumacetate
SD-transblot	50.0	mM	Tris-HCl pH 8.5

	40.0	mM glycine
	20.0	mM methanol
	0.04	‰ SDS
TAE (10x)	400.0	mM sodium acetate
	10.0	mM EDTA pH 8.0
TBE (10x)	890.0	mM Tris-HCl pH 8.0
	890.0	mM boric acid
	20.0	mM EDTA pH 8.0
TE (10/0.1)	10.0	mM Tris-HCl pH 7.5
	1.0	mM EDTA pH 8.0
Tris-glycine-SDS (10x)	248.0	mM Tris-HCl pH 7.5
	1918.0	mM glycine
	1.0	% SDS

2.1.9 Bacteria Strains and Cell Lines

2.1.9.1 Bacteria Strains

Strain	Genotype	Reference
E. coli XL-1 Blue	F'proAB lacI^{q}ZΔM15 Tn10 / endA1 supE44 thi-1 recA1 gyrA96 relA1 lac	Stratagene

2.1.9.2 Cell Lines

Cell line	Origin	Reference
HEK293	Human embryionic kidney fibroblasts	ATCC CTL 1573
Cos-7	immortalised green monkey kidney fibroblasts	ATCC CRL-1651
Bosc	Helper free retrovirus producing cells based on HEK 293 cells	W. S. Pear
Phoenix A	Helper free retrovirus producing cells based on HEK 293 cells	G.P. Nolan
Ovcar-3	Human ovarian adenocarcinoma	ATCC HTB-161
A-431	Human epidermoid carcinoma	ATCC CRL-1555
U-1242	Human glioblastoma	SUGEN, USA
Dal	Human mammary carcinoma	G. Natali
MDA-MB-231	Human mammary adenocarcinoma	ATCC HTB-26
MDA-MB-361	Human mammary adenocarcinoma	ATCC HTB-27

MCF-10A	Human mammary gland epithelia	ATCC CRL-10317
Daudi	Human Burkitt's lymphoma	ATCC CCL-213
IM-9	Human B lymphoblast	ATCC CCL-159
KG-1	Human acute myelogenous leukemia	ATCC CCL-246
HEL	Human erythroleukemia	ATCC TIB-180
NCI-H460	Human large cell lung carcinoma	ATCC HTB-177
NCI-H661	Human lung carcinoma	ATCC HTB-183
NT-2	Human teratocarcinoma	Stratagene
NIH/3T3	Mouse embryo fibroblst	ATCC CRL-1658
PC-12	Rat pheochromocytoma	ATCC CRL-1721
RIN-5AH-T2B	Rat insulinoma	Billestrup, 1985
Rat-1	Rat fibroblasts, immortalised	R. Friedrich
B-29	Mouse mammary carcinoma	ATCC CRL-6325
L-8A	Mouse mammary carcinoma	ATCC CRL-6363
MM2SCT	Mouse mammary carcinoma	ATCC CRL-6375
MM5MTC	Mouse mammary carcinoma	ATCC CRL-6378
MM5.1	Mouse mammary carcinoma	ATCC CRL-6380
MM5/C1	Mouse mammary carcinoma	ATCC CRL-6444
MM5MT	Mouse mammary carcinoma	ATCC CRL-6590

2.1.10 Antibodies

2.1.10.1 Commercial Antibodies

Antibody	Description	Reference
PY(4G10)	Mouse monoclonal anti-phosphotyrosine antibody	Upstate Biotechnology, USA
Flag	Rabbit polyclonal anti-Flag	Santa Cruz Biotech, USA
Flag	Mouse monoclonal anti-Flag	Sigma, Germany
EGFR	Rabbit polyclonal anti-EGFR (amino acid 1006-1116)	Santa Cruz Biotech, USA
EGFR	Sheep polyclonal anti-EGFR (GST-fusion protein of extracellular domain)	Upstate Biotechnology, USA
ERK1	Rabbit polyclonal anti-ERK1 (C-terminal domain of rat ERK2)	Santa Cruz Biotech, USA
ERK2	Rabbit polyclonal anti-ERK2 (C-terminal domain of rat ERK2)	Santa Cruz Biotech, USA
SHP2	Monoclonal anti-SHP2	Tranduction Laboratory, USA
Grb2	Monoclonal anti-Grb2	Upstate Biotechnology, USA

Caveolin-1	Rabbit polyclonal anti-caveolin-1	Santa Cruz Biotech, USA

The following HRP-conjugated secondary antibodies were used in immunoblot (Western blot) and were purchased from BioRad (Munich, Germany).

Antibody	Dilution
Goat anti-mouse	1 : 50'000
Goat anti-rabbit	1 : 50'000
Rabbit anti-sheep	1 : 20'000
Protein A	1 : 20'000

2.1.10.2 Home-Made Antibodies

Antibody	Description	Reference
FRS2-NT	Rabbit polyclonal anti-FRS2-NT, GST-FRS2-NT as antigen	This work
FRS2-CT	Rabbit polyclonal anti-FRS2-CT, GST-FRS2-CT as antigen	This work
FRS2-WT	Rabbit polyclonal anti-FRS2-WT, GST-FRS2-WT (full-length) as antigen	This work
SHP2	Rabbit polyclonal anti-SHP2-CT Peptide as antigen	R. Lammers, This department.
Gab1	Rabbit polyclonal anti-Gab1-NT GST-Gab1 fusion protein as antigen	A. Lankenau, This department.

2.1.11 Commercial Purified Proteins

Protein	Description	Reference
$p13^{suc1}$	Recombinant yeast $p13^{suc1}$ GST fusion protein agarose conjugated	Upstate Biotechnology, USA
ERK2, active	Recombinant mouse ERK2 GST fusion protein, phosphorylated	Upstate Biotechnology, USA
ERK2, unactive	Recombinant mouse ERK2 GST fusion protein, unphosphorylated	Upstate Biotechnology, USA
MEK1, acitvated	Recombinant human MEK1 GST fusion protein, phosphorylated	Upstate Biotechnology, USA
MEK1, unacitvated	Recombinant human MEK1 GST fusion protein, unphosphorylated	Upstate Biotechnology, USA

2.1.12 Plasmids and Oligonucleotides

2.1.12.1 Original Plasmids

Plasmid	Description	Reference
pGEX5X-1/ pGEX5X-3/ pGEX-2T	Expression vector of GST fusion-proteins, Amp^r	Amersham Biotechnology, Freigurg, Germany
pRK5	Expression vector, Amp^r, CMV promotor, SV40 poly A, high copy plasmid	Genentech, San Francisco, USA
pcDNA3	Expression vector, Amp^r, Neo^r, CMV promotor, BGH poly A, high copy plasmid	Invitrogen, Netherlands
pEGFP-N3	Expression vector, Kan^r, Neo^r, CMV promotor, SV40 poly A,	Clontech, Palo Alto, USA
pLXSN	Retroviral expression vector, Amp^r, Neo^r, ori of PBR322, 5'-LTR and 3' LTR of MoMuLV, SV40 promoter	Miller & Rosman, 1989

2.1.12.2 Plasmids with Inserts

Plasmid	Insert	Reference
pRK5-FRS2	Full-length cDNA of human FRS2 (amino acids 1-508)	This work
pRK5-FRS2-Flag	Full-length cDNA of human FRS2 (amino acids 1-508), carboxyl terminal Flag-tag	This work
pcDNA3-FRS2-Flag	Full-length cDNA of human FRS2 (amino acids 1-508), carboxyl terminal Flag-tag	This work
pGEX-5X-3-FRS2-WT	Full-length cDNA of human FRS2 (amino acids 1-508)	This work
pGEX-5X-1-FRS2-PTB	cDNA of human FRS2 (amino acids 1-163)	This work
pGEX-5X-3-FRS2 -Myr/ΔPTB	cDNA of human FRS2 (amino acids 1-9 + 153-508)	This work

pGEX-5X-1-FRS2-ΔPTB	cDNA of human FRS2 (amino acids 156-508)	This work
pGEX-2T-FRS2-NT	cDNA of human FRS2 (amino acids 1-240)	This work
pGEX-5X-1-FRS2-CT	cDNA of human FRS2 (amino acids 326-508)	This work
pGEX-2T-CR	cDNA of human FRS2 (amino acids 238-345)	This work
pGEX-5X-3-ΔCR	cDNA of human FRS2 (amino acids Δ240-351)	This work
pcDNA3-FRS2-G2A	cDNA of human FRS2, G2A myristylation site mutant	This work
pEGFP-N3-FRS2-WT	Full-length cDNA of human FRS2, C-terminal GFP	This work
pEGFP-N3-FRS2-ΔC1	cDNA of human FRS2, (amino acids 1-428),	This work
pEGFP-N3-FRS2-ΔC2	cDNA of human FRS2, (amino acids 1-345)	This work
pEGFP-N3-FRS2-ΔC3	cDNA of human FRS2, (amino acids 1-185)	This work
pcDNA3-HA-ERK2	cDNA of mouse ERK2, amino terminal HA-tag	H. Daub, This department
pRK5-SHP2	cDNA of human SHP2	W. Vogel This department
pRK5-SHP2-C459A	cDNA of human SHP2, C459A mutant, inactive	W. Vogel This department
pGEX-5X-2-α-Heregulin	cDNA of human heregulin	C. Wallasch This department

2.1.13 Oligonucleotides (selection)

For cDNA cloning of full-length FRS2:

GCG AAT TCC ATG GGT AGC TGT TGT AGC TGT C
Sense starting at nucleotide 1, EcoRI

GCG GAA TTC TCT GAA GAA GCC **ATG** GGT AGC TGT TGT AGC TG
Sense starting at nucleotide 1, including Kozak consensus sequence, EcoRI

CCG ACT GAC CTC GAG TCA CAT GGG CAG ATC AGT ACT ATT GT
Antisense ending at nucleotide 1527, XhoI

CCG ACT GAC CTC GAG TCA *CTT GTC GTC ATC GTC TTT GTA GTC* CAT GGG CAG ATC AGT ACT ATT
Antisense ending at nt 1527 including *Flag-tag* sequence

For generation of truncation and deletion mutants:

For CT mutant:
GCG AAT TCT CCA CCA GTA CCT CAG ATA CCC
Sense starting at nt 979, EcoRI

For PTB mutant:
CCG ACT GAC CTC GAG CGG ATG GGA TGA AGC ATC TCC
Antisense ending at nt 489, XhoI

For ΔPTB mutant:
CGA CCC GGG GAT GCT TCA TCC CAT CCG
Sense starting at nt 472, smaI

CCA CCC GGG ACA GCT ACA ACA GCT ACC
Antisense starting at nt 4, smaI

For ΔCR mutant:
CCG GAT CCA TCT TTG CCT CCT GTT TGG
Sense starting at nt 1057, BamHI

2.2 Methods

2.2.1 Preparation, Analysis and Enzymatic Treatment of DNA

2.2.1.1 Plasmidpreparation for Analytical Purpose

Small amounts of DNA (2- 10 µg) from 2- 3 ml bacteria culture were prepared for analytical purposes according to the alkaline lysis method.

2.2.1.2 Plasmidpreparation for Preparative Purpose

A pure quality of plasmid DNA was needed for transfection into mammalian cells. The plasmid purification kit of QIAGEN was used to purify plasmids for this purpose according to the manufacturers protocols.

2.2.1.3 Restriction Digestions of DNA-Fragments

1.5 units/µg of restriction enzyme were incubated with DNA in 30 µl of the recommended incubation buffer at 37 °C for 2 hours.

2.2.1.4 Dephosphorylation of 5'-Ends of DNA-Fragments

Dephosphorylation of the 5'-ends prevented the religation of plasmids with sticky ends. 1 - 3 µg of cut plasmids was incubated in 10 - 20 µl of 1x phosphatase buffer (50 mM Tris-HCl pH 8.0, 0.1 mM EDTA pH 8.5) with 1 unit calf intestine phosphatase at 37°C for 60 min.

2.2.1.5 Gel Electrophoresis of DNA-Fragments

Double stranded DNA-molecules between 0.5 and 10 kb were separated in 0.5- 2 % horizontal agarose gels. The running buffer was 1 x TAE. The DNA-sample was mixed with 1/6 volume of 6 x DNA loading buffer. The electrophoresis was performed between 50-100 volts for 1 - 2 hours. After the separation the gel was stained for 10 min in 1 x TAE/ethidiumbromide (2mg/ml) and destained for 10 min in 1 x TAE buffer.

2.2.1.6 Isolation of DNA-Fragments from Agarose Gels

DNA-Fragments were isolated from agarose gels with the QIAquick gel extraction kit of QIAGEN according to the manufacturers instructions. The principle of the kit is the adsorption of DNA fragments (100 bp - 10 kb) to silica-gel membranes in the presence of high concentration of chaotropic salts, which modify the structure of water. The elution is performed under basic conditions and low salt concentrations.

2.2.1.7 Ligation of DNA-Fragments into Plasmid Vectors

The T4-DNA-ligase was used to synthesize the phosphodiester-bonds between the 5'-phosphate and the 3'-hydroxy-group of polynucleotides. 20 - 200 ng of cut vector were incubated with a 2 - 5 fold molar excess of the insert fragment in 10 µl 1x T4-DNA-ligase buffer (66 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 5 mM DTT, 1 mM ATP) and 1 unit T4-DNA-ligase overnight at 16 °C. The whole ligation reaction was used for transformation of bacteria as described.

2.2.1.8 Chemical Transformation of Bacteria

Competent *E. coli* bacteria were prepared according to the method of Chung and Miller (Chung and Miller, 1993). A bacteria preculture was grown overnight. After 12 hours, inoculated in 250 ml LB and grown until the mid-log phase (OD_{600} = 0.3 - 0.5). The bacteria were harvested, resuspended in 12 ml cold TSB-buffer (2 x TY media, 12.5% PEG 6000, 0.125 mM $MgSO_4$, 0.125 mM $MgCl_2$, 0.06 % DMSO) and incubated on ice for 30 min. Aliquots were stored at - 80 °C. For the transformation, 20 µl 5 x KCM (500 mM KCl, 150 mM $CaCl_2$, 250 mM $MgCl_2$) and 10 µl of the ligation mix were added to 70 µl ddH_2O . The competent bacteria were thawed on ice and 100 µl were added to the sample. The reaction was incubated on ice for 20 min and then transferred to room temperature for another 10 min. After addition of 400 µl LB-media the bacteria were incubated at 37 °C for 60 min in a shaking incubator and subsequently plated out on agar plates containing the appropriated resistance drug. The transformation efficiency was greater than 5 x 10^6 clones/µg DNA.

2.2.1.9 Sequencing of Plasmids

For the verification of DNA sequences a modified protocol of the chain termination reaction according to Sanger was used (Sanger *et al.*, 1977). 2 µg of DNA were denatured in 20 µl TE(10/0.1) by the addition of 2 µl 2 M NaOH/2 mM EDTA at room temperature for 5 min. The sample was ethanol precipitated and the pellet resuspended in 14 µl 1 x annealing mix (40 mM Tris-HCl pH 7.5, 50 mM NaCl, 15 mM $MgCl_2$, 0.7 µM specific primer). The primer was allowed to anneal for 5 min at 37 °C and at room temperature for another 5 min. The template was labeled by addition of 6 µl T7 DNA pol mix (50 mM DTT, 1 µM dTTP/dCTP/dGTP, 0.25 mCi/ml [α-^{35}S]-dATP, 1 unit T7 DNA polymerase) for 2 min at room temperature. 2.5 µl of this mixture were aliquoted to each of the 4 termination mixtures (80 µM dNTP (all nucleotides except X and 8 µM ddXTP) and incubated at 42 °C for 5 minutes. 2 µl formamide dye (deionized formamide, 1 mg/ml xylene cyanol, 1 mg/ml bromophenol blue, 10 mM EDTA pH 8.0) were added and the reaction was incubated at 90 °C for 10 min. The fragments were resolved on a denaturing 6 % acrylamide/7.6 M urea gel.

2.2.2 Analysis of RNA

The ubiquity of contaminating RNases and the concomitant difficulties in ensuring that an RNA preparation remains reasonably undegraded requires specific precautions. All the solutions were prepared with MilliporeTM-water and the glassware was baked at 200 °C for two hours. Gloves were used for all the procedures. The gloves were changed frequently. If necessary, the deionized water was treated with diethylpyrocarbonate (DEPC) to inhibit RNase activity.

2.2.2.1 Preparation of Total RNA

Total RNA from cell cultures were isolated with the RNeasy mini kit of QIAGEN according the manufacturers instructions. The cells were lysed in a denaturing guanidinium isothiocyanate containing buffer and homogenized with a QIAshredder

column. The RNA was then purified in spin columns containing special silica-gel membranes.

2.2.2.2 Preparation of Poly $(A)^+$-RNA

The monolayer cells were washed once with 1 x PBS and 3.5 ml guanidine solution (4 M guanidine solution, 20 mM sodium acetate, 0.5 % Sarkosyl and 0.1 mM DTT) for 10^8 cells were added to the dish to lyse the cells immediately. The viscous lysate was recovered by scraping the dish with a rubber policeman and removed with a 6-ml syringe with a 20-G needle. 1.5 ml 5.7 M CsCl were place in a ultracentrifuge tube and 3.5 ml of cell lysate were laid on top of the CsCl solution to create a step gradient. The sample was centrifuged for at 150'000 g 24 hours. The supernatant was removed carefully with a Pasteur pipette and the pellet was allowed to air dry for 5 to 10 min and then resuspended in 100 µl H_2O at room temperature. 0.5 g oligo (dT9) cellulose powder was added to 1 ml of 0.1 M NaOH and the slurry was poured into a silanized Pasteur pipette that was plugged with silanized glass wool. The column was equilibrated with 15 ml loading buffer (0.5 M LiCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, 0.1 % sodium dodecyl sulfate). The total RNA was heated to 70 °C for 10 min and LiCl was added to 0.5 M final concentration. The RNA solution was passed twice over the oligo (dT) column. The column was washed with 1 ml poly (A) loading buffer and with 2 ml middle wash buffer (0.5 M LiCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1 % sodium dodecyl sulfate). The poly $(A)^+$–RNA was eluted with 2 ml elution buffer (2 mM EDTA pH 8.0, 0.1 % sodium dodecyl sulfate). The eluted RNA was precipitated by adjusting the salt concentration to 0.3 M sodium acetate and addition of 2.5 volume ethanol. The sample was incubated on dry ice/ethanol for 30 min and resuspended in 30 µl TE-buffer.

2.2.2.3 Gel Electrophoresis of RNA

1.8 g agarose was dissolved in 130 ml H_2O and cooled to about 60 °C. Immediately before the gel was poured, 15 ml 10 x MOPS (200 mM 3-(N-morpholino)-propanesulfonic acid) and 4.56 ml formaldehyd were added. 5 - 10 µg total RNA were diluted in 11.25 µl H_2O and 38.75 µl sample buffer (20 mM MOPS, 5.7 % formaldehyde, 40 % formamide) were added. The samples were incubated at 55 °C for 15 min 5 µl loading buffer (40% formamide, 20 % glycerine, 2 mM EDTA pH 8.0, 0.1% bromophenol dye) was added and the samples were loaded onto the gel. The gel was run at 5 V/cm (running buffer 1 x MOPS) until the bromophenol dye has migrated two-thirds the length of the gel. The gel was stained with 1 x MOPS/10 µl EtBr for 20 min. A picture of the gel was taken on a UV transilluminator with a ruler laid alongside the gel, so that band positions could later be identified on the membrane. The gel was than destained 3 times for 10 min with 1 x MOPS.

2.2.2.4 Transfer of RNA onto Nitrocellulose Membrane

The RNA was transferred from the gel to a nitrocellulose membrane by upward capillary transfer (Thomas, 1980). Two glass dishes were filled with 20 x SSC (3 M NaCl, 0.3 M sodium citrate) and a glass plate, that forms the support for the gel was placed between the dishes. Three pieces of Whatman 3MM paper were presoaked in 2 x SSC (0.3 M NaCl, 0.03 M sodium citrate) and placed on the glass plate so that the ends submerge into both 20 x SSC buffer tanks. Three pieces of Whatman 3MM paper were cut to the same size as the gel, presoaked in 2 x SSC and placed into the middle of other

filter papers. The gel was placed upside down on the filter paper. A nitrocellulose membrane was wetted in 2 x SSC and placed on the surface of the gel. Air bubbles under the membrane were squeezed out. The membrane was covered with three layers of Whatman 3MM paper, which were presoaked in 2 x SSC. Whatman 3MM paper and paper towels were stacked on top to a height of 5 - 6 cm. A weight was added and the blot was left overnight. After blotting the membrane was placed between two sheets of Whatmann 3MM filter paper and baked in a vacuum oven at 80 °C for 2 hours.

2.2.2.5 Labeling of DNA Fragments with [a-^{32}P]-dATP

DNA probes were labeled with the "Megaprime^{TM} DNA labeling system" from Amersham pharamcia biotech. 25 ng of template DNA were placed in a microcentrifugation tube and 5 µl of random nonamer primers and H2O were added to 50 µl. The samples were denatured at 100 °C for 5 min. After cooling to room temperature 5 µl reaction buffer, 4 µl unlabelled dCTP, dGTP, dTTP 2 µl DNA polymerase I Klenow (2 u/µl) were added. The reaction was incubated at 37 °C for 10 min. The labeled DNA fragment was purified with a QIAnucleotide removal kit from QIAGEN according to the manufacturers instructions.

2.2.2.6 Hybridization of RNA-blots with Radioactive Probes

The membrane was blocked in prehybridization solution (50 % formamide, 5 x SSC, 5 x Denhard, 0,1 % SDS) containing 20 µg/ml denatured and sheared DNA for at 42 °C 1 hours. The prehybridization solution was changed once after 6 hours. The membrane was the incubated in hybridization solution (50 % formamide, 5 x SSC, 0.2 % SDS, 5 x Denhardt, pH 7.5 - 7.8) containing the denatured and labeled probed for 12 hours. After washing the membrane twice in 2 x SSC, 0.1 % SDS at 42 °C for 20 min and once with 0.2 x SSC, 0.1 % SDS for at 42 °C 15 min, autoradiography using intensifying screens was performed at - 80 °C for 12 - 60 hours. If the membrane was to be reprobed, the probe was removed by placing the membrane in boiling water containing 10 mM Tris pH 8.0, 0.1 % SDS. After 5 min the solution was slowly cooled down to room temperature by switching off the heat.

2.2.3 Amplification of RNA- and DNA-Fragments

2.2.3.1 Conversion of mRNA into Doubled-stranded cDNA

Purified mRNA can be used to produce cDNA with the help of the enzyme reverse transcriptase, which plays a crucial role in the replication of retroviruses. 2 µg poly (A)+-RNA were incubated with 1 µg random hexamer primer in a reaction volume of 13.5 µl and heated at 68 °C for 2 min. The first strand was synthesized by addition of 0.5 µl RNase inhibitor (40 units/ml), 1 µl AMV reverse transcriptase (25 units/µl), 1 µl 10 mM dNTPs (endconcentration 500 µM) and a 5 x buffer to the endconcentration of 30 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 5 mM DTT, 100 mM KCl. The reaction (volume 20 µl) was incubate on ice for 5 min and at 42 °C for 60 min. The second strand synthesis was then performed by addition of 2 µl 10 mM dNTPs (endconcentration 500 µM), 1.2 µl RNaseH (1unit/µl), 1.3 µl DNA-Polymerase I (5

units/µl) and a 5 x buffer to the endconcentration of 30 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 5 mM DTT, 100 mM KCl. The reaction (volume 60 µl) was incubated at 15 °C for 60 min and at 22 °C for another 60 min. After addition of 90 µl H_2O to a final volume of 150 µl, the reaction was phenol/chloroform extracted and precipitated by the addition of 1/10 volumes 3 M sodiumacetate pH 9.0 and 3 volumes ethanol and incubation in a ethanol/dry ice bath for 15 min. The pellet was washed with ice-cold 70% ethanol and resuspended in 20 µl H_2O.

2.2.3.2 PCR-Amplification of DNA and cDNA-Fragments

The method of the polymerase chain reaction (PCR) techniques allows the exponential amplification of a specified DNA sequence *in vitro*.. In a repeating cycle, doubled stranded DNA is separated by heat denaturation, the primers anneal to their binding sites that flank the target region and a polymerase can synthesize new strands of DNA, complementary to the template (Mullis *et al.*, 1986; Mullis and Faloona, 1987). Each PCR reaction was performed in a total reaction volume of 50 µl containing 0.1 µg template DNA, 1x TaKaRa PCR buffer, 2.5 µM $MgCl_2$, 0.5 µM of each primer, 200 µM dNTP and 0.1 – 0.75 units TaKaRaTaq.
The following cycle was used in a Techne thermo cycler:

	94°C 3 min
25x	[94°C, 30 sec—>T°C, 60 sec —> 72°C, 60sec]
	72°C, 10 min
	4 °C

The annealing temperature T was calculated as T= 2 ((A+T) + 2 (G+C)) -5, where the A, T, G and C correspond to the number of each respective eoxyribonuclotides in the oligonucleotide). An elongation time of 60 sec was used for the synthesis of a fragment of one kilobase.
The amplification was verified by running an analytical agarose gel with an aliquot of the reaction.

2.2.3.3 Purification of PCR Products

The PCR-products were purified with the QIAquick PCR purification kit of QIAGEN according to the manufacturers instructions. The principle of the kit is the adsorption of DNA fragments (100 bp- 10 kb) to silica-gel membranes in the presence of high concentration of chaotropic salts, which modify the structure of water. The elution is performed under basic conditions and low salt concentrations.

2.2.3.4 Subcloning of PCR Fragments

PCR-products were subcloned by using primers with recognition sites for restriction enzymes. After restriction digest and gel purification the fragments were ligated into the required plasmids.

2.2.4 Rabbit Polyclonal Antibodies

2.2.4.1 Production of Antibodies

GST-fusion proteins were used as antigens for production of rabbit polyclonal antibodies. 0.5 mg of antigen was mixed with the same volume of complete Freund's adjuvant and injected subcutaneously into a rabbit. For booster immunization, 0.5 mg of antigen were mixed with the same volume of incomplete Freund's adjuvant and injected four, eight and ten weeks after the priming immunization. Prior to the priming immunization and each booster immunization, the animal was bled and the titer of the serum was tested in immunoprecipitations and immunoblotting as described. The preimmune bleed was used as a control to ensure that the antibody activity detected in later bleeds was due to the immunization.

2.2.4.2 Purification of Antibodies

Polyclonal antibodies were immunopurified on an antigen column to isolate specific antibodies from a mixed pool. CNBr-activated sepharose 4B beads were swollen for 15 min in 1 mM HCl which preserves the activity of the reactive groups and washed on a sintered filter (porosity G3) with the same solution. The gel is then washed with coupling buffer (0.1 M $NaHCO_3$ pH 8.3, 0.5 M NaCl) and immediately transferred to solution of the ligand (GST-fusion protein used as antigen). About 5-10 mg protein were used per milliliter gel. The mixture was rotated end-over-end for 2 hours at room temperature. The beads were then pelleted and resuspended in 1 M ethanolamine pH 8.0 to block the residual active groups by adding an excess of small primary amino groups. After washing the beads three times with coupling buffer, they were transferred to a column. The column was first washed with 10 bed-volumes of 10 mM Tris-HCl pH 7.5, followed by 10 bed-volumes of 100 mM glycine-HCl pH 2.5 and 10 bed-volumes of 10 mM Tris-HCl pH 8.8. Then 10 bed-volumes of 100 mM triethylamine pH 11.5 were added and the column was equilibrated with 10 mM Tris-HCl pH 7.5 until the pH was stable. The polyclonal serum was centrifuged at 5'000 rpm for 5 min to remove any debris, diluted 1:10 in 10 mM Tris-HCl pH 7.5 and passed three times through the column. The column was then washed three times with 20 bed-volumes of 10 mM Tris-HCl pH 7.5 followed by 20 bed-volumes of 500 mM NaCl, 10 mM Tris-HCl pH 7.5. The antibodies that were bound by acid-sensitive interactions were eluted by passing 10 bed-volumes of 100 mM glycine-HCl pH 2.5 through the column and collected in 1 bed-volume 1 M Tris-HCl pH 8.0. The column was washed with 10 mM Tris-HCl pH 8.8 until the pH was stable and the remaining antibodies bound by base-sensitive interactions were eluted by passing 10 bed-volumes of 100 mM triethylamine pH 11.5 through the column and collected in 1 bed-volume 1 M Tris-HCl pH 8.0. If the column was to be reused it was washed with 10 mM Tris-HCl pH 7.5 and stored at 4°C. The antibody fractions were combined and dialyzed against PBS containing 0.02 % sodium azide.

2.2.5 Techniques of Mammalian Cell Culture

2.2.5.1 General Cell Culture Techniques

All used mammalian cell lines were cultured in incubators (Heraeus, B5060 Ek/CO2) at 5-7 % CO_2, 37°C and a saturated water vapor atmosphere. All cell culture work was performed aseptic in a sterile hood (Heraeus, Laminair). The cells were passaged regularly and the media was changed every other day. In addition, the culture were checked routinely for infection with mycoplasma. The cells were frozen in a media containing 90 % FCS and 10 % DMSO for long term storage in liquid nitrogen. A Neubauer counting chamber was used for counting the cells.

2.2.5.2 Test of Contamination with Mycoplasma

The contamination of cell cultures with mycoplasma influences the expression of cell surface proteins, the transduction of extracellular signals, the metabolism and other processes. Because of these influences on the experimental results the cultures were regularly checked for infection with mycoplasma. Subconfluent cells on 6 cm plates were fixed with methanol, washed twice with PBS and incubated with the DNA-staining reagent bisbenzimid (0.1 mg/ml in PBS) at 37 °C for 15 min. The cultures were then examined in a fluorescence microscope for the presence of mycoplasma.

2.2.5.3 Transfection of Mammalian Cells by Lipofectamine

Lipofectamine® is a polycationic transfection reagent. It binds DNA and its lipophilic property allows the transfer of DNA across the cell membrane into cells. Cos-7 cells were seeded at a density of 180'000 (80'000) cells/well in 6-well plates (12-well plate) 24 hours before transfection. Two solutions were prepared for the transfection: For solution A, 10 µl Lipofectamine® were diluted in 90 µl DMEM without serum (4 µl Lipofectamine® and 36 µl DMEM without serum) and 1.5 µg (0.8 µg) of plasmid DNA were diluted in 100 µl (40 µl) DMEM without serum for solution B. Both solutions were carefully mixed and incubated at room temperature for 45 min. The cells were washed once with 2 ml (1 ml) DMEM without serum during this incubation time. 800 µl (320 µl) of DMEM without serum were added to the transfection mixtures. The reaction was carefully mixed and added onto the cells, from which the media was removed before. After an incubation of 4 hours in the incubator 1000 µl (400 µl) DMEM with 20% FCS were added to the cells. The cells were incubated for another 12-20 hour. For starvation, the media was replaced with DMEM without serum overnight.

2.2.5.4 Transfection of Mammalian Cells by Calcium Phosphate-DNA Precipitation

A solution of calcium chloride, plasmid DNA and N-N-bis(2-hydroxyethyl)-2-amino-ethanesulfonic acid (BES) buffer is added to the a plate of cells. During a overnight incubation under an atmosphere of 3 % CO_2 a calcium phosphate-DNA precipitate forms. This precipitate adheres to the cell surface and is taken up by the cells. 0.5 x 10^6 cells/10 cm tissue culture plate were seeded in 5 ml complete medium the day before transfection. 10 µg plasmid DNA were diluted in 360 µl H_2O, 20 µl $CaCl_2$ and 200 µl 2xBBS (50 mM N-N-bis(2-hydroxyethyl)-2-amino-ethanesulfonic acid (BES), 280 mM NaCl, 1,5 mM Na_2HPO_4, pH 6.95) were added. The solution was vortexed and incubated at room temperature for 15 min. The calcium phosphate-DNA solution was added dropwise onto the medium-containing plate. The cells were then incubated at 3 %

CO_2 atmosphere for 12 - 24 hours. For starvation, the media was replaced with DMEM without serum for 24 hours.

2.2.5.5 Control of Transfection Efficiency

An expression plasmid of the β-galactosidase was transfected into the cells as described. 48 hours after transfection, the cells were washed once with PBS and fixed with 3% paraformaldehyd solution at room temperature for 10 min. The cells were washed twice with PBS and permeabilized with 0.2 % Triton X-100 in PBS for 10 min. After four wash steps with PBS, the transfected cells were stained with X-gal solution (2 mg/ml in DMSO) at 37°C for 60 min.

2.2.5.6 Retroviral Genetransfer into cells

Ecotrophic virus containing supernatant released from transfected BOSC 23 cells was used to infect NIH3T3 fibroblasts. After sterile filtration through 0.45 µm filter, the supernatant was added to subconfluent cells (10^5cells/6-cm dish) for 4 hours in the presence of polybrene (4 µg/ml). If necessary different dilutions or several infections were performed sequentially. After infection the media was changed and the cells were directly used for experiments or grown for at least 14 days in medium containing antibiotics for selection of stable cell line.

2.2.6 Metabolic Radiolabeling of Proteins

2.2.6.1 Radiolabeling with ^{35}S-Methionine

To label proteins radioactively with [^{35}S]-methionine, the cells were washed once with PBS and starved in DMEM without methionine and FCS for 24 hours. After the first 5 hours, 40 µCi/ml [^{35}S]-L-methionine were added to the media.

2.2.6.2 Radiolabeling with ^{32}P-Orthophosphate

To label proteins with [^{32}P]-phosphate, the cells were washed once with PBS and starved for 18 hours in DMEM without FCS. The cells were washed again with PBS, the media replaced with phosphate free DMEM and after 1 hour 150 µCi/ml [^{32}P]-orthophosphate were added for another 5 hours.

2.2.7 Purification of GST-Fusion Proteins

Eucaryotic proteins can be expressed in bacteria as glutathione-S-transferase (GST) fusion proteins. In many cases these fusion proteins are soluble and can be affinity purified with glutathion-sepharose after lysis of the bacteria. The purified fusion proteins can be used in precipitation experiments or for antibody production.

2.2.7.1 Expression of GST-Fusion Proteins

3 ml LB-media containing the required antibiotics were inoculated with 2-3 freshly transformed bacteria colonies at 37 °C overnight. The next morning 10 ml of this preculture were transferred into 1000 ml LB-media containing antibiotics and further incubated until the OD_{600} was between 0.4 - 0.5. The expression of the fusion proteins was induced by addition of isopropyl-β-thiogalactopyranoside (IPTG) to a final concentration of 1 mM IPTG, the bacteria were further grown for 3 hours and collected by centrifugation.

2.2.7.2 Purification of GST-Fusion Proteins

Bacteria from a 1000 ml liquid culture were collected by centrifugation at 6000 rpm for 5 min. The pellet was washed once with ice-cold PBS and resuspended in 10 volumes of ice-cold lysis PBS containing 0.5 mM EDTA, pH 8.0, 1 mM benzamidine, 150 ml aprotinin, 300 ml PMSF and 0.5 mM DDT. After disrupting the cell walls using a probe sonicator with a 2 mm-diameter probe, Triton X-100 was added to a final concentration of 1 % and the probe was incubated on ice for 10 min. The insoluble debris was removed by centrifugation at 10'000 g. The supernatant was incubated with 0.5 ml of a 50 % slurry of glutathion-agarose beads at 4 °C for 12 hours. The beads were washed three times with 50 ml ice-cold PBS, loaded onto a column and the fusion proteins were eluted with a buffer containing 50 mM Tris-HCl pH 8.0 and 5 mM reduced glutathion. The first three bed-volumes were collected and analyzed by SDS-PAGE. After the estimation of the concentration of the fusion protein with a BSA-standard, aliquots were frozen in 20 % glycerol at - 20 °C.

2.2.8 Precipitation and Detection of Proteins

2.2.8.1 Stimulation and Lysis of Cells

Different signal transduction pathways were investigated upon stimulation of cell with different growth factors. Before stimulation, the cells were starved in DMEM without serum for 18-24 hours. Cellular proteins were brought into solution with the detergent Triton X-100. This detergent lysis the cell membrane and leaves the nucleus and other intracellular compartments intact. Membrane and cytoskeletal fragments are pelleted by centrifugation and the supernatant is used for subsequent experiments. The cells were washed once with ice cold PBS and lysed on ice in an appropriate volume lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10 % glycerin, 5 mM EDTA pH 8.0, 1% Triton X-100), to which 10.0 µg/ml aprotinin, 1.0 mM PMSF, 100.0 mM sodiumfluorid, 10.0 mM p-nitrophenylphosphat 20.0 mM sodiumpyrophosphate, 2.0 mM sodium-orthovanadate pH 10.0 were freshly added. Alternatively, RIPA (Radio Immuno Protection Assay) buffer eas used in stead (1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate pH7.2, 2 mM EDTA, 50 mM sodium fluoride, and 0.2 mM sodium vanadate along with 100 U/ml aprotinin added freshly) was used. The volume was adjusted to the size of the cell culture plate. After 10 min the cell lysate was collected in an Eppendorff-tube and the insoluble fragments were pelleted by centrifugation (13'000 rpm, 4 °C, 10 min). The supernatant was collected in

an new Eppendorff-tube and used either for immunoprecipitation as described or as whole cell lysate.

2.2.8.2 Pierce BCA Protein Estimation Assay

The Micro BCA Protein Assay Kit from Pierce was used to determine the protein concentration of the crude cell lysates. The kit is based on the Biuret reaction, in which proteins react with Cu^{2+} in an alkaline medium to produce Cu^{1+}. The Cu^{1+} ions then react with bicinchonic acid (BCA) yielding a water-soluble, purple complex exhibiting a strong absorbance at 562 nm. The kit is not sensible to detergents (e.g. Triton X-100), which are used to lyse cells. The assay was performed in microtiter plates according to the manufacturer recommendation. A bovine serum albumin concentration series (100 - 2000 µg protein/ml) was used as standard.

2.2.8.3 Immunoprecipitation of Proteins

In immunoprecipitations antibodies, which were coupled to protein A-sepharose, were used for the enrichment of proteins from total cell lysate. Protein A is a membrane protein of the bacteria *Staphylococcus aureus*, which binds specifically to the Fc-domain of certains immunoglobulins (Chenais *et al.*, 1977). For the immunoprecipitation, the cell lysate was diluted with an equal volume of HNTG-buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10 % glycerin, 5 mM EDTA pH 8.0, 0.1 % Triton X-100), 30 µl 50 % protein A-sepharose and 1-5 µg monoclonal antibody or 2-10 µl polyclonal antibody were added. The samples were rotated at 4 °C for at least 4 hours end-over-end. The protein A-sepharose beads were pelleted by centrifugation (13'000 rpm) and washed three times with HNTG-buffer. After addition of 50 µl 2 x Laemmli-buffer, the beads were boiled at 100 °C for 5 min to denature the proteins.

2.2.8.4 *In vitro* Binding Assay with GST-Fusion Proteins

5 µg GST-fusion protein were incubated with 40 µl glutathion-sepharose in 600 µl HNTG-buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10 % glycerin, 5 mM EDTA pH 8.0, 0.1 % Triton X-100) and rotated end-over-end at 4 °C for 1 hour. The sepharose beads were pelleted by centrifugation (13'000 rpm) and washed once with HNTG-buffer. An equal volume of cell lysate and HNTG-buffer were added to the beads and the samples were processed as described in 2.2.11.3.

2.2.8.5 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Proteins

The SDS-polyacrylamide-gel electrophoresis allows the separation of proteins according to their molecular weight (Laemmli, 1970). The detergent sodium-dodecyl-sulfate binds and denatures proteins. The binding of the dodecyl-sulfate, results in a negative, total charge of the protein independent of the specific amino acid composition, but correlated to its molecular weight. The negative charge determines the migration of the protein through the gel. 7.5, 10 or 15 % SDS-polyacrylamide gels or gradient gels with a continuous polyacrylamide gradient from 7.5 to 12.5 % were used, dependent on the molecular weight of the proteins. The stacking gels contained 4 % polyacrylamide. The SDS-gel electrophoresis was performed as described in Current Protocol in Molecular Biology (Ausubel F. M., 1988). After electrophoresis, the gels were either stained as described in 2.6.7 the proteins were blotted onto nitrocellulose as described in 2.6.8. The following proteins were used as molecular weight markers:

Protein	Molecular Weight (kDa)
Myosin	200
β-galactosidase	116
Phosphorylase b	97
Bovine serum albumin	66
Ovalbumin	45
Carboanhydrase	29
Trypsin-inhibitor	21
Lysozyme	14

2.2.8.6 Staining and Fixation of Polyacrylamide Gels

The gels were stained for 5 min in Coomassie-solution (1 % Coomassie G250, 10 % acetic acid, 40 % methanol) and destained with 40 % methanol, 10 % acetic acid. The destained gels were placed on 3 MM Whatmann-paper and dried at 80°C under vacuum. If the proteins were radioactively labeled, the gels were exposed to films.

2.2.8.7 Transfer of Proteins onto Nitrocellulose Membrane

For immunodetection, the proteins were blotted onto nitrocellulose membranes. The transfer was performed with transblot-SD-buffer (50.0 mM Tris-HCl pH 7.5, 40.0 mM glycine, 20 % methanol, 3.75 x 10^{-5} % SDS) at 0.8 mA/cm^2 nitrocellulose membrane in a semidry blot aperture for 2 hours. After the transfer, the proteins were stained with Ponceau S (2 g/liter in 2 % TCA), the bands of the molecular weight standard were marked and the membrane was destained and blocked with 1 x NET-gelatine (50.0 mM Tris-HCl pH 8.0, 5 mM EDTA pH 8.0, 150 mM NaCl, 0.5 % Triton X-100 0.25 % gelatine).

2.2.8.8 Immunoblotting of Proteins (Western blot analysis)

Proteins, that are immobilized on nitrocellulose membranes can be detected with antibodies. In a first step the immobilised protein (antigen) is recognized by a specific monoclonal or polyclonal antibody (primary antibody). In a second step the primary antibody is recognized by a species specific secondary antibody, to which a horse radish peroxidase is coupled. In alkaline medium luminol (a diacylhydrazide) is oxidized by horse radish peroxidase and hydrogen peroxide. Immediately following oxidation, the luminol is in an excited state which then decays to ground state via a light emitting pathway, which can be detected by exposure to a film. The nitrocellulose membrane was incubated for one hour with three changes of 1 x NET-gelatine (50.0 mM Tris-HCl pH 8.0, 5 mM EDTA pH 8.0, 150 mM NaCl, 0.5 % Triton X-100. 0.25 % gelatine) to block unspecific binding sites of antibodies. The membrane was incubated with the primary antibody (1:500 - 1:10000 dilution in 1 x NET-gelatin) at room temperature for 3 hours or at 4°C overnight. After removal of the primary antibody, the membrane was washed 3 times for 20 min with 1 x NET-gelatine at room temperature and incubated with the secondary antibody for one hour. The membrane was washed 3 times for 20 minutes with 1 x NET-gelatine before detection with a ECL-kit from NEN. The membrane was incubated in a 1:1 mixture of both ECL-reagents for one minute. Afterwards, the membrane was wrapped with plastic film, and the air bubbles between the membrane and the plastic film were removed by gently smoothing with a tissue paper. The membrane was exposed to the film for different time periods. When the membrane should be reprobed with another antibody, the bound antibodies were

removed by stripping in 62.5 mM Tris pH 6.8, 100 mM β-mercaptoethanol, 2% (w/v) SDS at 50 °C for 1 hour.

2.2.9 *In vitro* Kinase Assay

2.2.9.1 *In vitro* Kinase Assay with Precipitated FRS2

Ovcar-3 cells were serum starved overnight and stimulation with 25 ng/ml EGF or aFGF. The lysates were subjected to immunoprecipitations with pre-immunserum or anti-FRS2 antibody as described in 2.2.8.3. After wash the immunoprecipitates 3 times with 0.75 ml HNTG-buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerin, 5 mM EDTA pH 8.0, 0.1% Triton X-100) and once equilibrated with 0.4 ml equilibration buffer (20 mM HEPES/NaOH pH 7.5, 1 mM DTT, 10 mM MgCl2, 0.2 mM orthovanadate), 30 µl reaction buffer (20 mM HEPES/NaOH pH 7.5, 1 mM DTT, 10 mM $MgCl_2$, 200 µM orthovanadate, 5 µM p-nitrophenylphosphate, 0.5 mg/ml myelin basic protein (MBP), 50 µM ATP) and 1 µCi [γ-^{32}P]-ATP were added to each precipitates. The samples were incubated in a shaker at room temperature for 10 min and the kinase reaction was stopped by addition of 20 µl 3 x Laemmli buffer. After SDS-PAGE, the gel was dried and detected by autoradiography.

2.2.9.2 *In vitro* Kinase Assay with Precipitated ERK2 MAP Kinase

The transiently expressed hemaglutinin-tagged MAP kinase HA-ERK2 was immunoprecipitated from cell lysat. The precipitates were washed 3 times with 0.75 ml HNTG-buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerin, 5 mM EDTA pH 8.0, 0.1% Triton X-100) and once equilibrated with 0.4 ml equilibration buffer (20 mM HEPES/NaOH pH 7.5, 1 mM DTT, 10 mM MgCl2, 0.2 mM orthovanadate). 30 µl reaction buffer (20 mM HEPES/NaOH pH 7.5, 1 mM DTT, 10 mM $MgCl_2$, 200 µM orthovanadate, 5 µM p-nitrophenylphosphate, 0.5 mg/ml myelin basic protein (MBP), 50 µM ATP) and 1 µCi [γ-^{32}P]-ATP were added to the precipitates. The samples were incubated in a shaker at room temperature for 10 min and the kinase reaction was stopped by addition of 20 µl 3 x Laemmli buffer. The probes were resolved by gradient (7-12.5%) SDS-PAGE. The gel was dried and detected by autoradiography.

2.2.9.3 *In Vitro* Kinase Assay with Purified ERK2 and MEK1

For each reaction, 1µg (7.5 pmol) of inactive ERK2 (unphosphorylated), 100ng (1.5 pmol) of active ERK2 (phosphorylated), 0.2µg (3 pmol)of inactive MEK1, and 125ng (1.75 pmol) active MEK1 were used according to the manufacture recommendation. Each of the purified ERK2 and MEK1 proteins was incubated with 2µg of purified GST-FRS2 fusion proteins in presence of 1 µCi [γ-^{32}P]-ATP in assay buffer (20 mM HEPES pH 7.5, 25 mM β-mercaptoethanol, 5 mM EGTA, 1 mM sodium ortovanadate, 1 mM DTT, 15 mM $MgCl_2$, 100µM ATP) for 30 min at 30°C. The activity of the purified kinases were controlled using myelin basic protein (MBP) as substrate for ERK2, and for MEK1 a two-step kinase assay were performed using unphosphorylated ERK2 as the first and MBP as second substrates. The reaction was stopped by addition

of Laemmli buffer, and the probes were solved by gradient (7-12.5%) SDS-PAGE. The gel was dried and followed by exposure of autoradiography.

2.2.9.4 *In-Gel* Kinase Assay

The lysates from stimulated and untreated cells were subjected to immunoprecipitation with anti-FRS2 antibody. The immunoprecipitates were then resolved by SDS-PAGE where 0.5 mg/ml myelin basic protein (MBP) was embedded as substrate. The gel was then fixed (20% isopropanol, 50 mM Tris pH 8.0) for 1 hour at room temperature, washed three times (50 mM tris pH8.0, 1 mM dithiothreitol), denatured (6 M guanidiniumhydrochloride, 20 mM dithiothreitol, 2 mM EDTA, 50 mM Tris pH 8.0) for 1 hour at room temperature, and renatured (50 mM Tris pH 8.0, 0.04% Tween 40, 1mM dithiothreitol, 2 mM EDTA) for 16 hours at 4°C. After 1 hour washing (40 mM HEPES pH 8.0, 1 mM dithiothreitol, 10 mM $MgCl_2$, 0.1 mM EGTA) at room temperature, the gel was incubated in kinase buffer (40 mM HEPES pH 8.0, 5 µCi/ml [γ-^{32}P]-ATP, 40 µM ATP, 10 mM $MgCl_2$, 0.1 mM EGTA) for 1 hour at room temperature. Finally, the gel was washed extensively in 5% trichloroacetic acid and 1% sodium pyrophosphate, followed by drying and autoradiography

2.2.10 Phosphoamino Acid Analysis

[^{32}P]-labeled phosphoamino acids can be identified by partial hydrolysis of the protein in HCl followed by two-dimensional thin layer electrophoresis. Cells were labeled with [^{32}P]-ortho-phosphate. After precipitation of the protein of interest, it was transferred to a polyvinyldifluorid membrane using the same technique as for immunoblotting (2.2.11.8). The band of interest was located by autoradiography and excised of the membrane with a clean razor bladder. The piece of membrane was submerged in 6 M HCl and incubated at 110 °C for 60 min. The liquid hydrolysate was transfered to a fresh tube and dried in a Speedvac evaporator. The sample was resuspended in 10 µl H_2O and spotted onto a cellulose thin-layer chromatography plate which was then carefully wetted with the respective electrophoresis buffer. A first dimension electrophoresis was performed at pH 1.9 (580 mM formic acid, 1360 mM glacial acetic acid) and 1.5 kV for 20 min, followed by a second dimension electrophoresis at pH 3.5 (870 mM glacial acetic acid, 0.5 % (v/v) pyridine, 0.5 mM EDTA) and 1.3 kV for 16 min. After the first run the plate was dried at 60 °C for 15 min rotated 90 ° counterclockwise for the second run. The plates were dried and exposed at - 80 °Cusing an intensifying screen.

2.2.11 Detergent-free Purification of Caveolin-rich Membrane Fractions

According to the method described by Song *et al.* (Song *et al.*, 1996). After two washes with ice-cold phosphate-buffered saline, Ovcar-3 cells (two confluent 150-mm dishes) were scraped into 2 ml of 500 mM sodium carbonate, pH 11.0. Homogenization was carried out sequentially in the following order using a loose-fitting Dounce homogenizer (10 strokes), a Polytron tissue grinder (three 10-s bursts), and a sonicator (three 20-s bursts). The homogenate was then adjusted to 45% sucrose by the addition of 2 ml of 90% sucrose prepared in MBS (25 mM Mes, pH 6.5, 0.15 M NaCl) and placed at the bottom of an ultracentrifuge tube. A 5-35% discontinuous sucrose gradient was formed above (4 ml of 5% sucrose/4ml of 35% sucrose; both in MBS containing 250 mM sodium carbonate) and centrifuged at 32,000 rpm for 20-22 h in an SW40T rotor (Beckman Instruments, Palo Alto, CA). From the top of each gradient, 1-ml gradient fractions were collected to yield a total of 13 fractions. Gradient fractions were separated by SDS-PAGE. A light-scattering band confined to the 5-35% sucrose interface was observed that contained caveolin but excluded most other cellular proteins.

2.2.12 Immunofluorescence

Immunofluorescence allows for an examination of the subcellular localization of proteins. Cells are first fixed, then permeabilised with a mild detergent to allow the antibody access to subcellular compartments within the cell. Unspecific binding sites are blocked with PBG, and then the cells are immersed in a solution that contains antibody recognising the desired protein (the primary antibody). The sample is washed a second time and incubated with a second antibody that recognises the primary antibody (the secondary antibody). This antibody is coupled to a fluorophore. The emitted light of the fluorophore betrays the subcellular localization of the target protein when examined by fluorescence microscopy while exposing the sample to light at the excitation wavelength of the fluorophore.
Adherent cell cultures were seeded on sterile, uncoated glass cover slips in a small cell culture well. At 50% confluency, the cells were washed with PBS and fixed in 4 % paraformaldehyde for 20 min at room temperature. Rinsed twice with PBS, the cells were permeabelized with 0.2 % Triton X-100 for ten minutes. After washing twice with PBS, the aldehyde groups were quenched with 100 mM glycin, 0.1 % $NaBH_4$ for 20 min. The cells were extensively washed with PBS and blocked for 60 min with 5 % BSA (bovine serum albumin), 2% NGS (normal goat serum), 0.1 % fish gelatine. The cells were incubated with the primary antibody (1:100 - 1:1'000) for 12 hours at 4 °C, or 2 hours at room temperature. The cells were washed again several times in PBS to remove residual unbound antibody and subsequently incubated with Cy3-konjugated secondary antibody (Molecular Probes, Leiden, Netherlands) for 2 hours at RT. All stages following application of the secondary antibody were performed in the dark. Cells were finally incubated with DAPI in PBS for 10 minutes at RT, the cells washed once more and mounted upside down onto a glass slide in Permafluor® (Molecular

Probes, Leiden, Netherlands). Samples were visualised and photographed with a Zeiss Axiovert X-100 Digital Confocal Microscope.

2.2.13 Computational Analysis of Sequences

Program	URL	Reference
BLAST	www.ncbi.nlm.nih.gov/BLAST	(Altschul *et al.*, 1990)
Prosite	www.expasy.org/prosite	(Sibbald *et al.*, 1991)
Scansite	www.scansite.mit.edu	(Yaffe *et al.*, 2001)

3. RESULTS

3.1 Phosphoprotein p90 Associates with SHP2

SHP2 is a ubiquitously expressed cytoplasmic tyrosine phosphatase and is involved in diverse signaling pathways initiated by growth factors (Adachi *et al.*, 1992; Freeman *et al.*, 1992; Ahmad *et al.*, 1993; Feng *et al.*, 1993; Lechleider *et al.*, 1993a; Vogel *et al.*, 1993). Via its tandem SH2 domains, SHP2 targets not only to activated RTKs, but also to tyrosyl-phosphorylated signaling intermediates, such as IRS-1, Gab1, Gab2, gp130 and SIRPs (Holgado-Madruga *et al.*, 1996; Kharitonenkov *et al.*, 1997; Symes *et al.*, 1997; Gu *et al.*, 1998).

In an attempt to identify interaction partners of SHP2, rat pheochromocytoma PC12 cells were stimulated with EGF, FGF, NGF and interleukin-6 (IL-6). SHP2 immunoprecipitates were analyzed for the presence of phosphotyrosine proteins (Figure 7). SHP2 of 68 kDa became tyrosine phosphorylated upon stimulation with any of the four ligands and coprecipitated several other proteins. The 170 kDa phosphoprotein in EGF or NGF stimulated cells was likely the receptor tyrosine kinase (RTK) of the respective ligand, since SHP2 is known to interact physically with a variety of ligand-activated RTKs (Feng *et al.*, 1993; Kazlauskas *et al.*, 1993; Lechleider *et al.*, 1993b; Vogel *et al.*, 1993). The tyrosine phosphorylated bands of 116 and 97 kDa are possibly the adaptor proteins Gab1 and Gab2, respectively (Holgado-Madruga *et al.*, 1996; Gu *et al.*, 1998). A phosphoprotein of 90 kDa coprecipitated with SHP2 upon FGF or NGF stimulation but not upon treatment with EGF or IL-6. This 90 kDa band resembled the protein SNT (suc1-associated neurotrophic target), which was reported as a 78-90 kDa polypeptide that is rapidly tyrosine phosphorylated only in response to neurotrophic factor stimulation in PC12 cells (Rabin *et al.*, 1993). Meanwhile, it was proposed that SNT might be identical to the protein designated FRS2 (FGF receptor substrate 2), which becomes phosphorylated on tyrosine residues and binds to Grb2 in response to FGF stimulation (Kouhara *et al.*, 1997). Thus, the observation of this SHP2-binding protein p90 raised the question whether this protein is FRS2/SNT.

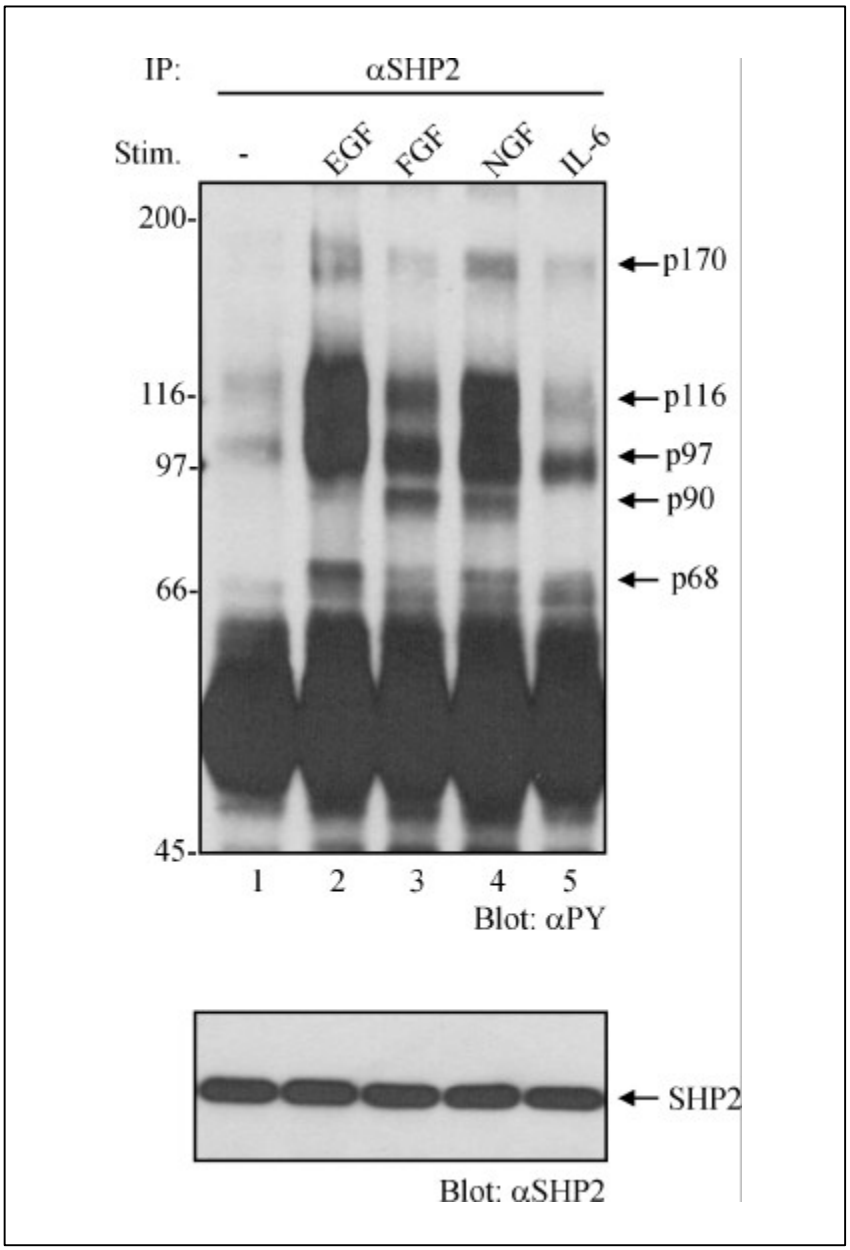


Figure 7: Phosphoprotein p90 associates with SHP2.
PC12 cells were serum starved overnight and stimulated with the indicated ligands for 5 min at 37°C or left untreated. The cell lysates were subjected to immunoprecipitation with anti-SHP2 antibody. The immunoprecipitates were resolved by SDS-PAGE followed by immunoblotting with anti-phosphotyrosine antibody (PY). Lane 1, untreated; lane 2, 50 ng/ml EGF; lane 3, 50 ng/ml FGF; lane 4, 50 ng/ml NGF; lane 5, 50 ng/ml interleukin-6 (IL-6). Molecular weights are indicated on the left in kilodaltons.

3.2 Molecular Cloning and Expression of Human FRS2

At the beginning of this study, only the murine FRS2 amino acid sequence was published (Kouhara *et al.*, 1997). As the focus of this study was the function of FRS2/SNT in human cells, a BLAST search (TBLASTN) was performed in order to identify the corresponding human DNA sequences. Specific oligonucleotide primers were designed according to the genomic DNA sequence obtained (GenBank access number U91327), and used for PCR amplification of human placenta cDNA. The full-length cDNA of human FRS2 was then cloned into expression plasmids pcDNA3 or pRK5. The sequence was checked by DNA sequencing and found to be identical with the sequence of human FRS2$_\alpha$/SNT-1 published later (GenBank access number AF036718). The open reading frame (ORF) of 1527 bp encodes a protein of 508 amino acids with a predicted molecular weight of 56 kDa. The amino acid sequences of murine and human FRS2 share 95% identity. FRS2 contains a consensus myristylation sequence (MGXXXS/T) (Resh, 1994) at the amino-terminus of the molecule (MGSCCS), as well as a stretch of 130 amino acid (residues 11-140) with 29% sequence identity to the phosphotyrosine-binding (PTB) domain of IRS-1 (O'Neill *et al.*, 1994). In addition, FRS2 harbours four binding sites for Grb2 (pYXNX), Tyr^{196}, Tyr^{306}, Tyr^{349}, Tyr^{392}, and two for SHP2, Tyr^{436} and Tyr^{471} (see Figure 9).

FRS2 cDNA was subcloned into a pcDNA3 expression plasmid, coding for the protein with a fused Flag-epitope at the C-terminus. Protein expression by this construct was checked in human embryo kidney (HEK) 293 cells by transient transfection. The cells were stimulated with sodium pervanadate or left untreated. The cell lysates were subjected to immunoprecipitations with anti-Flag antibody (Figure 8). Immunoblot with anti-phosphotyrosine and anti-Flag antibodies revealed that human FRS2 migrated at 92-95 kDa and was tyrosine phosphorylated in pervanadate treated cells. This is in agreement with earlier report on murine FRS2 (Kouhara *et al.*, 1997).

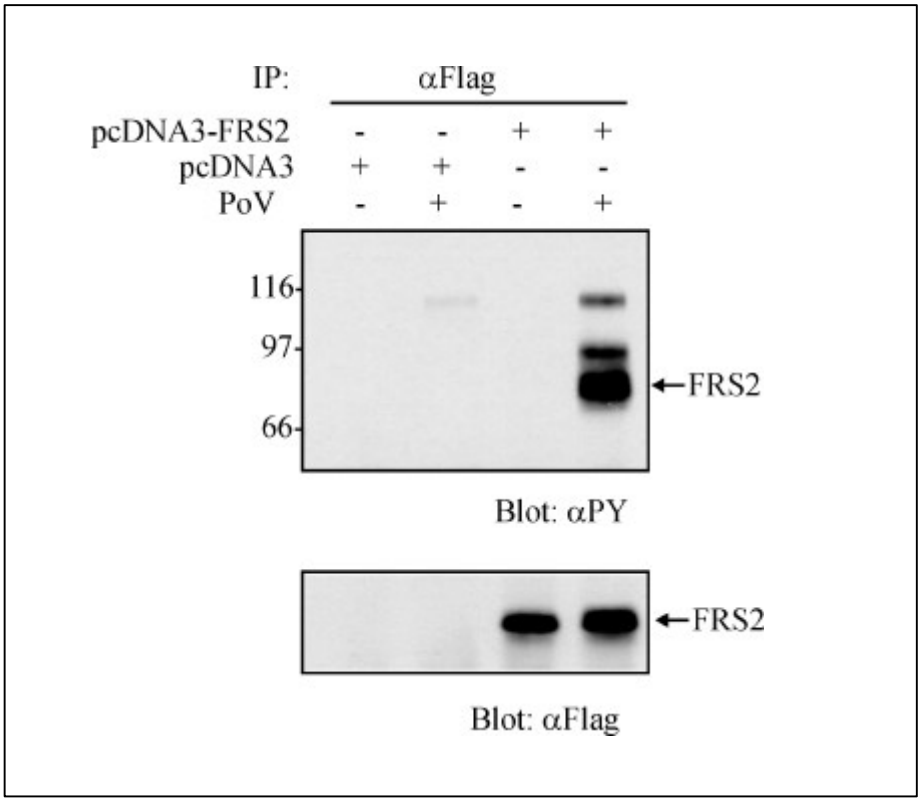


Figure 8: Expression of epitope-tagged FRS2 in HEK293 cells.
HEK293 cells were transfected with either pcDNA3-FRS2-Flag or the empty vector. The transfectants were serum starved overnight and stimulated with 100 nM sodium pervanadate (PoV) for 5 min at 37°C. Cell lysates were subjected to immunoprecipitations with anti-Flag antibody. The immunoprecipitates were resolved by SDS-PAGE followed by immunoblotting with anti-phosphotyrosine (PY) or anti-Flag antibodies. Molecular weights are indicated on the left in kilodaltons.

3.3 Production of Specific Anti-FRS2 Antibodies

3.3.1 Production of Anti-FRS2 Polyclonal Antibodies

Highly specific antibodies were necessary for biochemical characterization of endogenous FRS2. Thus, rabbit polyclonal anti-FRS2 antibodies were produced. Full-length FRS2 as well as two fragments encoding N-terminus and C-terminus were subcloned into pGEX vectors in frame with the vector encoded GST peptide: FRS2-WT

(aa 1-508), FRS2-NT (aa 1-240) and FRS2-CT (aa 326-508) (Figure 9). The GST-FRS2 fusion proteins were expressed in *Escherichia coli*, purified with glutathione sepharose and used as antigens for the immunization of rabbits.

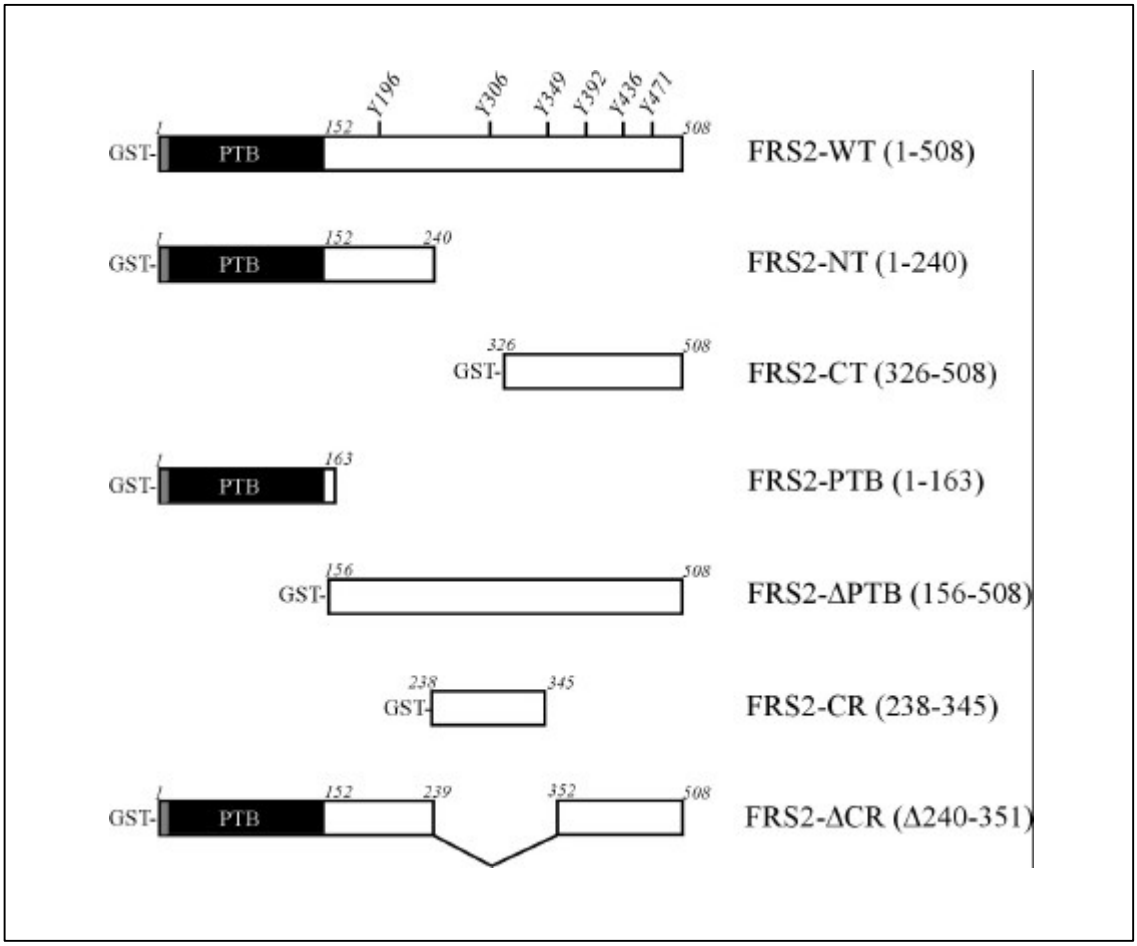


Figure 9: Schematic presentation of GST-FRS2 fusion proteins.
Schematic presentation of all GST-FRS2 fusion proteins used in this study. The myristylation site is shown as grey box, PTB domain as black box. The phosphotyrosine sites that serve as binding sites for Grb2 and SHP2 are also indicated.

3.3.2 Specificity Determination and Purification of the Antibodies

The specificity of the obtained antibodies were first checked in immunoprecipitation. BHK21 cells were transiently transfected with pRK-FRS2-WT or the empty vector and stimulated with sodium pervanadate or left untreated. Cell lysates

were subjected to immunoprecipitation with either pre-immunserum or anti-FRS2 antibodies and analysed by immunoblot for tyrosine phosphorylation. As shown in Figure 10, the anti-FRS2-CT antibody specifically recognizes the tyrosine phosphorylated FRS2 in both endogenous and overexpressed form when compared to the pre-immunserum. Results from other tests demonstrated that all three antibodies are able to precipitate endogenous FRS2, however, the anti-FRS2-NT and anti-FRS2-CT antibodies showed higher affinity toward the protein than the anti-FRS2-WT antibody (data not shown). Therefore the anti-FRS2-CT antibody was chosen for most of the subsequent experiments.

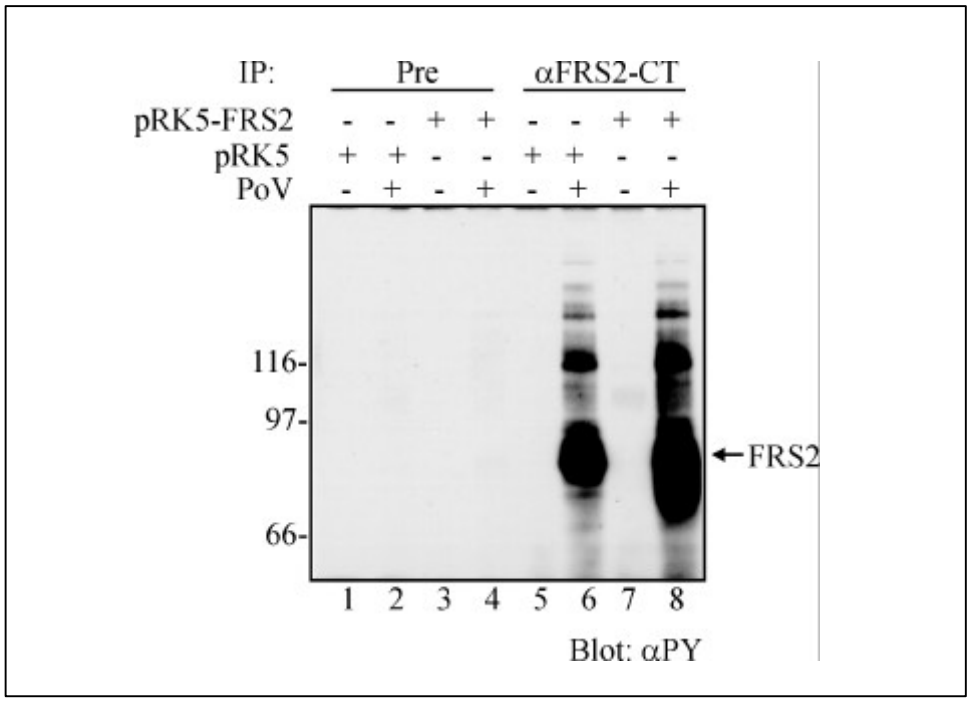


Figure 10: Test of anti-FRS2 antibody.
BHK21 cells were transiently transfected with pRK-FRS2 or the empty vector, stimulated with 100nM sodium pervanadate (PoV) for 5 min at 37°C. The cell lysates were subjected to immunoprecipitation with either pre-immunserum or anti-FRS2 antibody followed by immunoblot with anti-PY antibody. Molecular weights are indicated on the left in kilodaltons.

Since the antibodies in form of antisera failed to detect the endogenous FRS2 in immunoblot (data not shown), affinity purification was carried out with antigen columns containing GST-FRS2 fusion proteins coupled to CNBr-activated sepharose beads. The purified antibodies demonstrated the desired detection capability in

immunoblot (data not shown) and were used for all subsequent immunoblot analysis (see below).

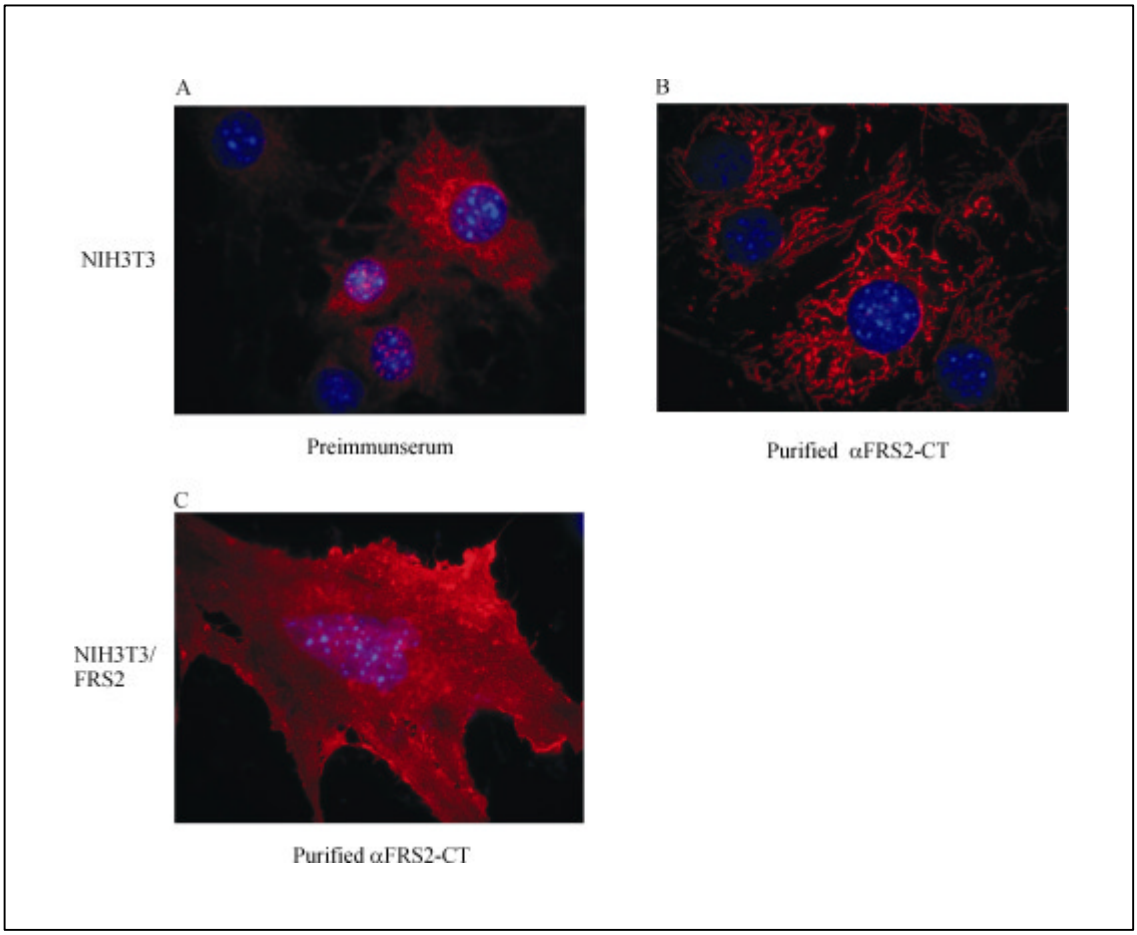


Figure 11: Immunofluoresence microscopy of FRS2.
(**A**) NIH3T3 cells were incubated with pre-immunserum or (**B**) purified anti-FRS2 antibody. (**C**) NIH3T3 cells were transiently transfected with pRK-FRS2 and incubated with purified anti-FRS2 antibody. Cell nuclei were stained with DAPI.

The antibodies were also tested for immunofluoresence microscopy (Figure 11). The images obtained from NIH3T3 cells incubated pre-immunserum or purified anti-FRS2-CT antibody displayed similar staining pattern (Figure 11A, B). These unspecific cross-reactions indicate that the purified antibody is not sufficient to detect endogenous FRS2. However, the antibody is capable of immunoflouresence detection when FRS2 is overexpressed in NIH3T3 cells. In accordance with the earlier report (Kouhara *et al.*, 1997), the protein is clearly localized to the plasma membrane of the transfected cells (Figure 11C).

3.4 p90/FRS2 Complexes with SHP2 and Grb2 upon TrkA Activation

FRS2 was identified as a tyrosyl phosphoprotein that coprecipitates with the adaptor protein Grb2 in FGFR signaling (Wang *et al.*, 1996; Kouhara *et al.*, 1997), while SNT was first detected as a $p13^{suc1}$-binding tyrosine phosphorylated protein downstream of the NGF receptor TrkA (Rabin *et al.*, 1993). To test whether FRS2 shares the suc1-binding properties of SNT, the expression plasmid pRK-FRS2-WT was transiently transfected into HEK293 cells followed by precipitation with $p13^{suc1}$-conjugated agarose beads which were reported to bind SNT (Rabin *et al.*, 1993; Wang *et al.*, 1996). As shown in Figure 12A, a tyrosine phosphorylated protein specifically precipitated with $p13^{suc1}$ from pervanadate treated, FRS2-transfected cells. The apparent molecular weight of 92-95 kDa indicated that SNT and FRS2 are likely identical.

To verify whether FRS2 also associates with Grb2 upon activation of TrkA, expression vectors for FRS2 along with wild-type or kinase-inactive TrkA were cotransfected transiently into HEK293 cells. As expected, the activated TrkA was able to phosphorylate FRS2 on tyrosine residues (Figure 12B, upper panel). Reblot with a specific anti-Grb2 antibody revealed an association of tyrosine phosphorylated FRS2 and Grb2 (Figure 12B, middle panel). This result indicates that in addition to FGF-induced signaling, FRS2 transmits signal from the NGF-activated TrkA by recruiting the adaptor protein Grb2. Thus, this is a further evidence indicating that FRS2 and SNT are the same protein.

As described before, the initial observation in this study was the association of a tyrosyl phosphoprotein p90 with SHP2 upon NGF and FGF stimulation (Figure 7). When the membrane was reprobed with anti-SHP2 antibody, the tyrosine phosphatase was readily detected along with tyrosine phosphorylated FRS2 (Figure 12B, bottom panel). Thus, the tyrosyl phosphoprotein p90 was FRS2. Together, these data establish that, upon activation of TrkA, FRS2 becomes tyrosine phosphorylated and is able to associate with the adaptor protein Grb2 as well as the tyrosine phosphatase SHP2. The interaction between FRS2 and SHP2 was confirmed later by others also in FGFR signaling pathway, where FRS2 was indirectly linked to Grb2 through SHP2 (Hadari *et al.*, 1998; Meakin *et al.*, 1999).

A minor tyrosine phosphorylated protein of 116 kDa that coprecipitated with FRS2, which was also observed in Figures 8 and 10, possibly corresponds to the docking protein Gab1. The relatively weak 116 kDa band from cells without exogenously expressed FRS2 was possibly Gab1 that coprecipitated along with the endogenous FRS2. However, immunoblot with a polyclonal antibody against Gab1 was not able to confirm this hypothesis (data not shown). This is possibly owing to the low efficiency of the available antibody, as an indirect association of FRS2 and Gab1 through Grb2 was reported later (Ong *et al.*, 2001).

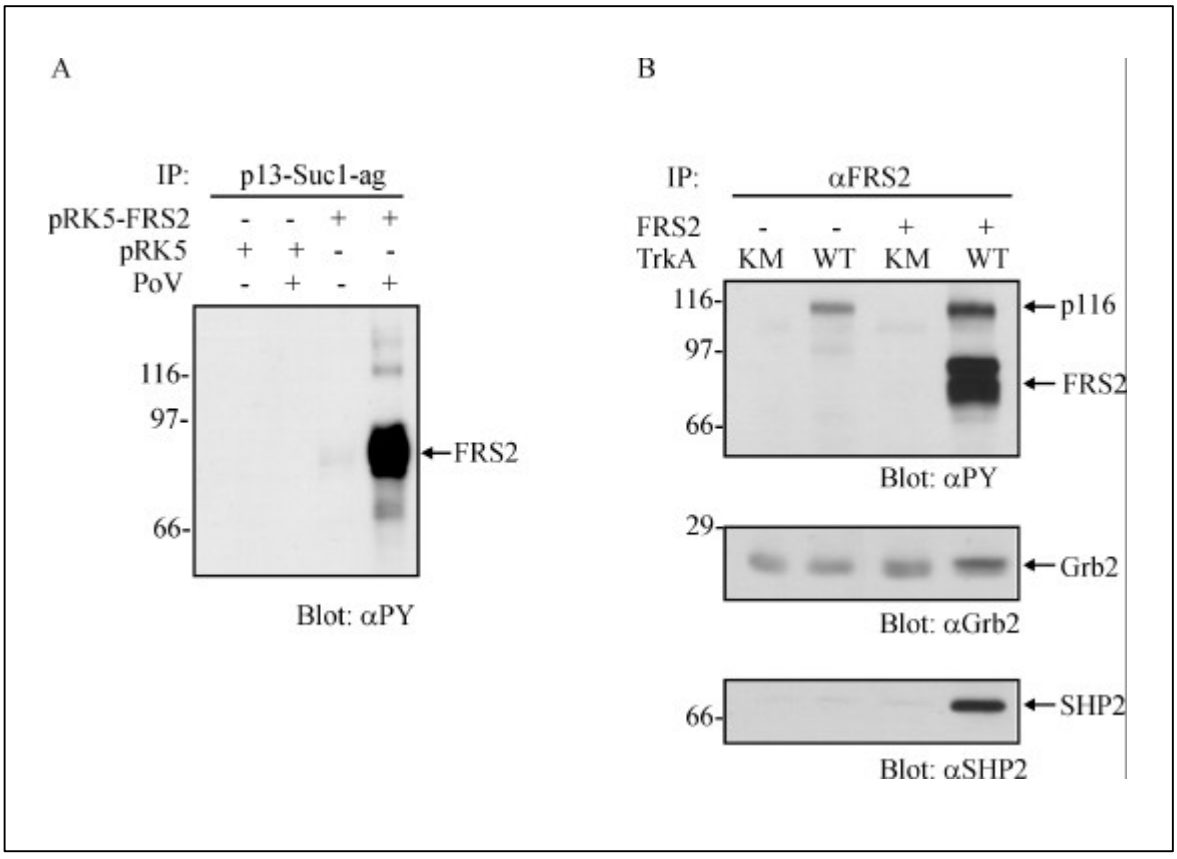


Figure 12: FRS2/SNT associates with Grb2 and SHP2 upon TrkA activation.
(**A**) HEK293 cells were transfected with either pRK5-FRS2 or the empty vector. The transfectants were serum starved overnight and stimulated with 100 nM sodium pervanadate (PoV) for 5 min at 37°C. Cell lysates were subjected precipitation with $p13^{Suc1}$ agarose conjugate. The precipitates were resolved by SDS-PAGE and transferred to nitrocellulose membrane followed by immunoblot with anti-PY antibody. (**B**) HEK 293 cells were cotransfected with pRK-FRS2 or the empty vector along with wild type (WT) or kinase inactive (KM) TrkA. The cell lysates were subjected to immunoprecipitation with anti-FRS2 antibody and followed by immunoblotting with the indicated antibodies. Molecular weights are indicated on the left in kilodaltons.

3.5 FRS2 is Ubiquitously Expressed and Associates with Multiple Proteins

3.5.1 Northern Blot Analysis of FRS2 in Mammary Carcinoma Cell Lines

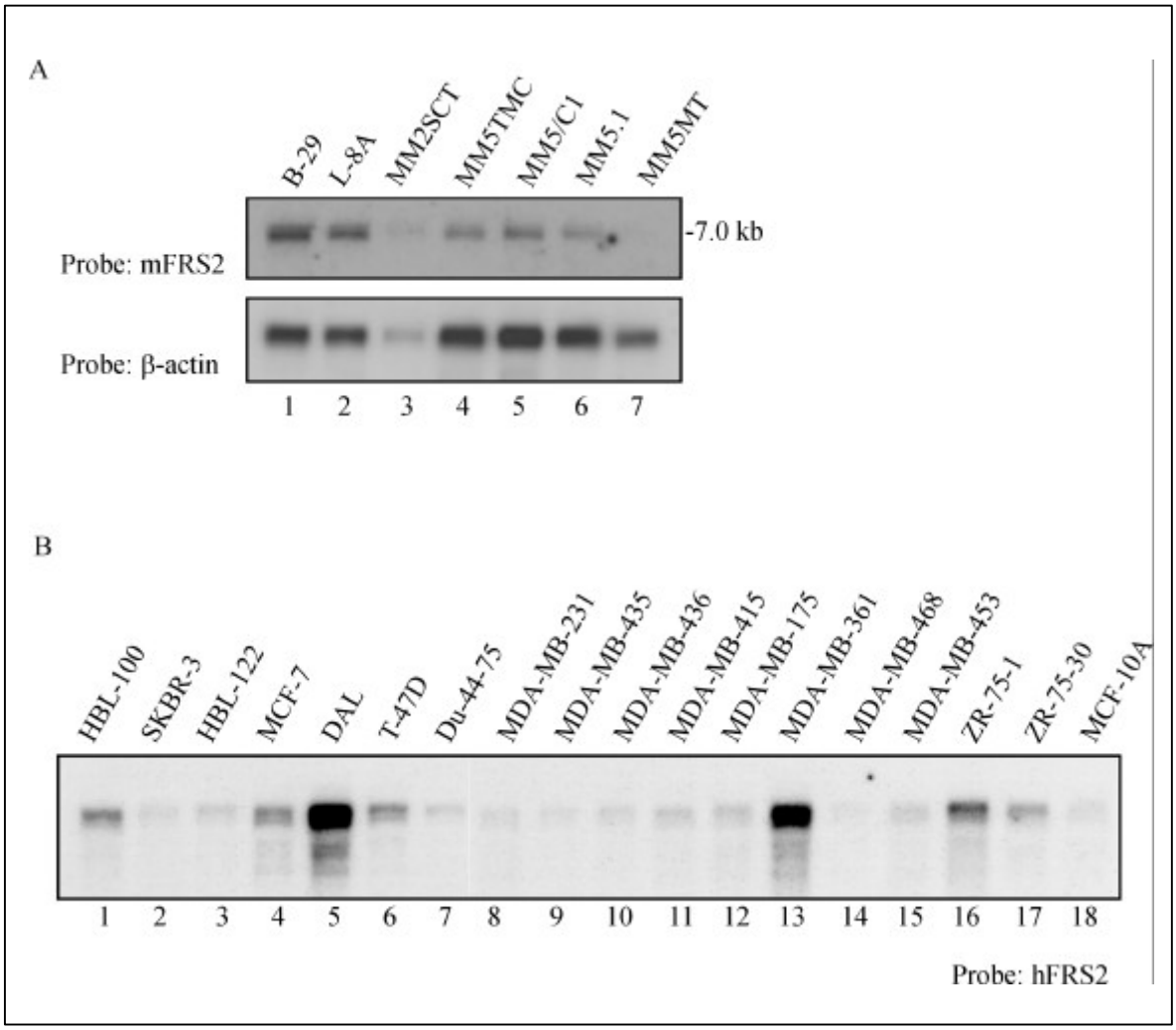


Figure 13: Northern blot analysis of FRS2 mRNA.
(**A**) Northern blot analysis of mRNA from 7 mouse mammary carcinoma cell lines with a PCR fragment of mouse FRS2 amplified from L-8A cDNA as probe. Rehybridazition of the membrane with a β-actin specific probe served as loading control. (**B**) Northern blot analysis of mRNA from 18 human mammary carcinoma cell lines with full-length human FRS2 cDNA as probe.

The tissue distribution of mouse FRS2 mRNA has been reported to be most abundant in brain, kidney, lung, ovary and testis (Kouhara *et al.*, 1997). In order to select a cell model, expression of endogenous FRS2 at the RNA level was examined. The mRNA from 7 mouse and 18 human mammary carcinoma cell lines was isolated and subjected to Northern blot analysis using PCR fragments of either murine or human FRS2 as probe. In 6 out of 7 mouse mammary carcinoma cell lines, FRS2 mRNA of 7 kb was detected at comparable levels (Figure 13A). In MM5MT cells (lane 7) FRS2 mRNA was not detectable.

In the human mammary carcinoma cell lines (Figure 13B), the FRS2 mRNA expression level was low in 11 out of 18 cell lines, whilst it was moderately expressed in 5 cell lines, i.e. HBL-100, MCF-7, T-47D, ZR-75-1 and ZR-75-30 (lane 1, 4, 6, 16, 17), and was highly expressed in 2 cell lines, DAL and MDA-MB-361 (lane 5 and 13). Comparable amounts of mRNA were loaded on the gel as visualized under UV-light after gel electrophoresis (data not shown).

In summary, transcription of the FRS2 gene was detected in the vast majority of the mammary carcinoma cell lines. Thus, FRS2 may be involved in growth regulation of these cells and variations of its expression levels may reflect differences in the transformation status of the individual tumor lines.

3.5.2 Detection of Tyrosine Phosphorylated Endogenous FRS2

In order to obtain an overview of the endogenously expressed FRS2, a total of 24 cell lines were analysed (listed in Material). The cells are of different tissue origins, including fibroblast, mammary carcinoma, ovarian adenocarcinoma, epidermoid carcinoma, glioblastoma, lung carcinoma, as well as hematopoietic cells. The cells were grown to 70-80% confluence, serum starved overnight and treated with sodium pervanadate for 5 min at 37°C or left untreated. Cell lysates were subjected to immunoprecipitation with both anti-FRS2-NT and CT antibodies. The immuno-precipitates were resolved by SDS-PAGE followed by immunoblotting with anti-PY antibody. The representative blots of human epidermoid carcinoma A-431, human ovarian adenocarcinoma Ovcar-3 and human teratocarcinoma NT-2 cells are shown in

Figure 14, where endogenous FRS2 migrated as a tyrosine phosphorylated fuzzy band of 92-95 kDa. Together with data from other cell lines (not shown), these results confirmed that FRS2 is ubiquitously expressed. The variations of the expression levels are cell line-dependent rather than tissue-specific.

3.5.3 Screening for Potential FRS2 Interaction Partners

In search of FRS2 interaction partners, several strategies were applied. The yeast 2-hybrid system, which is a sensitive method for protein-protein interaction studies, could not be employed. This was due to an auto-activation reaction where the bait (FRS2) protein/DNA-binding domain bound directly to the activation domain encoded by the pray vector thereby leading to transcription of the reporter gene. Thus, biochemical methods utilizing the specific anti-FRS2 antibodies as well as the GST-FRS2 fusion proteins were applied.

3.5.3.1 Coimmunoprecipitation with Endogenous FRS2

The expression analysis described in the previous section also revealed that FRS2 coprecipitated with several phosphoproteins (Figure 14). The pattern of association phosphoproteins varies in different cell lines, suggesting that FRS2 interacts with different partners depending on the cellular context. There are common interaction partners of FRS2, such as p68 and p116/118 that possibly represent SHP2 and Gab1, respectively. Moreover, depending on the antibodies used in immunoprecipitations, the pattern of FRS2-associated proteins differs within the same cell line. For example, in Ovcar-3 cells, two bands of 156 and 120 kDa are detected in the immunocomplex with the anti-FRS2-CT antibody that are not coprecipitated with the anti-FRS2-NT antibody. These variations may be due to the different epitopes recognized by each antibodies, or competition of interacting proteins and the antibodies for binding sites on FRS2.

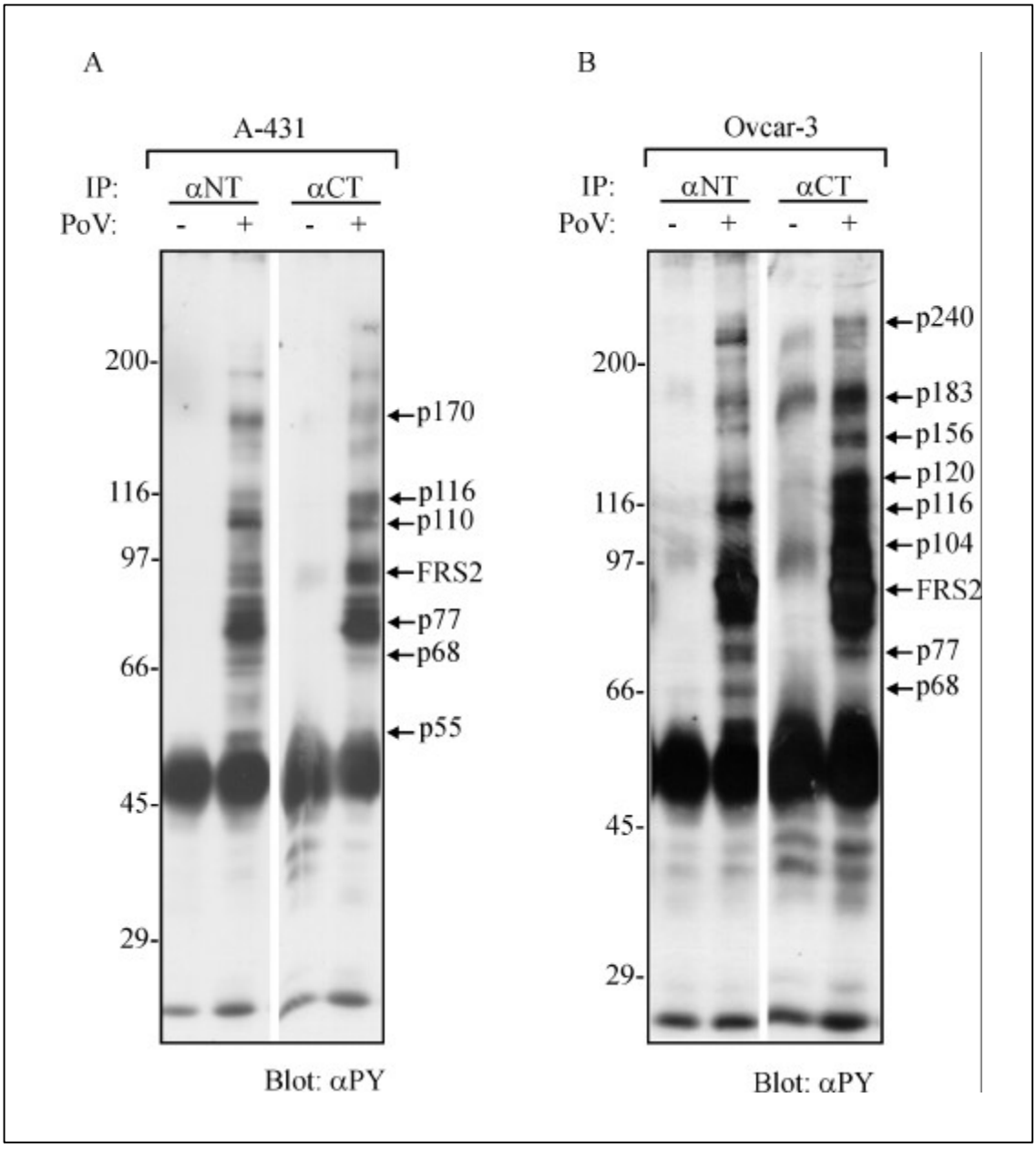
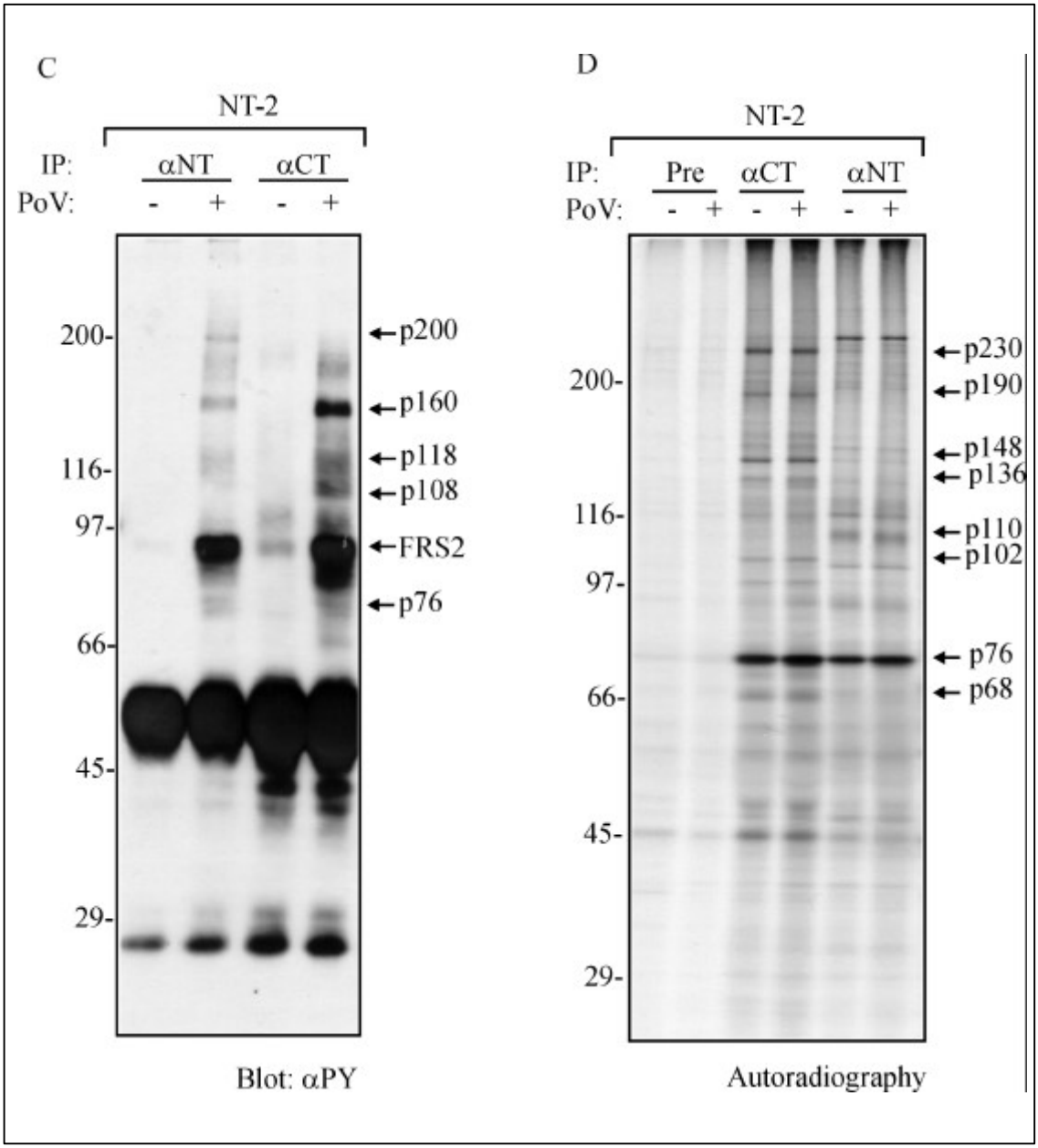


Figure 14: FRS2 is ubiquitously expressed in a variety of cell lines.
A-431 (**A**), Ovcar-3 (**B**), and NT-2 (**C**) cells were serum starved overnight and stimulated with 100 nM sodium pervanadate (PoV) for 5 min at 37°C. After lysis, the cell lysates were subjected to immunoprecipitations with anti-FRS2 antibodies. The immunoprecipitates were resolved by SDS-PAGE followed by immunoblotting with anti-phosphotyrosine (PY) antibody.



(**D**) NT-2 cells were serum starved overnight in methionine free medium supplied with 20 µCi/ml [^{35}S]-methionine, and stimulated with 100 nM sodium pervanadate for 5 min at 37°C, or left untreated. The lysates were subjected to immunoprecipitation with pre-immunserum (Pre), anti-FRS2-CT (αCT) or anti-FRS2-NT antibody (αNT). The immunoprecipitates were resolved by SDS-PAGE (7 %-12.5 %) followed by autoradiography. Molecular weights are indicated on the left in kilodaltons.

In order to study non-phosphoproteins in association with endogenous FRS2 that are not detectable on anti-PY immunoblots, A-431, NT-2, Ovcar-3, U-1242, PC12, MM5MTC, MM5.1 and MM5/C1 cells were metabolically labelled with ^{35}S-methionine

overnight, and either stimulated with sodium pervanadate or left untreated. The cell lysates were subjected to a two-step immunoprecipitation: first pre-clear with pre-immunserum then precipitation with anti-FRS2 antibodies. The precipitates were separated by SDS-PAGE followed by autoradiography. A representative result from human teratocarcinoma NT-2 cells is shown in Figure 14D. The autoradiography displayed a number of proteins in constitutive association with FRS2 independent of stimulation. The pattern of binding proteins again differ dependent on the antibody used. More bands are observed in the immunocomplex with the anti-FRS2-CT antibody including p230, p190, p148, p136 and p68, whereas p110 and p102 are only detected in the anti-FRS2-NT antibody immunoprecipitates. This suggests that different regions of FRS2 have different interaction partners. Additionally, in agreement with the results from the immunoblots, patterns of FRS2-associated proteins vary from cell line to cell line (data not shown).

3.5.3.2 In Vitro Binding Assay with GST-FRS2 Fusion Proteins

In further search of FRS2 interaction partners, *in vitro* binding assays with GST-FRS2 fusion proteins as affinity reagents were performed with lysates of several cell lines. The cells were either metabolically labelled with ^{35}S-methionine overnight or serum starved overnight without radioactivity, before stimulation either with sodium pervanadate or left untreated. The lysates were pre-cleared with glutathione sepharose prior to incubation with glutathione sepharose bound GST or GST-FRS2 fusion proteins as affinity reagents. The precipitates were analysed by SDS-PAGE followed by autoradiography or anti-PY immunoblot. The autoradiographs from ^{35}S-methionine labelled cells did not show any significant difference between treated and untreated cells (data not shown). In contrast, the immunoblot revealed several tyrosine phosphorylated proteins coprecipitated with different GST-FRS2 fusion proteins. The results from Ovcar-3 cells are presented in Figure 15. Since the fusion proteins represented different parts of FRS2, they bound to diverse proteins. GST-FRS2-NT associated p170 and GST-FRS2-CT associated p42 might correspond to EGFR and ERK2, respectively (Figure 15).

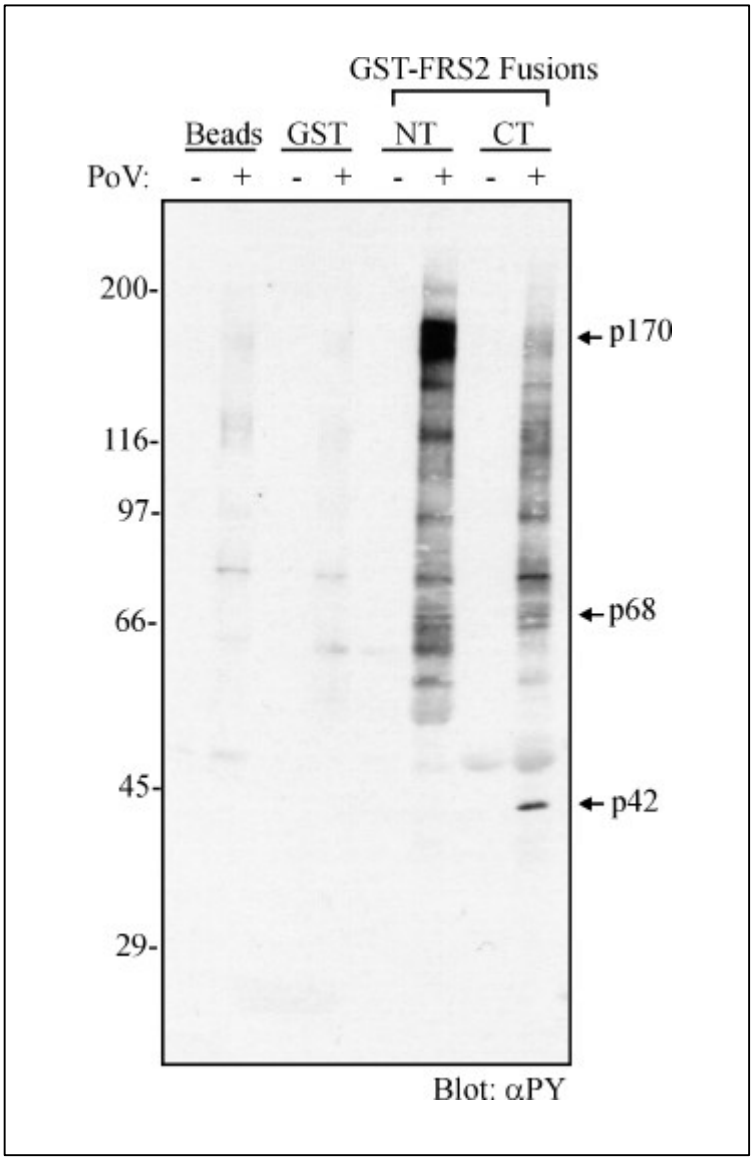


Figure 15: *In vitro* binding assay with GST-FRS2 fusion proteins.
Ovcar-3 cells were serum starved overnight and stimulated with 100 nM sodium pervanadate for 5 min at 37°C or left untreated. The cell lysates were pre-cleaned with glutathione sepharose alone before incubated with 1µg GST or GST-FRS2 fusion proteins bound to glutathione sepharose. Precipitates were resolved by SDS-PAGE followed by immunoblotting with anti-PY antibody. Molecular weights are indicated on the left in kilodaltons.

3.5.4 FRS2 Is Localized within Caveolae

Caveolae are vesicular invaginations of the plasma membrane. Functionally, caveolae were first implicated in cellular transport processes and more recently in signal transduction related events. The chief structural proteins of caveolae are the caveolins. Caveolins form a scaffold onto which many classes of signaling molecules can assemble to generate preassembled signaling complexes (Okamoto *et al.*, 1998; Couet *et al.*, 2001; Deurs *et al.*, 2003). These caveolin-interacting proteins include G-protein α subunits, Ras, Src family tyrosine kinases, EGFR, PDGFR, TrkA, insulin receptor, MAP kinases, PKA, and PKC isoforms (Kim and Kim, 1997; Liu *et al.*, 1997; Engelman *et al.*, 1998a; Engelman *et al.*, 1999; Smart *et al.*, 1999; Deurs *et al.*, 2003). In addition to concentrating these signal transducers within a distinct region of the plasma membrane, caveolin binding may functionally regulate the activation state of caveolae-associated signaling molecules (Okamoto *et al.*, 1998; Couet *et al.*, 2001; Razani and Lisanti, 2001; Anderson and Jacobson, 2002).

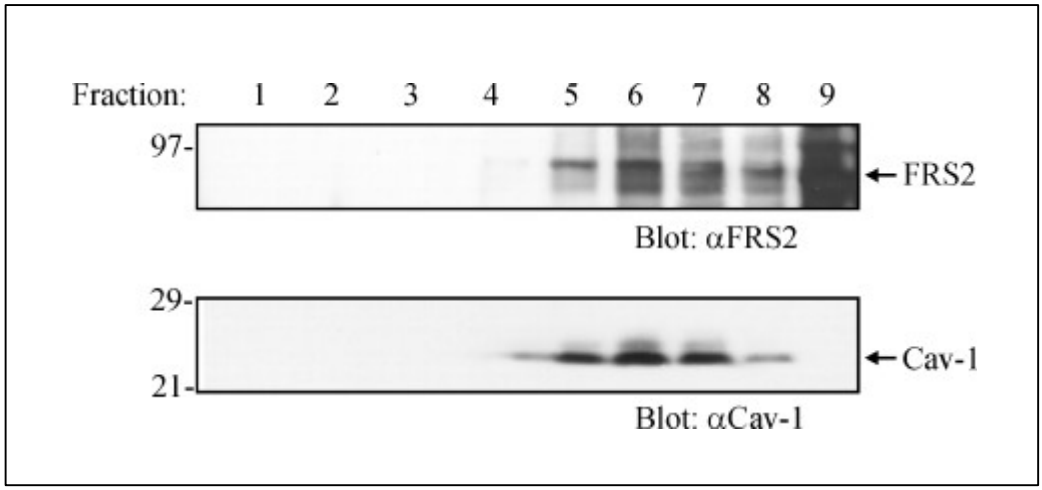


Figure 15: Caveolae localization of FRS2.
Ovcar-3 cells were used to prepare caveolin-enriched membrane fractions by detergent-free density-gradient ultra centrifugations as described in Methods. The fractions were resolved by SDS-PAGE followed by immunoblot with anti-FRS2 and anti-caveolin-1 (Cav-1) antibodies. Molecular weights are indicated on the left in kilodaltons.

FRS2 is anchored to cell membrane through its N-terminal myristylation and interacts directly with FGFR and TrkA. Thus, this docking protein is likely to be

localized within caveolae. To corroborate this hypothesis, Ovcar-3 cells were used to prepare caveolin-enriched membrane fractions by detergent-free density-gradient ultra centrifugations in presence of sucrose as described in Methods. Totally 13 fractions were obtained and analysed by SDS-PAGE followed by immunoblot with anti-FRS2 antibody. As shown in Figure 16, FRS2 was readily detected in fractions 5 to 8. Due to the high concentration of sucrose, proper separation of fractions 9-13 by SDS-PAGE was not possible (fractions 10-13 not shown). However, since the signal of FRS2 peaked in fractions 6 and 7, then declined substantially in fraction 8, it was not likely to be detected in the last fractions. Reblotting of the same membrane with a specific anti-caveolin-1 antibody revealed that this caveolae characteristic protein was present in the same fractions as FRS2. The EGFR was also found in these fractions (data not shown) which is in agreement with its reported caveolae localization (Engelman *et al.*, 1998b). Together, these data suggest that FRS2 is very likely located within caveolae, and therefore might interact with other signaling molecules present in this organelle. Similar findings were also reported by other research groups later (Davy *et al.*, 2000; Ridyard and Robbins, 2003).

3.6 FRS2 Mediates EGF-induced Signaling Pathway

3.6.1 EGF Induces Tyrosine Phosphorylation of FRS2

In the previous experiments attempting to identify FRS2 interaction partners, a phosphorylated protein of 170 kDa which might correspond to EGFR, was detected in different cell lines through different approaches. To investigate whether FRS2 is indeed involved in EGF-induced signaling pathway, the human epidermoid carcinoma line A-431 and NIH3T3 cells stably expressing EGFR (NIH3T3/EGFR) were treated with either EGF or acidic FGF (aFGF). Cell lysates were analysed by anti-FRS2 immunoprecipitation followed by immunoblotting. As expected, FRS2 became strongly tyrosine phosphorylated upon FGF stimulation in both cell lines (Figure 17, upper panel lane 3, 6). Interestingly, EGF also induced tyrosine phosphorylation of a 90 kDa protein (Figure 17, upper panel lane 2, 4) that was recognized by the anti-FRS2 antibody (Figure 17, bottom panel). Thus, EGF is able to induce a relatively moderate FRS2 tyrosine phosphorylation in addition to NGF, BDNF, FGF, GDNF and VEGF (Xu *et al.*, 1998; Easton *et al.*, 1999; Meakin *et al.*, 1999; Kurokawa *et al.*, 2001; Melillo *et al.*, 2001; Stoletov *et al.*, 2002).

3.6.2 FRS2 Complexes with SHP2, Grb2 and Activated EGFR

Tyrosine phosphorylated FRS2 is able to bind the tyrosine phosphatase SHP2 and the adaptor protein Grb2 upon stimulation with FGF or NGF (Figure 12) (Kouhara *et al.*, 1997; Ong *et al.*, 1997; Hadari *et al.*, 1998; Meakin *et al.*, 1999). Therfore, in response to EGF stimulation, the tyrosine phosphorylated FRS2 is likely to complex with these two molecules as well. The reblot with anti-SHP2 antibody clearly demonstrates that SHP2 coprecipitated with FRS2 in a ligand-stimulation-dependent manner (Figure 17, upper middle panels). Grb2 was also associated with tyrosine phosphorylated FRS2 (data not shown and see below).

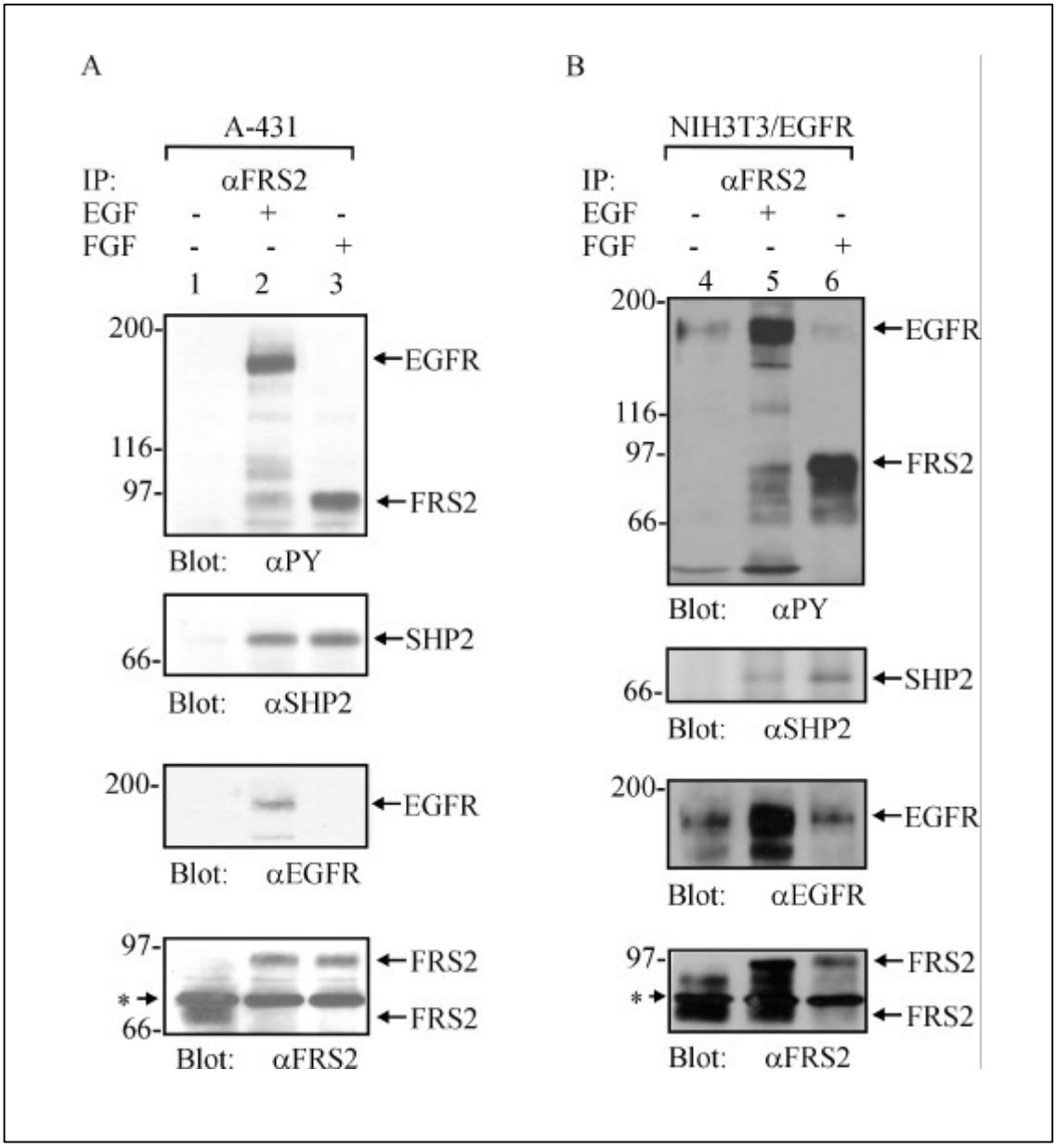


Figure 17:The activated EGFR induces FRS2 tyrosine phosphorylation.
(**A**) A-431 and (**B**) NIH3T3/EGFR cells were serum starved overnight and stimulated with 25 ng/ml EGF or aFGF for 5 min. The lysates were subjected to immunoprecipitations with anti-FRS2 antibody. The immunoprecipitates were resolved by SDS-PAGE followed by immunoblotting with indicated antibodies. Molecular weights are indicated on the left in kilodaltons. *Unspecific band due to an antibody cross-reaction with an unidentified protein.

In addition to FRS2, EGF induced tyrosine phosphorylation of several other proteins that coprecipitated with FRS2. The predominant 170 kDa tyrosine phosphorylated protein detected solely after EGF stimulation was readily recognized by an antibody against EGFR (Figure 17, lower middle panels). The amount of EGFR

coprecipitated with FRS2 increased substantially upon EGF stimulation, which indicates that tyrosine phosphorylation of the receptor is essential for the association of EGFR with FRS2. The minor tyrosine phosphorylated bands of 116 kDa and 54 kDa that were experimentally detected only in EGF stimulated NIH3T3/EGFR cells represent most likely adaptor proteins Gab1 and Shc, respectively, which are known to be tyrosine phosphorylated upon EGF stimulation and to coprecipitate with the activated EGFR (Ruff-Jamison *et al.*, 1993; Holgado-Madruga *et al.*, 1996).

In the FRS2 immunoprecipitates from FGF treated cells small amounts of coprecipitated EGFR were detected as in the unstimulated NIH3T3/EGFR control. In contrast to EGF stimulation there was no evidence for FGFR coprecipitation in either FGF treated A-431 or NIH3T3/EGFR cells, suggesting a lower affinity of the interaction of FRS2 with FGFR relative to that with the EGFR, since cDNA microarray analysis revealed that the receptors are expressed at comparable levels in the A-431 cells (data not shown). Interestingly, in addition to the inverse extent of association and tyrosine phosphorylation of EGFR and FGFR with FRS2, the amount of associated SHP2 in the anti-FRS2 immunoprecipitate was only moderately enhanced in FGF versus EGF stimulated cells. These observations suggested a clear and distinct involvement of FRS2 in EGFR and FGFR signal transduction.

Together, these results demonstrate for the first time that FRS2 undergoes EGF induced tyrosine phophorylation, and coprecipitates with the activated EGFR. Moreover, the tyrosine phosphorylated FRS2 is able to recruit the tyrosine phosphatase SHP2 and the adaptor protein Grb2. These results suggesting a role of FRS2 in EGF-induced signal transduction pathway.

3.6.3 FRS2 Associates with Activated EGFR through its PTB Domain

As shown in the last experiment, the activated EGFR associates with the tyrosine phosphorylated FRS2 upon EGF stimulation. To map the EGFR binding site of FRS2, bacterially expressed GST-FRS2 fusion proteins (Figure 9) representing the full-length FRS2 (GST-FRS2-WT), the N-terminal PTB domain (GST-FRS2-PTB), the truncation mutant lacking the PTB domain (GST-FRS2-ΔPTB), the N-terminal mutant (GST-

FRS2-NT), and the C-terminal mutant (GST-FRS2-CT) were compared for their ability to bind EGF. When these proteins were employed as affinity reagents for *in vitro* binding assays using A-431 cell lysates, GST-FRS2-WT, GST-FRS2-PTB and GST-FRS2-NT specifically bound to the ligand activated EGFR (Figure 18, lane 5, 8, 17), whereas GST alone or the fusion proteins lacking the PTB domain failed to associate with the receptor. It thus appeared that the PTB domain is responsible for the interaction of FRS2 with the activated EGFR, which is in agreement with the observation from the before described experiment in Ovcar-3 cells (Figure 15). This is also a result in accordance with findings reported for the association of FRS2 with receptor tyrosine kinases FGFR1 and TrkA (Xu *et al.*, 1998; Meakin *et al.*, 1999). In addition, the GST-FRS2 fusion proteins failed to "pull-down" FGFR, despite the reported constitutive association (Lin *et al.*, 1998; Xu *et al.*, 1998; Ong *et al.*, 2000) (data not shown).

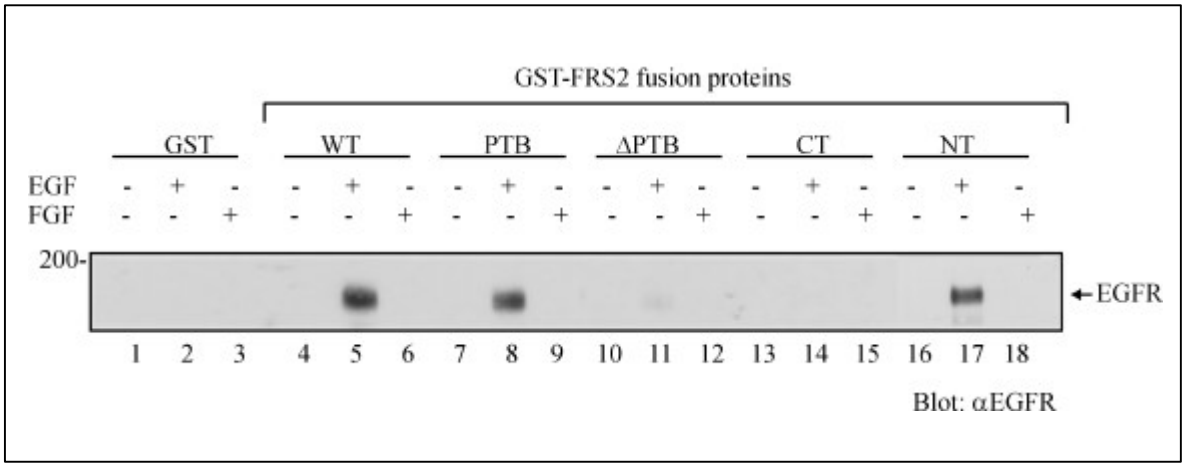


Figure 18: FRS2 binds to the activated EGFR through its PTB domain.
A-431 cells were serum starved overnight and stimulated with 25 ng/ml EGF or aFGF. The lysates were subjected to *in vitro* binding assays with 1µg glutathione sepharose bound GST or GST-FRS2 fusion proteins as indicated, resolved by SDS-PAGE and followed by immunoblotting. Molecular weight is indicated on the left in kilodalton.

3.6.4 FRS2 Enhances the MAP Kinase Activity in EGFR Signaling

It is well established the that tyrosine phosphorylated FRS2 links the FGF and NGF receptor activations to the Ras/MAPK signaling cascade. To examine the function of FRS2 in EGFR signaling, an *in vitro* MAP kinase assay was carried out after cotransfecting equal amounts of expression vectors for HA-tagged ERK2 along with increasing amounts of FRS2-FLAG vectors into Cos-7 cells which express detectable levels of endogenous EGFR. After stimulation with EGF or FGF, ERK2 was immunoprecipitated by anti-HA antibody. The precipitates were divided and either subjected to *in vitro* MAP kinase assay using myelin basic protein (MBP) as substrate, or resolved by SDS-PAGE to monitor the amount of expressed ERK2 (Figure 19A, upper and middle panels). Crude lysates were additionally immunoblotted with anti-Flag antibody (Figure 19A, bottom panels). As shown in Figure 19A, while the amount of transfected FRS2 increased, the MAP kinase activities elevated proportionally in response to both EGF and FGF stimulation. The results from two separate experiments summarized in Figure 19B demonstrate that FRS2 enhances both EGF and FGF induced ERK2 activation in a dose-dependent manner, indicating a positive role of FRS2 in relaying signals from the activated EGFR to the MAP kinase cascade. The moderate induction of ERK2 catalytic activity by FGF when compared to EGF is possibly due to the relatively low FGFR expression level in Cos-7 cells. Thus, FRS2 provides an alternative route in linking EGFR to the Ras/MAPK signaling cascade.

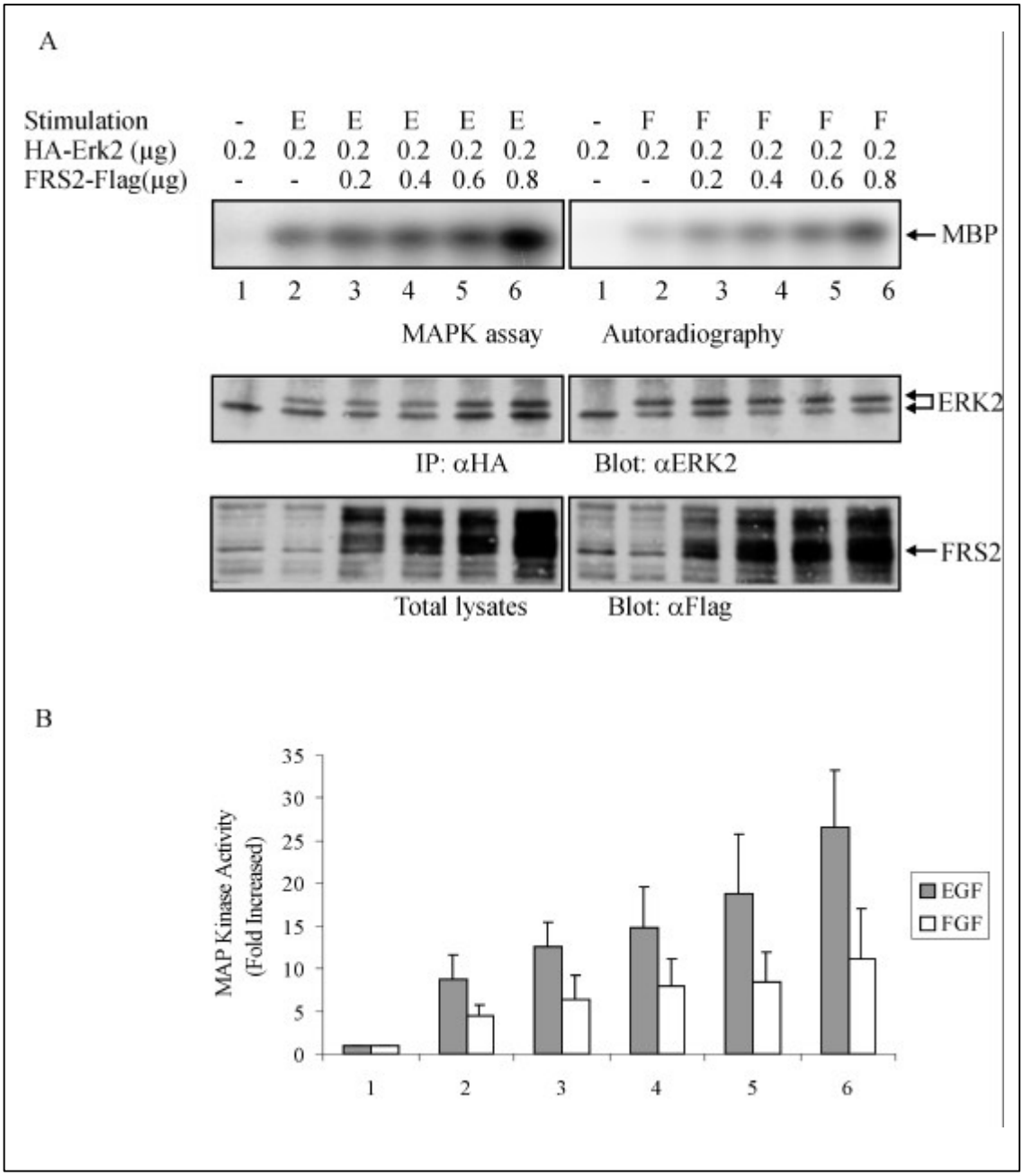


Figure 19: FRS2 enhances EGF- and FGF-induced MAP kinase activity.
(**A**) Cos-7 cells in 6-well plates were cotransfected with 0.2 µg HA-ERK2 and 0.2-0.8 µg FRS2-Flag, stimulated with 25 ng/ml EGF or aFGF. The cells were lysed and the lysates subjected to immunoprecipitation with anti-HA antibody followed by MAP kinase assays using MBP as substrate (upper panel). Equal amounts of lysates were also subjected to immunoprecipitation with anti-HA antibody followed by immunoblotting with anti-ERK antibodies (middle panel). Total crude lysates were resolved by SDS-PAGE followed by immunoblotting with anti-Flag antibody (bottom panel). (**B**) Quantification of *in vitro* MAP kinase assays.

3.7 FRS2 is Serine/Threonine Phosphorylated

3.7.1 Different Growth Factors Induce FRS2 Migration Shift

Upon EGF and FGF stimulation, a significant alteration of FRS2 electrophoretic mobility in SDS-PAGE was observed in Figure 17 (bottom panel). In untreated cells, FRS2 has an apparent molecular weight of 70 to 75 kDa, which significantly shifted to 92-95 kDa in EGF or FGF treated cells. This migration shift of FRS2 was mediated by the RTK activation of the respective growth factors, since it was completely abolished by pre-incubation of cells with the specific RTK inhibitors AG1478 against EGFR and SU5402 against FGFR (data not shown).

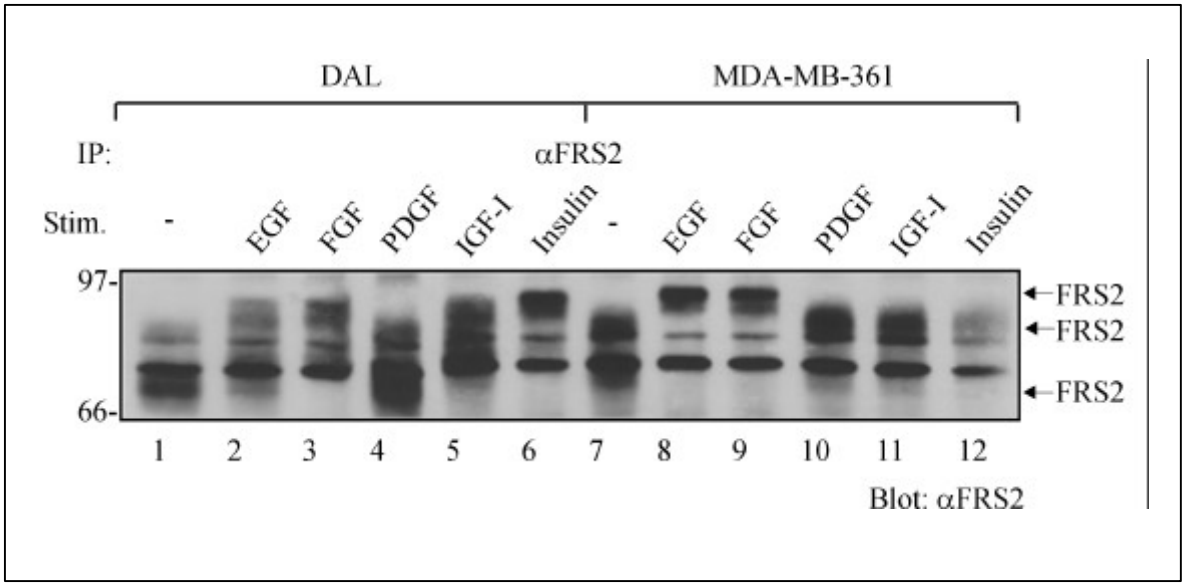


Figure 20: Induction of FRS2 migration shift by multiple growth factors.
DAL and MDA-MB-361 cells were serum starved overnight and stimulated with different ligands for 5 min at 37°C. The lysates were subjected to immunoprecipitations with anti-FRS2 antibody. The immunoprecipitates were resolved by SDS-PAGE followed by immunoblotting with anti-FRS2 antibody. Lane 1 and 7, untreated; lane 2 and 8, 25 ng/ml EGF; lane 3 and 9, 25 ng/ml aFGF; lane 4 and 10, 50 ng/ml PDGF; lane 5 and 11, 50 ng/ml IGF-I; lane 6 and 12, 100 nM insulin. Molecular weights are indicated on the left in kilodaltons.

To explore the effects of other growth factors on FRS2 migration shift, two human mammary carcinoma cell lines, DAL and MDA-MB-361, that express FRS2 at relatively high level (Figure 13B), were stimulated with EGF, heregulin, FGF, PDGF, IGF-I or insulin. As shown in Figure 20, FRS2 had an apparent molecular weight of 72-

75 kDa in untreated DAL cells, while it migrated as an 80-85 kDa protein in untreated MDA-MB-361 cells. This observation of various electrophoretic mobilities of FRS2 in different cell lines suggests that FRS2 is differentially modified in a cell line-dependent manner. While EGF and FGF induced FRS2 migration shift in both cell lines, IGF-1 and insulin were only able to alter FRS2 migration in DAL cells (Figure 20, lanes 5 and 6), but not in MDA-MB-361 cells (lanes 11 and 12). PDGF had no significant effect on FRS2 in either cell line (lanes 4 and 10), which may be due to a low or even lack of the PDGFR expression. In contrast, PDGF was able to induce FRS2 migration shift in U-1242 cells (data not shown), a human glioblastoma lineage known to express the PDGFR (Farooqui *et al.*, 1999). Moreover, IGF-I and insulin induced FRS2 migration shift was also observed in A-431 and Ovcar-3 cells (data not shown). FRS2 thus is involved in considerably more signal transduction pathways than previously thought.

3.7.2 FRS2 Associates with a Kinase that Phosphorylates MBP

The observation of the FRS2 migration shift upon stimulation with multiple growth factors further emphasized the need to identify the underlying modification. Although abolished by RTK inhibitors, the extent of this shift is so drastic that it is unlikely to be caused solely by tyrosine phosphorylation. Immunoblot with specific anti-ubiquitin antibody showed that the low FRS2 gel mobility was not due to ubiquitination (data not shown). Additionally, deglycosylation treatment of the FRS2 immunocomplexes with endoglycosidase had no effect on the shift (data not shown). Another plausible explanation of this ligand-induced post-translational modification of FRS2 is phosphorylation on serine and threonine residues.

According to computational analysis of the protein sequence with Scansite (Yaffe *et al.*, 2001) and Prosite (Sibbald *et al.*, 1991), FRS2 contains more than 30 potential serine/threonine phosphorylation sites for PKA, PKG, PKC, PKB, and CaMKII throughout the protein (see Figure 25). Thus, the remarkable shift in electrophoretic mobility after growth factor stimulation shown in Figure 17 and 20 is possibly caused by serine/threonine in addition to tyrosine phosphorylation.

This hypothesis was first examined by determining whether FRS2 complexes with a serine/threonine kinase in immunoprecipitation. An *in vitro* kinase assay was performed with lysates from Ovcar-3 cells stimulated with either EGF or FGF. After immunoprecipitation with the anti-FRS2 antibody or pre-immunserum, the immunoprecipitates were subjected to *in vitro* kinase assay with myelin basic protein (MBP) as substrate. It is known that MBP serves as a substrate for many serine/threonine kinases including PKC, PKA, and MAPK (Vartanian *et al.*, 1989; Rossomando *et al.*, 1991; Eyster *et al.*, 1993). Indeed, the autoradiography in Figure 21 revealed that MBP became significantly phosphorylated when incubated together with anti-FRS2 immunoprecipitates. Moreover, specific immunocomplexes of EGF or FGF stimulated samples showed an elevated MBP phosphorylation as compared to FRS2-precipitates of untreated cells. These data thus demonstrate that a serine/threonine kinase might be constitutively associated with FRS2 and become activated upon ligand stimulation, suggesting that it might phosphorylate FRS2 on serine/threonine residues.

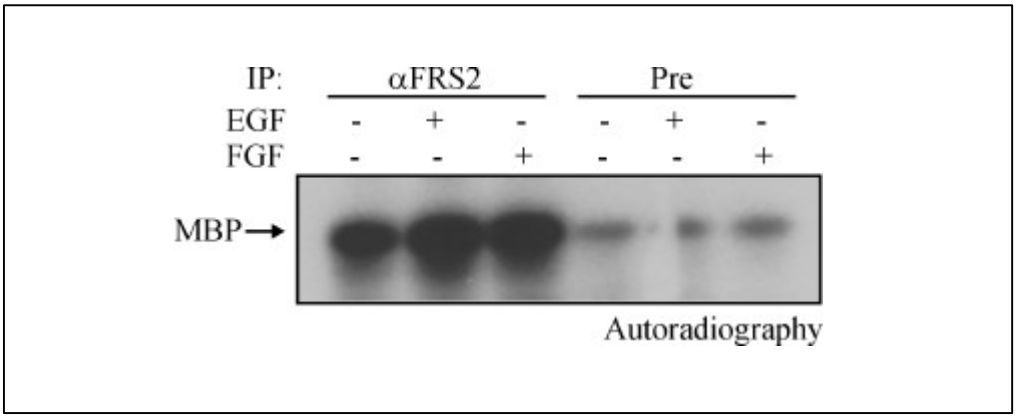


Figure 21: FRS2 complexes with a kinase that phosphorylates MBP.
Ovcar-3 cells were serum starved overnight and stimulated with 25 ng/ml EGF or aFGF. The lysates were subjected to immunoprecipitations with pre-immunserum or anti-FRS2 antibody. The immunoprecipitates were then subjected to an *in vitro* kinase assay with MBP as substrate. After SDS-PAGE, the gel was dried and phosphorylated MBP was detected by autoradiography.

3.7.3 EGF and FGF Induce Serine/Threonine and Tyrosine Phosphorylation of FRS2

In addition to the above described results and computational prediction of phosphorylation sites, another two lines of evidence support the hypothesis of FRS2 serine/threonine phosphorylation. First, it is known that inhibition of serine/threonine phosphatases by okadaic acid or calyculin A leads to activation of a number of serine/threonine kinases, and subsequent phosphorylation of their substrates (Ishihara *et al.*, 1989; Gopalakrishna *et al.*, 1992; Song and Lavin, 1993). Incubation of unstimulated cells with calyculin A resulted in retarded FRS2 electrophoretic mobility, implying that FRS2 might be a substrate of serine/threonine kinases (data not shown). Second, treatment of immunoprecipitated FRS2 with alkaline phosphatase abolished the ligand-induced migration shift (data not shown), confirming that this shift might be due to serine/threonine phosphorylation.

To corroborate this hypothesis, phosphoamino acid analysis of FRS2 was carried out with A-431 cells that were metabolically labelled with ^{32}P-orthophosphate and treated with EGF or FGF. The lysates were subjected to immunoprecipitation with anti-FRS2 antibody and resolved by SDS-PAGE followed by transfer to polyvinylidene difluoride (PVDF) membrane. After autoradiography, the band of interest was cut out and subjected to acid hydrolysis as described in Methods. The phosphoamino acids were then separated by 2-D thin-layer electrophoresis and detected by autoradiography. As shown in Figure 22A, FRS2 appeared as a diffuse band on SDS-PAGE with an apparent molecular weight of 70-75 kDa in untreated cells, and shifted to 90-95 kDa after EGF or FGF stimulation. The subsequent phosphoamino acid analysis revealed that in untreated cells FRS2 was constitutively phosphorylated on serine as well as threonine residues, but not on tyrosine residues. Upon stimulation with either EGF or FGF, extensive phosphorylation of FRS2 on serine and threonine residues and relatively low levels of tyrosine phosphorylation were observed (Figure 22B).

Thus, analysis of FRS2 phosphorylation *in vivo* further supports the hypothesis that the migration shift of FRS2 is largely the result of enhanced phosphorylation on serine and threonine residues.

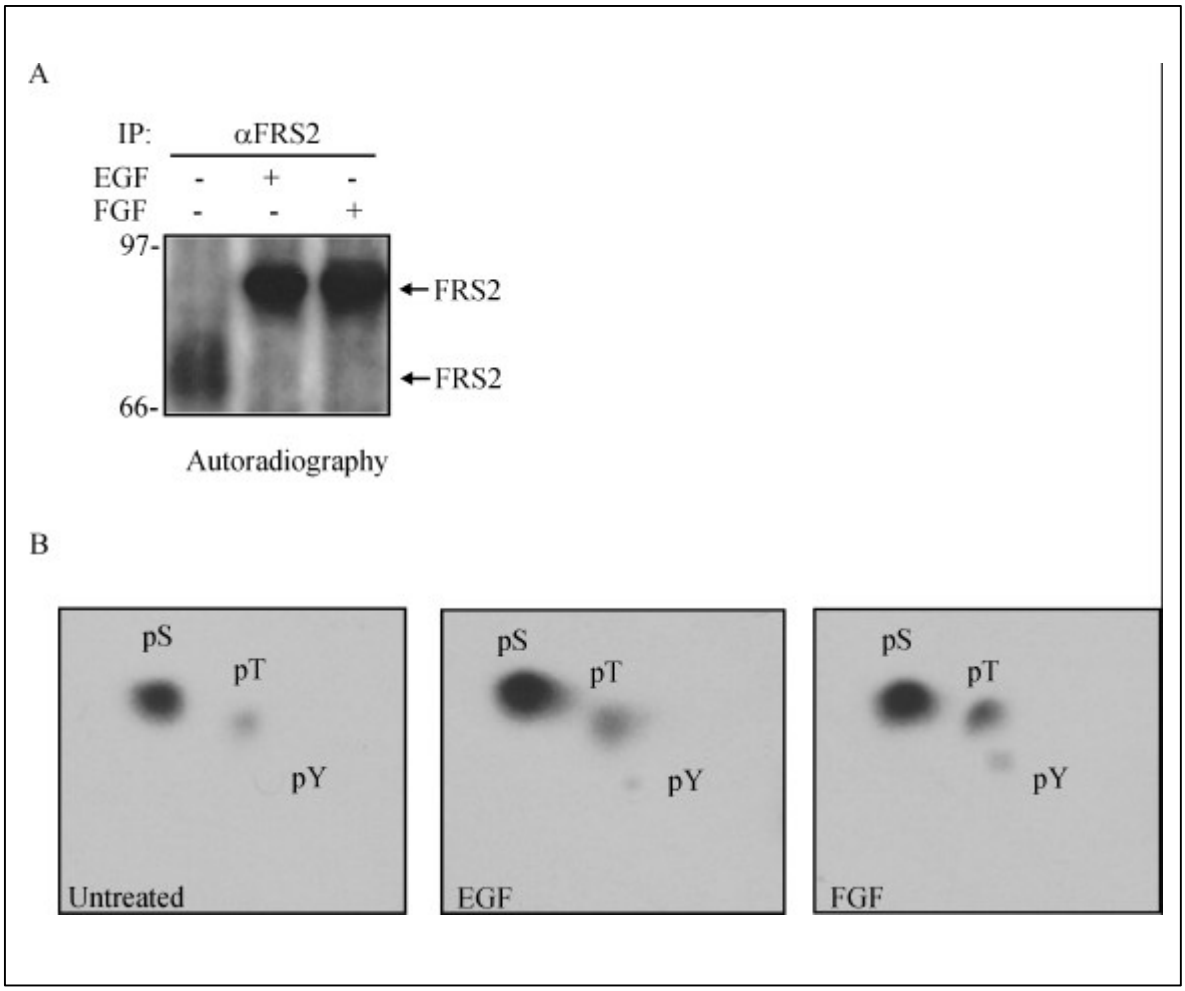


Figure 22: FRS2 is phosphorylated on both serine/threonine and tyrosine residues. A-431 cells were serum starved overnight and metabolically labeled with 150 μCi/ml ^{32}P-orthophosphate in phosphate and serum free medium for 4 hours and stimulated with 25 ng/ml EGF or aFGF for 5 min or left untreated. The lysates were subjected to immunoprecipitations with anti-FRS2 antibody. (**A**) The immunoprecipitates were resolved by SDS-PAGE, transferred to PVDF membrane and deteced by autoradiography. Molecular weights are indicated on the left in kilodaltons. (**B**) The specific bands were excised and subjected to phosphoamino acid analysis by two-dimensional thin layer eletrophoresis as described in Method.

3.7.4 Pharmacological Inhibition of FRS2 Migration Shift

In order to identify regulator of FRS2 serine/threonine phosphorylation, a set of enzyme inhibitors were utilized in pharmacological interference experiments (summarized in Table 1). Briefly, as outlined in Figure 23, Ovcar-3 cells were stimulated in the presence of the inhibitors and the FRS2 migration shift was analysed by immunoprecipitation followed by immunoblotting with the FRS2-specific antibody.

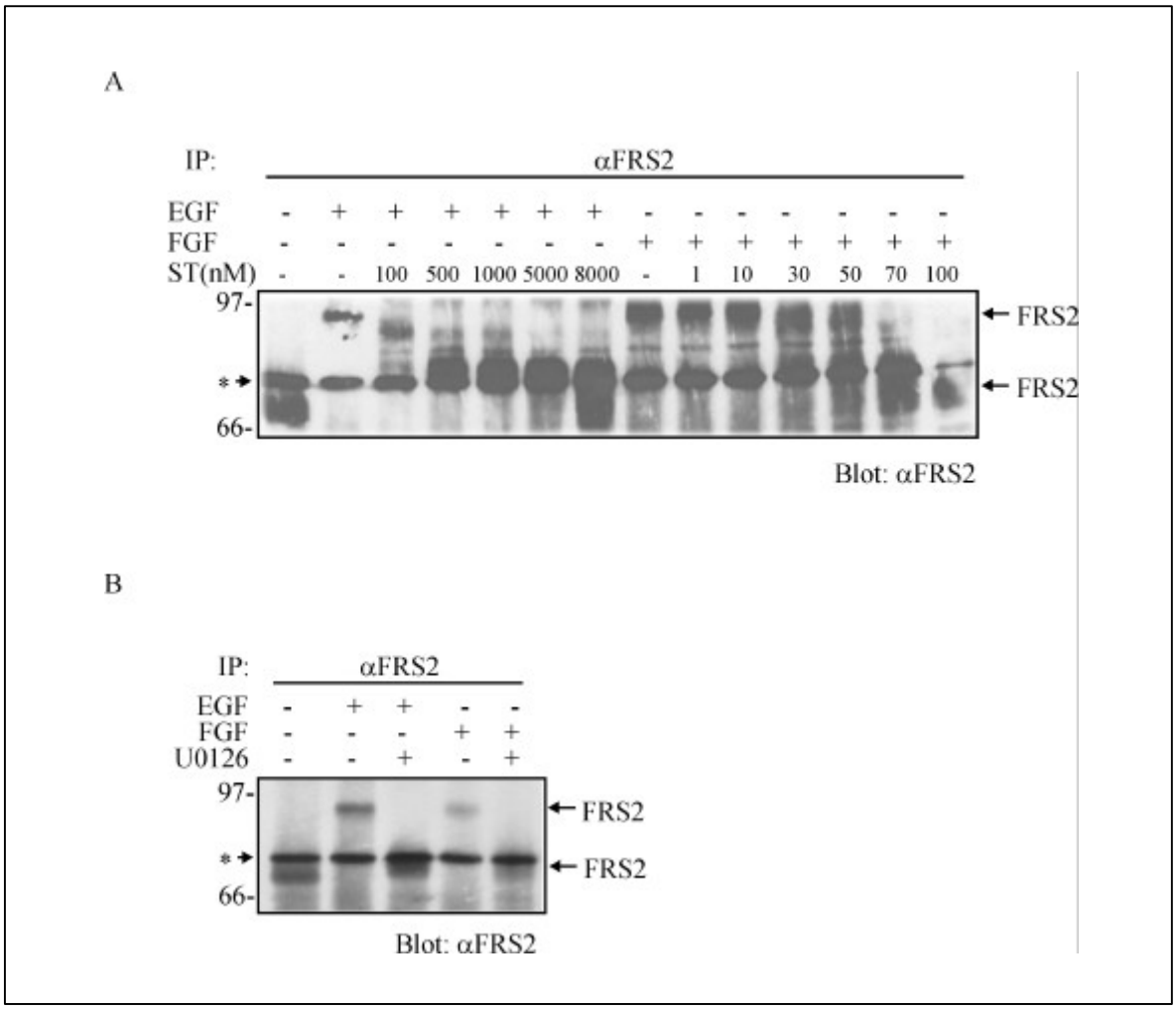


Figure 23: Pharmacological interference experiments.
Ovcar-3 cells were serum starved overnight and incubated with inhibitors for 20 min at 37°C before stimulation with 25 ng/ml EGF or aFGF for 5 min. The lysates were subjected to immunoprecipitations with anti-FRS2 antibody. The immunoprecipitates were resolved by SDS-PAGE followed by immunoblotting with anti-FRS2 antibody. (**A**) Treatment with broad sprectrum serine/threonine kinase inhibitor staurosporine (ST) at different concentrations. (**B**) Treatment with 5 µM MEK1/2 inhibitor U0126. Molecular weights are indicated on the left in kilodaltons. *Unspecific band due to an antibody cross-reaction with an unidentified protein.

As mentioned before, RTK inhibitors abolished FRS2 shift in response to their specific ligands, EGF (AG1478), aFGF (SU5402), and PDGF (AG1295). Inhibition of other enzymes, such as Src kinase and PLCγ which might be indirectly involved in FRS2 serine/threonine phosphorylation, gave negative results (data not shown). The broad spectrum serine/threonine kinase inhibitor staurosporine was able to abolish the migration shift of FRS2. However, while for FGF stimulation a concentration of 70 nM was sufficient, 8 µM was required in EGF stimulation (Figure 23A). This different concentration requirement implies that either different kinases are responsible for the serine/threonine phosphorylation of FRS2, or some upstream kinase is inhibited in the FGF-induced signaling pathway. Serine/threonine kinase inhibitors specific for PKA, PKC, PKG, CaMK II, MLCK and cdc2 were not able to affect FRS2 serine/threonine phosphorylation (data not shown). The PI3K inhibitor wortmannin, which prevents activation of PKB, was also ineffective (data not shown).

Table 1: Kinase Inhibitors Tested on FRS2 Mobility Change.

Name	***Specificity***	***Concentration***	***Effect*** [a]
AG1478	EGFR	250 nM	+ (EGF)
SU5402	FGFR	20 µM	+ (aFGF)
AG1295	PDGFR	10 µM	+ (PDGF)
PP1	Src	20 µM	- [b]
U73122	PLC γ	10 µM	-
Staurosporine	PKC, PKA, PKG, MLCK, CaMK-II	8 µM (EGF) 70 nM (aFGF)	+
H-89	PKA	5 µM	-
GÖ6976	PKC	5 µM	-
PKG inhibitor	PKG	5 µM	-
KN-93	CaMK II	5 µM	-
ML-7	MLCK	5 µM	-
Roscovitine	Cdc2	50 µM	-
Wortmannin	PI-3K	200 nM	-
SB202190	p38 kinase	10 µM	-
SB600125	JNK	10 µM	-
U0126	**MEK1/2**	**5 µM**	**+**

[a] Effect on FRS2 migration shift: +, inhibition of the ligand-induced shift; -, no inhibition of the shift in presence of ligand and inhibitor. [b] FGFR kinase activity was inhibited at 10 µM.

It has been reported that docking protein Gab1 is associated with activated ERK2 and acts as a substrate for the kinase (Roshan *et al.*, 1999; Yu *et al.*, 2001; Yu *et al.*, 2002), and IRS-1 is a substrate of JNK (Aguirre *et al.*, 2000; Aguirre *et al.*, 2002) . To examine the possible role of MAP kinases in EGF- or FGF-induced FRS2 serine/threonine phosphorylation, inhibitors against the MAP kinase family members were tested. While the specific inhibitors against JNK (SB600125) and p38 kinase (SB202190) did not abrogate serine/threonine phosphorylation of FRS2 (data not shown), the specific MEK1/2 inhibitor U0126, which prevents activation of ERK1 and ERK2 (Favata *et al.*, 1998), completely abolished the FRS2 gel migration shift upon both EGF and FGF stimulation (Figure 23B). These results demonstrate that ERK1/2 are responsible for FRS2 serine/threonine phosphorylation.

3.7.5 GPCR Agonists Induce FRS2 Serine/Threonine Phosphorylation

Besides the growth factors, G-coupled protein receptor (GPCR) agonists were tested for their ability to induce the FRS2 serine/threonine phosphorylation. Ovcar-3 cells were stimulated with GPCR agonists, carbachol, LPA and thrombin (Figure 24A, lanes 4, 6 and 8). The result demonstrated that the GPCR agonists are indeed able to induce the same migration shift of FRS2 as EGF (lane 2). It is well established that GPCR agonists can induce transactivation of the EGFR for cellular signal transduction (Daub *et al.*, 1996; Daub *et al.*, 1997; Prenzel *et al.*, 1999). To examine whether the GPCR agonists induced migration shift of FRS2 is mediated by transactivation of EGFR, the EGFR specific inhibitor AG1478 was utilized. When AG1478 was present, the migration shift of FRS2 was significantly reduced in GPCR agonist stimulation (Figure 24A, lane 5, 7 and 9), whereas it was completely abrogated in EGF stimulation (lane 3). This result implies that the FRS2 mobility change by GPCR stimulation is partially caused by transactivation of EGFR.

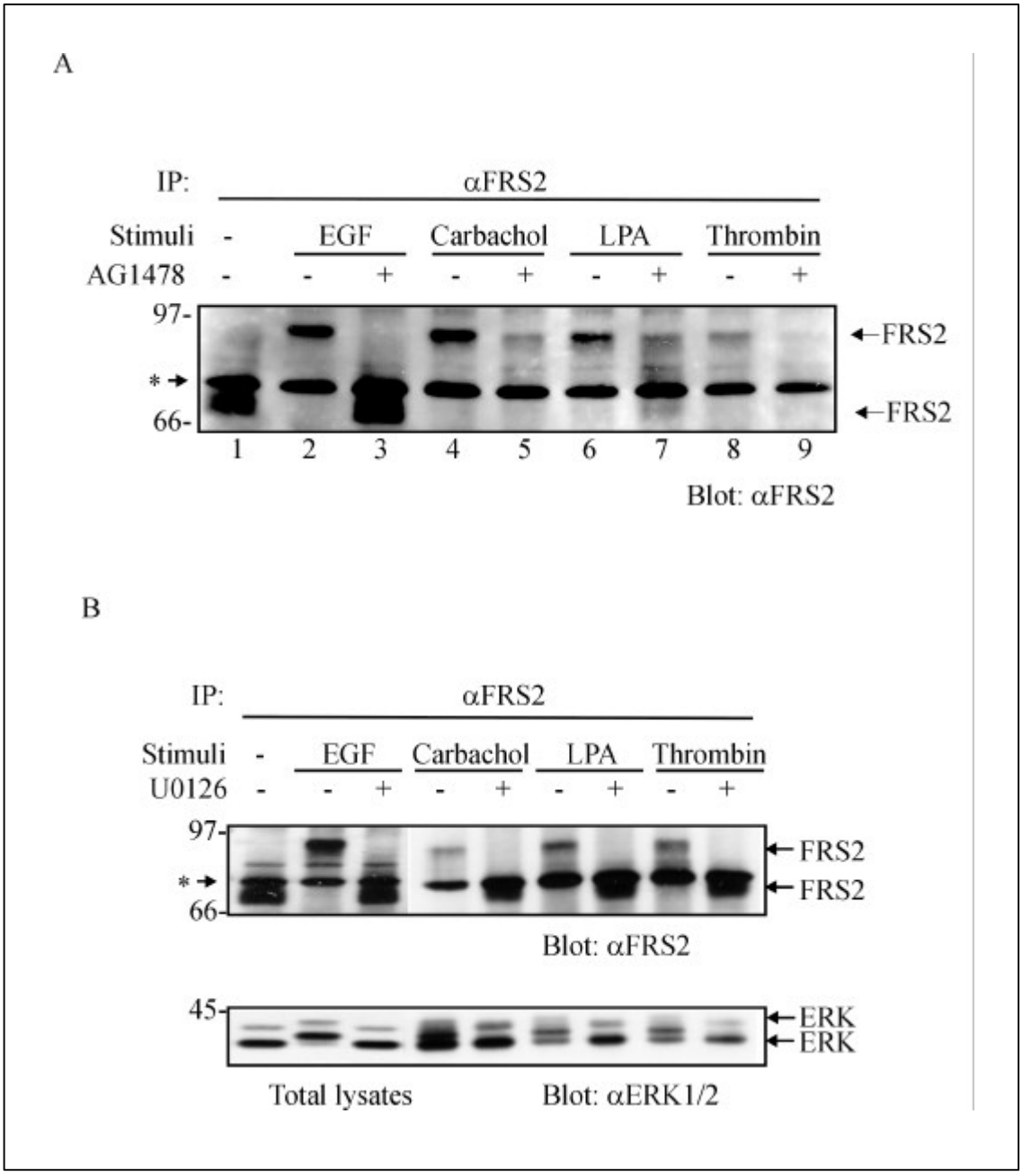


Figure 24: FRS2 is also involved in GPCR signaling.
Ovcar-3 cells were serum starved overnight and incubated with or without (**A**) 200 nM AG1478 or (**B**) 5µM U0126 for 20 min at 37°C before stimulation with GPCR agonists for 5 min at 37°C. Lane 1, untreated; lanes 2 and 3, 25 ng/ml EGF; lanes 4 and 5, 1 mM carbachol; lanes 6 and 7, 10 µM LPA; lanes 8 and 9, 2U/ml thrombin. The lysates were subjected to immunoprecipitations with anti-FRS2 antibody. The immunoprecipitates were resolved by SDS-PAGE followed by immunoblotting with anti-FRS2 antibody. Molecular weights are indicated on the left in kilodaltons. *Unspecific band due to an antibody cross reaction with an unidentified protein.

GPCRs are able to activate ERK through Src or PKC independent of the EGFR (Gutkind, 2000; Marinissen and Gutkind, 2001). To determine whether the GPCR induced FRS2 migration shift is also mediated by ERK activation, U0126 was tested on Ovcar-3 cells. As shown in Figure 24B, the pre-incubation with U0126 completely abrogated the carbachol, LPA and thrombin induced FRS2 migration shift as well as in the setting of EGF. Thus, it is clear that GPCR activation is also able to induce ERK mediated FRS2 serine/threonine phosphorylation, implicating that FRS2 is also involved in regulation of GPCR signaling.

Thus, the serine/threonine phosphorylation of FRS2 is induced in different cell lines by activation of different growth factor receptors as well as by GPCR agonists stimulations. The latter is partially mediated by transactivation through EGFR. The fact that this migration shift phenomenon occurs upon stimulations of a broad range of growth factors and GPCR agonists, implies that FRS2 is likely to be involved in multiple signal transduction pathways, and might play a role of central importance in the cellular signaling networks.

3.8 MAP Kinase and FRS2 form a Feedback Loop

3.8.1 ERK Phosphorylates FRS2 *in vitro*

The previous experiments demonstrated that FRS2 not only activates ERK (Figure 19 and 21), but also is a substrate of this serine/threonine kinase *in vivo* (Figure 22, 23 and 24). The minimal consensus sequence for phosphorylation by ERK consist of a serine or threonine followed by a proline (S/TP). In addition, a proline at position –2 is favourable, whereas a proline at position –1 is unfavourable (the phosphoacceptor S/T is position 0). The consensus ERK phosphorylation site is thus PXS/TP (Clark-Lewis *et al.*, 1991; Gonzalez *et al.*, 1991). Examination of the FRS2 primary sequence revealed 8 threonine residues as putative ERK phosphorylation sites, including ^{130}PRTP, ^{133}PRTP, ^{136}PTTP, ^{374}PKTP, ^{450}PQTP, ^{453}PKTP, ^{456}PTTP and ^{461}PQTP in different regions of the protein (Figure 25).

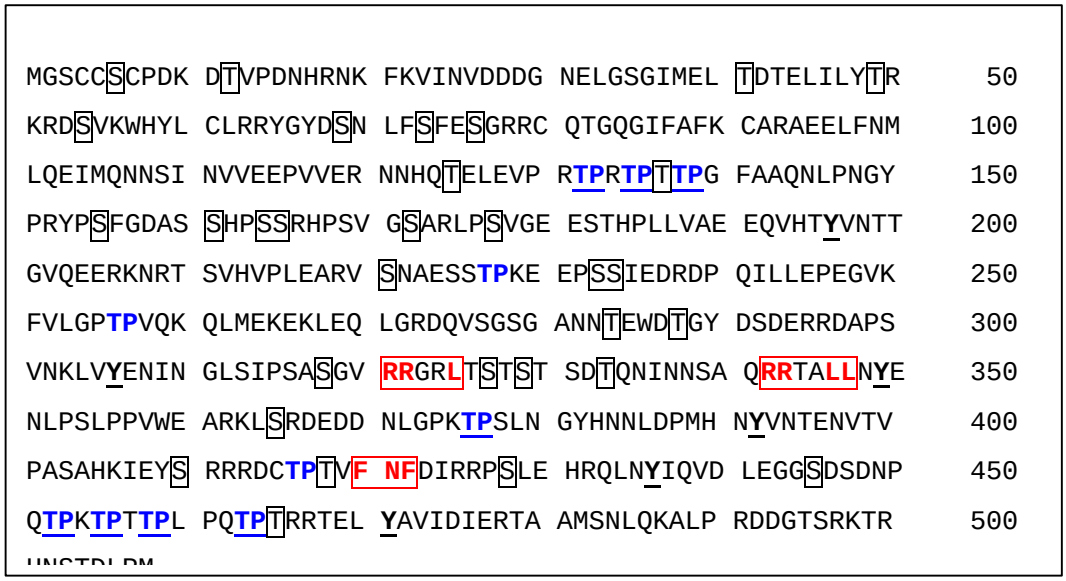


Figure 25: FRS2 phosphorylation and binding sites of ERK.
The minimal consensus of ERK phosphorylation S/TP are marked in bold blue and the putative ERK phosphorylation sites (PXS/T) are in addition underlined. The putative ERK docking sites are marked in red boxes. The FxF motif is marked in red bold box. Computer program predicted putative phosphorylation sites of other serine/threonine kinases are marked in boxes. The tyrosine phosphorylation sites are marked in underlined bold.

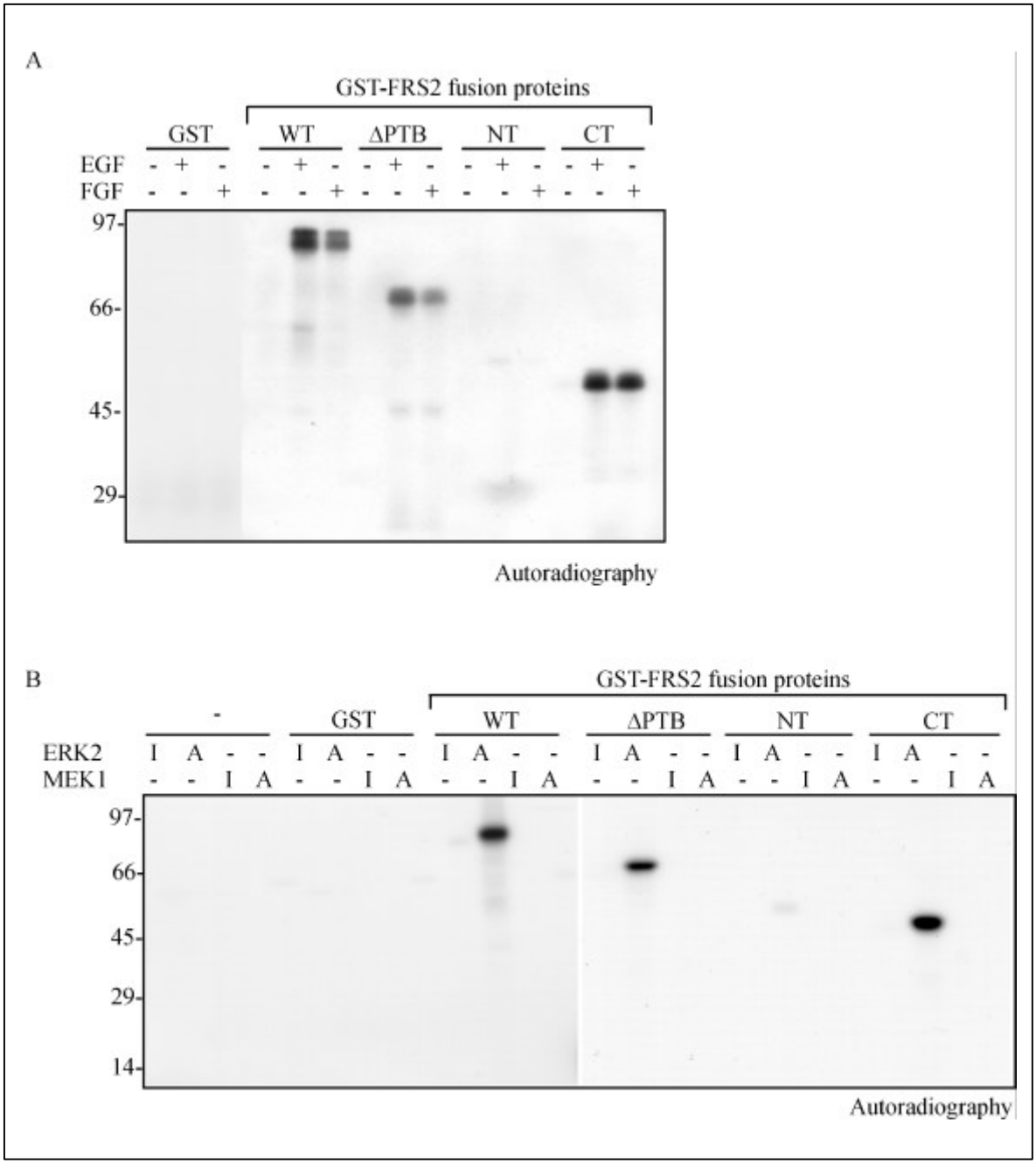


Figure 26: FRS2 is a substrate of ERK.
(A) Cos-7 cells were transiently transfected with HA-ERK2, and serum starved overnight before stimulation with either 25 ng/ml EGF or aFGF at 37°C for 5 minutes. The lysates were subjected to immunoprecipitations with anti-HA antibody, followed by *in vitro* MAP kinase assay using GST or GST-FRS2 fusion proteins as substrates. After gel electrophoresis, the gel was dried and exposed to film for autoradiography. (B) In vitro kinase saay with purified ERK2 and MEK1 and GST or GST-FRS2 fusion proteins as substrates. I: inactive (unphosphorylated). A: active (phosphorylated). Molecular weights are indicated on the left in kilodaltons.

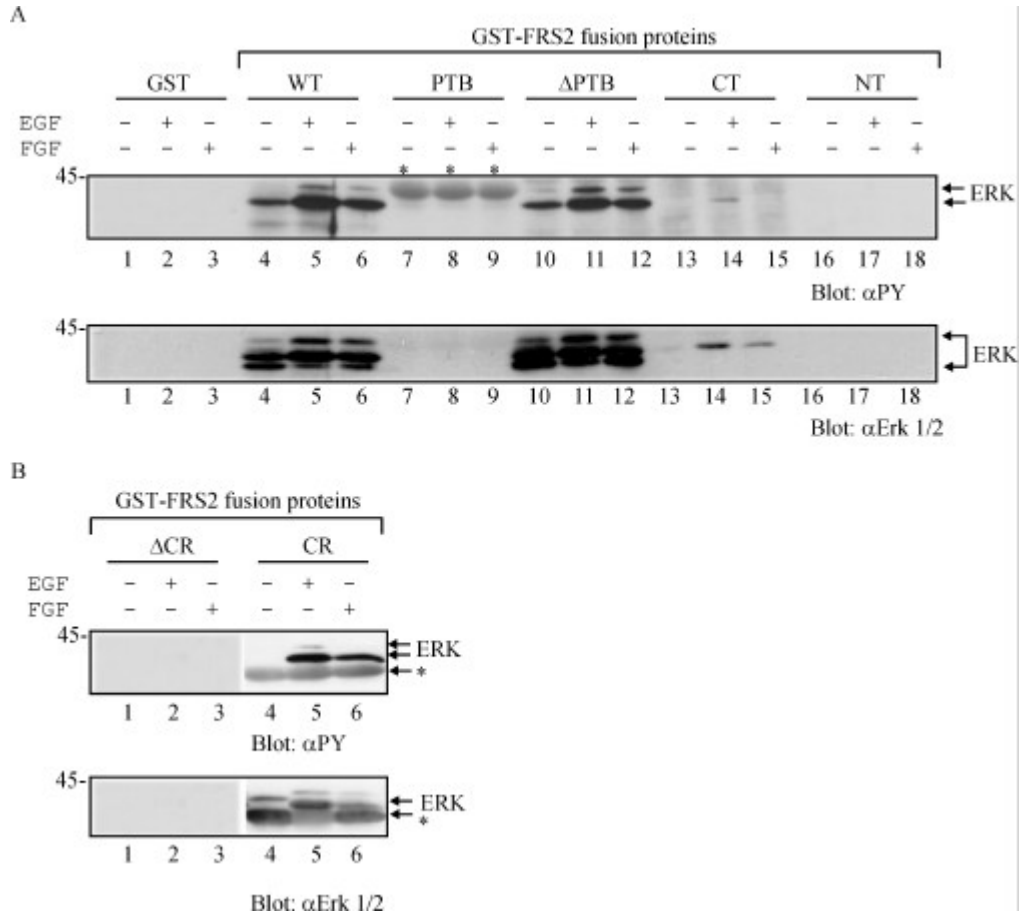
To test whether FRS2 is a direct substrate for ERK2 phosphorylation, *in vitro* MAP kinase assays were performed by incubating different GST-FRS2 fusion proteins (see Figure 9) with immunoprecipitated HA-tagged ERK2 from EGF or FGF treated and untreated Cos-7 cells in the presence of ^{32}P-γATP. SDS-PAGE followed by autoradiography revealed marked phosphorylation of GST-FRS2-ΔPTB, GST-FRS2-CT and GST-FRS2-WT, i.e. all carboxyl-terminus containing GST-fusion proteins (Figure 26A). In contrast, the amino-terminal portion of FRS2 including the PTB domain, GST-FRS2-NT, was not phosphorylated, despite of the three predicted ERK phosphorylation sites within this part of protein (Figure 25).

To exclude the possibility that FRS2 might be phosphorylated by another kinase that coprecipitates with ERK2, purified ERK2 and MEK1 were employed in *in vitro* kinase assays with the same set of GST-FRS2 fusion proteins as substrates. The result shown in Figure 26B corroborated the findings in Figure 26A and demonstrated that MEK1 is not able to phosphorylate FRS2. The activity of the purified kinases were controlled using MBP and unphosphorylated ERK2 as substrates (data not shown). The fact that phosphorylated GST-FRS2-WT migrates as a double band in Figure 26A, indicating that there is more than one ERK2 phosphorylation site and that differentially phosphorylated FRS2 molecules have different electrophoretic migration properties. These data establish that FRS2 is a substrate of ERK1/2 *in vitro*, and that the ERK phosphorylation site(s) appear(s) to be located in the C-terminal region of the molecule.

3.8.2 FRS2 Central Region Interacts Directly with ERK

The pharmacological interference experiments (Figure 23 and 24) and the *in vitro* MAP kinase assay described above demonstrated an involvement of ERK1/2 in serine/threonine phosphorylation of FRS2, however, it remained unclear whether a direct interaction between FRS2 and ERK1/2 exists. To address this issue, *in vitro* binding assays were performed with lysates of A-431 cells stimulated with EGF or FGF using different GST-FRS2 fusion proteins as affinity reagents. As shown in Figure 27A, ERK1/2 of 44 kDa and 42 kDa from stimulated and unstimulated cells coprecipitated with GST-FRS2-WT and GST-FRS2-ΔPTB. The phosphotyrosine and, to a lesser extent, the ERK1/2 signals were augmented upon ligand treatment of the cells. GST-FRS2-CT was in weak association only with the p42 ERK2 from the EGF stimulated cells as it was observed before (Figure 15). The GST-FRS2 fusions containing only the N-terminus of FRS2, GST-FRS2-PTB and GST-FRS2-NT, were not able to bind ERK1/2 (Figure 27A, upper panel). The association of GST-FRS2-WT and GST-FRS2-ΔPTB fusion proteins with ERK1/2 from untreated as well as stimulated cells suggests a constitutive association between specific parts of FRS2 and these kinases.

Comparison of the previous *in vitro* MAP kinase assay (Figure 26) and this binding assay reveals that the GST-FRS2 fusions that bind to ERK1/2 are identical with those that serve as ERK2 substrates (Figure 27C). The fusion proteins GST-FRS2-WT and GST-FRS2-ΔPTB associate with ERK and are substrates for ERK phosphorylation, while GST-FRS2-PTB and GST-FRS2-NT do not bind to ERK nor become phosphorylated. Thus, it seems that docking of ERK with FRS2 is a prerequisite for phosphorylation of the latter.



C

GST-FRS2 Fusion Proteins	*Association with ERK*	*Substrate of ERK*
GST-FRS2-WT (1-508)	+++	+
GST-FRS2-PTB (1-163)	-	-
GST-FRS2-ΔPTB (156-508)	+++	+
GST-FRS2-CT (326-508)	+	+
GST-FRS2-NT (1-240)	-	-
GST-FRS2-ΔCR (Δ240-351)	-	N.D.
GST-FRS2-CR (238-345)	++	N.D.

Figure 27: FRS2 central region binds to ERK1/2 *in vitro*.
(**A**), (**B**) A-431 cells were serum starved overnight and stimulated with 25 ng/ml EGF or aFGF. The lysates were subjected to *in vitro* binding assay with glutathione sepharose bound GST or GST-FRS2 fusion proteins as indicated, and resolved by SDS-PAGE followed by immunoblotting with the indicated antibodies. Molecular weights are indicated on the left in kilodaltons. *Unspecific bands due to antibody cross-reaction with GST-fusion proteins. (**C**) The association of GST-FRS2 fusion proteins with ERK1/2 are indicted as an estimation of anti-ERK1/2 reactive bands shown in (A). Whether these fusion proteins serve as ERK substrate is concluded from Figure 26.

The relatively weak interaction between the C-terminus-containing mutant, GST-FRS2-CT, and ERK1/2 when compared to GST-FRS2-WT and GST-FRS2-ΔPTB, together with the lack of interaction between GST-FRS2-NT and ERK1/2 suggest that the putative ERK binding site of FRS2 is likely located in the central region between the NT and CT truncation mutants plus the first residues from the C-terminus mutant. To examine this hypothesis, two additional fusion protein constructs CR (aa 238-345, Figure 9) encompassing the central region of FRS2, and its complimentary counterpart GST-FRS2-ΔCR (Δ240-352) were designed and employed. As shown in Figure 27B, the central-region-containing GST-FRS2-CR was able to bind ERK1/2, while GST-FRS2-ΔCR was not. Therfore, the central part of FRS2 contained the major docking region for ERK1/2.

In addition, an association of FRS2 and ERK1/2 was further confirmed by in-gel kinase assays performed with immunoprecipitated FRS2 (data not shown). This method was chosen to visualize the coprecipitated kinase that would otherwise be masked by the antibody light chain.

These experiments confirmed that the MAP kinases ERK1/2 constitutively associate with FRS2 through the ERK docking site in the central region of FRS2. Together with the fact that FRS2 is an upstream signaling mediator of ERKs, these data suggest a feedback loop between FRS2 and ERK1/2.

3.8.3 ERK Activation Reduces EGF- and FGF-Induced FRS2 Tyrosine Phosphorylation

The experiments so far have demonstrated a feedback mechanism where EGF- or FGF-induced tyrosine phosphorylation of FRS2 first leads to activation of ERK1/2 that is constitutively associated with the central region of FRS2 and in turn phosphorylates FRS2 on threonine residues. However, it is still unclear whether this feedback mechanism plays a positive or a negative regulatory role, i.e. whether the FRS2 threonine phosphorylation affects its tyrosine phosphorylation positively or

negatively. As mentioned before, phosphorylation on threonine residues by ERK1/2 resulted in a significant migration shift of FRS2 from ligand stimulated Ovcar-3 cells on SDS-PAGE, which was completely abolished by the presence of MEK1/2 inhibitor U0126 (Figure 23B).

The same effect on FRS2 migration shift was observed when A-431 cells were treated with this inhibitor prior to stimulation with EGF or FGF (Figure 28, upper panel). Remarkably, reprobing of the same membrane with the antibody against phosphotyrosine revealed that while inhibition of ERK activity abrogated FRS2 threonine phosphorylation, it significantly enhanced tyrosine phosphorylation of FRS2 in response to both EGF and FGF stimulation (Figure 28, upper middle panel, lane 3, 5). Thus, under normal physiological condition, FRS2 threonine phosphorylation down-regulates its tyrosine phosphorylation, demonstrating a negative feedback loop between ERK and FRS2 in the EGFR and FGFR signaling pathways.

To find out whether this increase of tyrosine phosphorylation has any effect on proteins known to interact with FRS2, the membrane was reprobed with specific antibodies against SHP2 and Grb2 (Figure 28, middle panels). Indeed, augmented tyrosine phosphorylation of FRS2 after blocking its threonine phosphorylation by ERK resulted in an elevated recruitment of SHP2 in EGF as well as FGF stimulated A431 cells . In contrast, FRS2-Grb2 complex formation increased only in response to FGF but not EGF stimulation. In correlation with this unchanged FRS2-Grb2 association, the amount of coprecipitated EGFR was not affected irrespective of the altered FRS2 phosphotyrosine content (Figure 28), which implicates that FRS2-Grb2 association is likely indirect through Grb2 interaction with the EGFR. It thus seems that FRS2 links EGFR activation with MAP kinase primarily through SHP2.

These results support a negative regulatory mechanism in which stimulation of MAPK activity via FRS2 leads to a reduction of FRS2 tyrosine phosphorylation. The resulting release of SH2 domain containing proteins from the RTK-FRS2 signaling complex might thereby down-regulate FRS2-mediated signaling pathways.

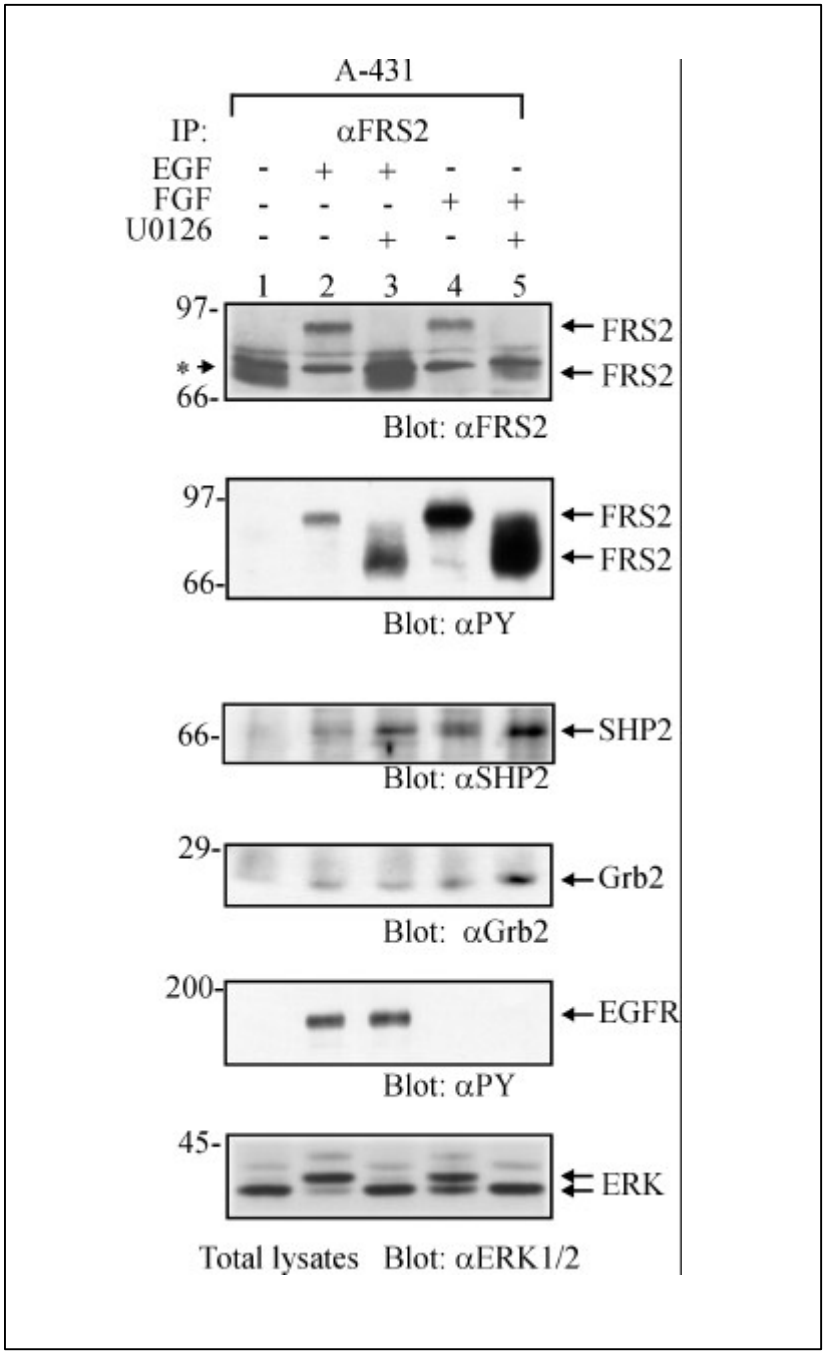


Figure 28: The FRS2 migration shift is abolished while tyrosine phosphorylation is enhanced upon inhibition of ERK activation.
A-431 cells were serum starved overnight and incubated with 5 µM U0126 for 20 min at 37°C before stimulation with 25 ng/ml EGF or aFGF for 5 minutes. The lysates were subjected to immunoprecipitations with anti-FRS2 antibody. The immunoprecipitates were resolved by SDS-PAGE followed by immunoblotting with the indicated antibodies. Crude lysates resolved by SDS-PAGE and immunoblotted with anti-ERK1/2 antibodies served as control. Molecular weights are indicated on the left in kilodaltons. *Unspecific band due to an antibody cross reaction with an unidentified protein.

3.8.4 Differential Regulation of FRS2 Tyrosine Phosphorylation

The FRS2 migration shift as a result of ERK-mediated threonine phosphorylation has been observed in responese to various stimuli in multiple cell lines, including EGF-treated Ovcar-3 (Figure 23 and 24), and PC12 cells (data not shown). However, only FGF but not EGF was able to induce FRS2 tyrosine phosphorylation in these cells, despite the relatively high expression levels of EGFR family members. Moreover, after pre-incubation with EGF, the FGF-induced FRS2 tyrosine phosphorylation was diminished in Ovcar-3 cells, but enhanced in PC12 cells (Figure 29). These results indicate that, tyrosine phosphorylation is not a prerequisite for threonine phosphorylation of FRS2 and may be positively (PC12 cells) or negatively (Ovcar-3 cells) regulated by co-presence of EGF and FGF depending on the cellular context. Thus, feedback loop of FRS2 and ERK represents a part of a complex regulatory mechanism involving additional cell type-dependent factors.

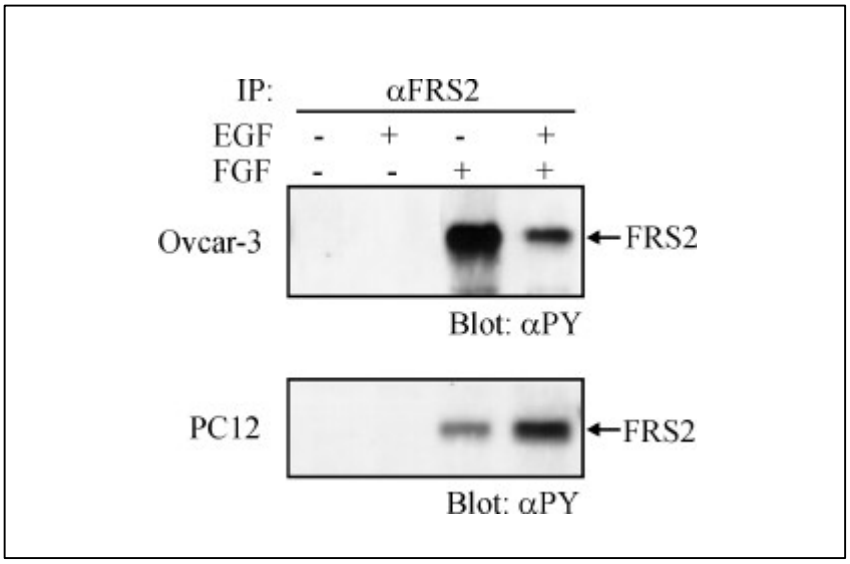


Figure 29: Differential regulation of FRS2 tyrosine phosphorylation by co-stimulation with EGF and FGF.
Ovcar-3 or PC12 cells were serum starved overnight and pre-incubated with 25 ng/ml EGF before stimulation with 25 ng/ml aFGF. The lysates were subjected to immunoprecipitations with anti-FRS2 antibody, resolved by SDS-PAGE and immunoblotted with anti-PY antibody.

3.9 Is FRS2 a Substrate of SHP2?

In the FRS2 and ERK1/2 mediated negative feedback loop, the activated EGFR or FGFR induce FRS2 tyrosine phosphorylation thereby activates ERK1/2, which in turn phosphorylates threonine residues on the docking protein and results in reduction of phosphorylation on tyrosine residues of FRS2. The dephosphorylation of phosphotyrosine may be archieved by the recruitment and activation of a tyrosine phosphatase. In this case, the tyrosine phosphatase SHP2 has been identified as interaction partner of FRS2 in response to both EGF and FGF stimulations. It has been reported that in FGFR signaling, SHP2 acts as a docking protein downstream of FRS2, nevertheless its catalytical activity is essential for sustained MAPK activity and for potentiation of FGF-induced PC12 cell differentiation (Hadari *et al.*, 1998).

To survey whether FRS2 is a substrate of SHP2, wild-type as well as catalytically inactive CA mutant of SHP2 along with FRS2 were transiently transfected into NIH3T3/EGFR cells and immunoprecipitations were performed with the anti-FRS2 antibody. Figure 30 shows that tyrosine phosphorylation of FRS2 somewhat increased in cells overexpressing SHP2 wild-type as compared to mock transfected cells in response to both EGF and FGF stimulation (Figure 30, upper panel), which correlates with enhanced association of FRS2 with SHP2 (Figure 30, upper middle panel). On one hand these results suggest that FRS2 is not a substrate of SHP2, on the other hand the expression of an enzymatically inactive SHP2-CA significantly increased FRS2 tyrosine phosphorylation, suggesting either a dominant negative effect or protection of phosphorylated tyrosine residues from the activity of another so far unidentified phosphatase. Thus, SHP2 seems to function as a downstream docking protein of FRS2 in both EGF and FGF induced signaling pathways, where it has been demonstrated to play a positive role in propagating signals from the respective RTK to the ERK MAP kinase (Hadari *et al.*, 1998; Feng, 1999; Qu *et al.*, 1999). Further experiments such as phosphatase assay are necessary to determine whether FRS2 is indeed a substrate of SHP2.

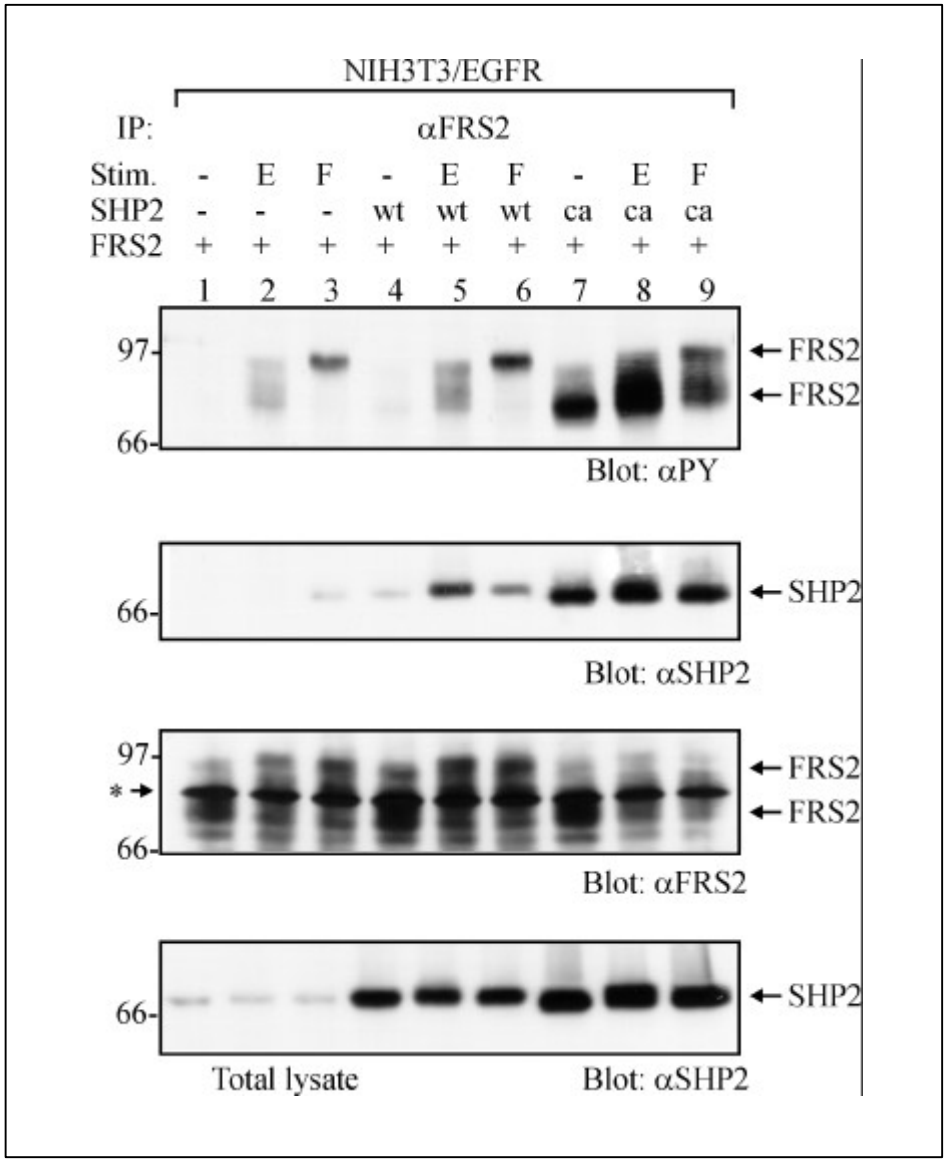


Figure 30: Effects of tyrosine phosphatase SHP2 on FRS2 phosphorylation.
Wild-type (wt) and catalytically inactive CA mutant SHP2 along with FRS2 were transiently transfected into NIH3T3/EGFR cells. Cells were serum starved overnight before stimulation with either 25 ng/ml EGF (E) or aFGF (F) at 37°C for 5 minutes. Immunoprecipitations were performed with anti-FRS2 antibody followed by immunoblotting with the indicated antibodies. Molecular weights are indicated on the left in kilodaltons. *Unspecific band due to an antibody cross reaction with an unidentified protein.

4. DISCUSSION

4.1 FRS2 in RTK-Mediated Oncogenic Signaling

Although the docking proteins lack intrinsic catalytic activity, it has been established that they function as platforms for the recruitment of signaling proteins in response to receptor tyrosine kinase (RTK) activation (Pawson and Scott, 1997; Hunter, 2000; Guy *et al.*, 2002). The docking protein FRS2 plays an important role in the signaling pathways induced by several growth factors including FGF, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF) and vascular endothelial growth factor (VEGF). FRS2 becomes tyrosine phosphorylated upon activation of the respective receptors, FGFR, TrkA, TrkB, Ret and VEGFR2, thereby creating docking sites for the phosphotyrosine binding adaptor protein Grb2 and tyrosine phosphatase SHP2 (Kouhara *et al.*, 1997; Hadari *et al.*, 1998; Xu *et al.*, 1998; Easton *et al.*, 1999; Meakin *et al.*, 1999; Kurokawa *et al.*, 2001; Melillo *et al.*, 2001; Stoletov *et al.*, 2002). FRS2 has also been suggested as a signal mediator downstream of IGF-IR and insulin receptor (Delahaye *et al.*, 2000; Kimpinski and Mearow, 2001). In this study, FRS2 was found to be ubiquitously expressed in a variety of cell lines of different tissue origin, including fibroblasts, mammary carcinoma, ovarian adenocarcinoma, epidermoid carcinoma, glioblastoma, lung carcinoma, as well as hematopoietic cells. Immunoprecipitations and *in vitro* binding assays demonstrated that upon tyrosine phosphorylation FRS2 associates with multiple phosphoproteins, among which the EGFR, SHP2, and ERK1/2 were identified (Figures 7, 12, 14, 15, 17, 18, 27 and 30).

It is well established that perturbations of RTK signaling are closely related with cancer (Blume-Jensen and Hunter, 2001). There are evidences showing that the EGFR family RTKs are involved in mammary and ovarian carcinoma, non-small-cell lung cancer, and colon cancer; FGFR in mammary and ovarian carcinoma, leukemia and gastric carcinoma; TrkA in neuroblastoma and thyroid carcinoma; IGF-IR in cervical cancer; Ret in medullary thyroid carcinoma, pheochromocytoma; insulin receptor in insulinoma; and VEGFR in tumor angiogenesis (Blume-Jensen and Hunter, 2001). Hence, the ubiquitous expression of FRS2 and its involvement in different RTK

signaling pathways imply that FRS2 may function as a mediator in oncogenic RTK signaling.

Several reports have indicated FRS2 involvement in human salivary adenocarcinoma, prostate tumor, as well as thyroid carcinogenesis triggered by Trk oncogenes (Zhang *et al.*, 2001; Wang, 2002; Ranzi *et al.*, 2003). FRS2 has also been found to be involved in parathyroid adenoma expressing an alternatively spliced variant of FGFR2 (Sakaguchi *et al.*, 1999). Increased FRS2 tyrosine phosphorylation was observed in correlation with ligand-independent up-regulation of transforming activity in transfectants expressing the variant FGFR2. The higher transforming activity was not accompanied by increased dimerization or overall autophosphorylation of the receptor. Moreover, FRS2 couples RET with the MAP kinase signaling cascade under both normal biological and pathological conditions, such as in papillary thyroid carcinomas (Melillo *et al.*, 2001). In human pancreatic cancer cells, FRS2 promotes mitogenic signaling generated by FGFR1/IIIc, which has the potential to enhance pancreatic ductal cell transformation (Kornmann *et al.*, 2002). Thus, there is an emerging role of FRS2 in RTK-mediated oncogenic signaling.

4.2 FRS2 in EGF- and FGF-Induced Signal Transduction

FRS2 directly binds to RTKs through its PTB domain. The association is constitutive for FGFR and VEGFR2, but dependent on receptor activation for TrkA, TrkB and Ret. The binding site on FGFR1 has been mapped to the juxtamembrane region lacking phosphotyrosine residues, whereas the binding site on VEGFR2 is still unknown (Xu *et al.*, 1998; Stoletov *et al.*, 2002). TrkA, TrkB and Ret associate with the FRS2 PTB domain through the known phosphorylation-dependent binding site of Shc (NPXpY) (Meakin *et al.*, 1999; Melillo *et al.*, 2001). Therefore it has been suggested that a dynamic balance of Shc or FRS2 binding may affect the RTK-mediated intracellular signaling for cell proliferation, differentiation and survival (Meakin *et al.*, 1999; Califano *et al.*, 2000; Kurokawa *et al.*, 2001; Melillo *et al.*, 2001).

This study demonstrated that, upon EGF stimulation of A-431 and NIH3T3 cells stably expressing EGFR (NIH3T3/EGFR), FRS2 becomes tyrosine phosphorylated and

binds directly to the activated EGFR through its PTB domain (Figure 17 and 18). Thus, as in the case of TrkA, TrkB and Ret, activation of the EGFR is necessary for its interaction with FRS2. Therefore, it is tempting to assume that FRS2 might also share the binding site on EGFR with Shc. The docking protein Shc contains a PTB domain as well as a SH2 domain, which enables Shc to bind EGFR at two phosphotyrosine residues (Batzer *et al.*, 1994; Batzer *et al.*, 1995). It is thus possible that the FRS2 PTB domain competes with the Shc PTB domain for the binding site pY^{1148} of EGFR, while the Shc SH2 domain binding site pY^{1173} is not challenged. The observation that Shc coprecipitates with FRS2 and EGFR supports this hypothesis (Figure 17B).

The failure to detect the EGF-induced FRS2 tyrosine phosphorylation in earlier studies might be explained by diverse cellular context, since the composition as well as the regulation of signaling molecules might vary in different cell lines. For example, in the cell lines PC12 and Ovcar-3, FRS2 tyrosine phosphorylation remained refractory to EGF stimulation. Moreover, FRS2 displayed a weaker tyrosine phosphorylation upon EGF stimulation in PC12 cells stably overexpressing the EGFR (PC12/EGFR) as compared to NIH3T3/EGFR cells (data not shown).

In contrast to FRS2 and EGFR association, the previously reported direct interaction of FRS2 with the FGFR could not be confirmed by immunoprecipitation with anti-FRS2 antibody or *in vitro* association experiments with purified GST-FRS2 fusion proteins (Figure 17 and data not shown). Nevertheless, FGF stimulation resulted in FRS2 tyrosine phosphorylation indicating that the FGFR transduced signal to FRS2 in the cell lines used. This demonstrates a significant difference in the interaction of FRS2 with the EGFR versus the FGFR. The functional consequence of this distinctive feature for FRS2 signal transmission in EGF and FGF treated cells is subject to further investigation. The ability of FRS2 to interact with nonphosphorylated FGFR raises the possibility that FRS2 is sequestered by FGFR in unstimulated cells and is only available for activation by FGF. Alternatively, the constitutive association of FRS2 PTB domain with the juxtamembrane domain of FGFR might be required to maintain the membrane-anchorage of FRS2. Since protein myristylation alone is usually insufficient for stable membrane association, an additional targeting signal might be necessary to generate a stable anchor to the cell membrane (Resh, 1994).

FRS2 is known to link growth factor-induced signal transduction pathways to the MAP kinase cascade, thus the effect of FRS2 on MAP kinase activity in EGFR signaling was examined. When increasing amounts of FRS2 were overexpressed in ERK2 transfected Cos-7 cells, a dose-dependent enhancement of MAPK activity after EGF stimulation was observed (Figure 19). This result suggests FRS2 as a positive regulator in EGF-induced signal transduction, hence providing an additional route, parallel to those via Grb2 and Shc, for signal transmission from the activated EGFR to the MAP kinase cascade. By this mechanism, FRS2 might contribute to the prolonged ERK activation that was reported to be necessary for EGF-induced differentiation of PC12/EGFR cells (Traverse *et al.*, 1994).

The exact mechanism underlying the EGF-induced and FRS2-promoted MAPK signal enhancement is still to be elucidated. Upon FGF stimulation, the enhancement of FRS2 tyrosine phosphorylation induced by ERK inactivation is accompanied by an increase of coprecipitated Grb2 and SHP2 (Figure 28). This is consistent with earlier reports demonstrating that the recruitment of both Grb2 and SHP2 by FRS2 is essential in mediating MAPK activation by FGF (Hadari *et al.*, 1998; Hadari *et al.*, 2001). In response to EGF stimulation, both Grb2 and SHP2 coprecipitated with FRS2. However, when the ERK activity was blocked, only the amount of SHP2, but not Grb2, in the multiprotein complex was elevated in correlation with enhanced FRS2 tyrosine phosphorylation. Concomitant with the unchanged amount of Grb2, the active EGFR coprecipitated was also not altered (Figure 28). The latter is due to independency of FRS2 tyrosine phosphorylation in the FRS2-EGFR interaction. Thus, the FRS2-Grb2 association observed upon EGF stimulation might be an indirect one, which is mediated through the direct interaction of Grb2 with the activated EGFR. This leads to the hypothesis that FRS2 might link the activated EGFR with MAPK signaling cascade primarily through SHP2, which has been shown to be positively engaged in the EGFR-ERK signaling cascade (Deb *et al.*, 1998; Stein-Gerlach *et al.*, 1998; Qu *et al.*, 1999). Shi *et al.* reported that upon EGF stimulation SHP2 is recruited into a multiprotein complex assembled on Gab1 and promotes Ras-Raf-MEK-ERK cascade activation by specifically dephosphorylating an unidentified phosphoprotein p90 in the complex (Shi *et al.*, 2000). As shown in this study, a tyrosine phosphorylated protein of 116 kDa very

likely corresponding to Gab1 coprecipitated with FRS2 and SHP2 in EGF stimulated cells, suggesting that the 90 kDa protein reported by Shi *et al.* might represent FRS2.

The fact that coexpression of a catalytically inactive SHP2-CA mutant and FRS2 results in a dramatic enhancement of FRS2 tyrosine phosphorylation, supports the hypothesis that FRS2 might be a substrate of SHP2 (Figure 30). Although the overexpression of wild-type SHP2 showed no dephosphorylation effect on tyrosine phosphorylated FRS2, the possibility cannot be excluded that under certain circumstances phosphorylated FRS2 serves as a substrate for SHP2. Alternatively, it is also possible that the inactive phosphatase mutant binds to FRS2 and thereby protects its phosphotyrosine sites from dephosphorylation by another so far unidentified phosphatase. One candidate might be the receptor-like PTP LAR, which inhibits FGF-induced MAPK activation partially through interfering with phosphorylation and Grb2 interaction of FRS2 in a human osteosarcoma cell line stably overexpressing the PTP (Wang *et al.*, 2000).

4.3 Serine/Threonine Phosphorylation of FRS2

Previous studies of FRS2 have been concentrated on its tyrosine phosphorylation, though FRS2 has more than 30 putative serine/threonine phosphorylation sites as predicted by computer programs such as Scansite and Prosite (Sibbald *et al.*, 1991; Yaffe *et al.*, 2001). In this study, a significant migration shift of FRS2 on SDS-PAGE upon EGF and FGF stimulation was observed. This shift resembles the decrease of gel mobility described for IRS-1 as a result of serine/threonine phosphorylation (Tanti *et al.*, 1994). *In vitro* kinase assays with FRS2 immunocomplexes revealed the presence of a kinase that was able to phosphorylate MBP (Figure 21). In addition, incubation of FRS2 with a serine/threonine phosphatase inhibitor resulted in retarded gel migration, while treatment with alkaline phosphatase abolished the migration shift. Together, all these observations suggested that the migration shift of FRS2 is due to phosphorylation on serine/threonine residues. Indeed, phosphoamino acid analysis confirmed that, in response to EGF or FGF stimulation the

phosphoserine and phosphothreonine contents of FRS2 was elevated significantly as compared to the basal level.

FRS2 is phosphorylated on serine as well as threonine residues in untreated cells. The function of this basal phosphorylation remains to be investigated. Interestingly, the basal serine/threonine phosphorylation of IRS-1 has been reported to play a positive role in its tyrosine phosphorylation by insulin and IGF-I receptors, whereas the same phosphorylation of IRS-2 has divergent effects on its interaction with these RTKs in a ligand-dependent manner (Greene and Garofalo, 2002). Hence, it is possible that, the effects of FRS2 basal serine/threonine phosphorylation on its tyrosine phosphorylation might differ in response to EGF or FGF stimulation. This might explain why EGF only induces relatively moderate tyrosine phosphorylation of FRS2 in A-431 cells when compared to FGF. This phenomenon is not likely to be caused by different amounts of the respective RTKs in the cells, since similar expression levels of EGFR and FGFR3 were detected by cDNA microarray analysis (data not shown). Thus, the different extents of FRS2 tyrosine phosphorylation upon EGF or FGF stimulation implicate distinct stimulus-dependent regulatory mechanisms for FRS2.

It is known that docking proteins such as Gab1, Gab2 and IRS-1 are phosphorylated on serine/threonine as well as on tyrosine residues. The serine/threonine phosphorylation of Gab1 is catalyzed by PKC_{α}, $PKC_{\beta1}$ and ERK, Gab2 by PKB, IRS-1 by PKC_{ζ}, PKB and JNK1 (Paz *et al.*, 1999; Roshan *et al.*, 1999; Aguirre *et al.*, 2000; Gual *et al.*, 2001; Liu *et al.*, 2001; Aguirre *et al.*, 2002; Greene and Garofalo, 2002; Lynch and Daly, 2002). To identify the upstream kinase of FRS2, specific inhibitors against these serine/threonine kinases were tested for their ability to affect the FRS2 migration shift. The PKC inhibitor had no effect, which is consistent with earlier report that, though FRS2 associates with atypical PKCs, PKC_{λ} and PKC_{ζ}, it is not a substrate of these kinases. In this case, the likely role of FRS2 is to serve as an anchoring protein to target the activated PKCs to the plasma membrane (Lim *et al.*, 1999).

4.4 FRS2-ERK Feedback Loop

The MEK1/2 inhibitor U0126 that prevents activation of ERK1/2 was the only specific inhibitor that completely abolished the FRS2 gel migration shift, indicating that ERK1/2 are the responsible kinases for FRS2 phosphorylation. FRS2 has 11 putative ERK threonine phosphorylation sites, when S/TP is considered the minimal consensus sequence for ERK phosphorylation (Clark-Lewis *et al.*, 1991; Gonzalez *et al.*, 1991; Davis, 1993; Songyang *et al.*, 1996). However, taken into account that a proline at position -2 is favorable, whereas a proline at position -1 is unfavorable, FRS2 has 8 putative threonine phosphorylation sites. The *in vitro* MAP kinase assay indicated that the phosphorylation sites are located at the C-terminus of FRS2, despite the three predicted phosphorylation sites at the N-terminus (Figure 25 and 26).

It is known that many MAP kinases form tight complexes with their substrates through docking sites within the substrate. These docking actions promote not only the specificity of substrate recognition, but also the efficiency and accuracy of substrate phosphorylation by MAP kinases (Gavin and Nebreda, 1999; Smith *et al.*, 1999; Whitmarsh and Davis, 1999; Sharrocks *et al.*, 2000; Chen *et al.*, 2001). A direct interaction between FRS2 and ERK1/2 was observed in *in vitro* binding assays with GST fusion proteins containing the central region of FRS2. This constitutive association of FRS2 and ERK1/2 enables a rapid and solid feedback regulation, as the FRS2 migration shift occurs almost simultaneously with the tyrosine phosphorylation (data not shown).

Analysis of the primary sequence of the FRS2 central region revealed two amino acid stretches, ^{321}RRGRL^{325} and ^{342}RRTALL^{347}, that resemble the previously identified MAPK binding consensus KIM (kinase interaction motif, L-X-X-K/R-K/R-X_{1-5}-L/I-X-L) (Pulido *et al.*, 1998; Zuniga *et al.*, 1999; Sharrocks *et al.*, 2000). Another known motif FxF, (^{419}FNF^{421}) that provides enhanced binding to the MAP kinase is found C-terminal to these putative docking site(s). Thus, FRS2 possesses all three determinants of MAP kinase-recognition modules mentioned in Introduction (Sharrocks *et al.*, 2000). Nevertheless, experimental evidence is still required to determine whether they are all necessary for complex formation.

In addition, subcellular distribution of ERKs to membrane (Kim and Kim, 1997; Liu *et al.*, 1997; Menice *et al.*, 1997; Chen *et al.*, 2001) as well as detection of ERKs in

isolated caveolae upon PDGF and EGF stimulation has been reported (Liu *et al.*, 1997; Engelman *et al.*, 1998a; Furuchi and Anderson, 1998; Kim *et al.*, 2000; Park *et al.*, 2000). FRS2 is membrane-anchored through its N-terminal myristylation site, and was found in caveolae (Figure 16) (Davy *et al.*, 2000; Ridyard and Robbins, 2003). Hence, it is fully possible that FRS2 and ERKs colocalize within caveolae, thereby fulfilling the spatial prerequisite for a direct association.

A comparison of the *in vitro* MAP kinase (Figure 26) and binding assays (Figure 27) reveals that the ERK-binding GST-FRS2 fusions are identical with those serving as substrates. This indicates that docking of ERK to FRS2 is a prerequisite for phosphorylation of the latter. ERK neither binds nor phosphorylates the N-terminus of FRS2, in spite of the three predicted ERK phosphorylation sites within this region. However, the possibility cannot be excluded that when brought into association with ERK, such as in the full-length protein, the N-terminus of FRS2 might serve as substrate of the kinase. The fact that the truncation mutant lacking the PTB domain (GST-FRS2-ΔPTB) became phosphorylated to a lesser extent in *in vitro* MAP kinase assay, might indicate a reduction of the three phosphorylation sites. Lax *et al.* recently reported that only replacement of all these 8 threonine residues by valine prevented the FGF-induced FRS2 migration shift, suggesting that there are 8 phosphorylation sites in the molecule (Lax *et al.*, 2002). Additionally, the central region of FRS2 is only partially presented by the C-terminus GST-FRS2 fusion construct which nevertheless, *in vitro* serves as substrate for MAP kinases. These experiments were performed in excess of the kinase, which might account for substrate recognition even in the absence of a perfect docking site.

The MAPKs ERK1/2 are downstream effectors as well as upstream regulators of FRS2, thus there is a feedback loop between these two proteins. Feedback regulation is known for docking proteins. For example, Gab1 and PI3K form a positive feedback loop in EGFR signaling (Rodrigues *et al.*, 2000). Upon EGFR activation, Gab1 is recruited to and subsequently phosphorylated by the receptor, which facilitates its association with downstream effectors such as the p85 subunit of PI3K. The activation of PI3K then leads to production of PtdIns(3,4,5)P3 (phosphatidylinositol 3,4,5-triphosphate), which further promotes membrane recruitment of Gab1 via its PH domain and additional enhancement of PI3K signaling. The termination of this positive

feedback loop is mediated by the tyrosine phosphatase SHP2 that dephosphorylates the p85 binding site of Gab1 (Zhang *et al.*, 2002). Another example is the self-attenuated mechanism between IRS-1 and PKCζ. In the insulin-induced signaling pathway, tyrosine-phosphorylated IRS-1 associates with the p85 subunit of PI3K that mediates the activation of PKCζ. This serine/threonine kinase subsequently phosphorylates IRS-1, thereby diminishing its tyrosine phosphorylation and reducing complex formation of IRS-1 and PI3K, finally inhibiting further activation of PKCζ itself (Liu *et al.*, 2001).

4.5 Regulation of the FRS2-ERK Negative Feedback Loop

As mentioned above, docking proteins such as Gab1, Gab2 and IRS-1 are known to be phosphorylated on tyrosine as well as serine/threonine residues in response to stimulation with growth factors, where serine/threonine phosphorylation indirectly influences protein function by potentiating or diminishing tyrosine phosphorylation (Paz *et al.*, 1997; Paz *et al.*, 1999; Roshan *et al.*, 1999; Aguirre *et al.*, 2000; Gual *et al.*, 2001; Liu *et al.*, 2001; Aguirre *et al.*, 2002; Greene and Garofalo, 2002; Lynch and Daly, 2002). Hence, tyrosine phosphorylation of such a protein is like an on-off switch of its activity, while the phosphorylation on serine/threonine residues are more like fine-tuning for the magnitude and duration of its function.

Moreover, even for one docking protein, serine/threonine phosphorylation induced by different growth factors could lead to divergent regulations of its tyrosine phosphorylation state. One such example comes from the studies of Gab1. Yu *et al.* have recently demonstrated that EGF-mediated ERK activation in mouse IMCD-3 cells results in diminished tyrosine phosphorylation of Gab1 and consequently reduced binding to PI3K (Yu *et al.*, 2002). Conversely, the same group had reported earlier that HGF stimulation of the same cells leads to ERK activation, which enhances Gab1-PI3K interaction without affecting Gab1 tyrosine phosphorylation (Yu *et al.*, 2001). In addition, the ERK phosphorylation sites on Gab1 are different in EGF- and HGF-induced signaling (Yu *et al.*, 2001; Yu *et al.*, 2002). Hence, the relay of signals to downstream effectors by docking proteins is quite versatile and likely to depend on the

nature of the stimulus as well as the cell physiological context. Similarly, the FRS2-ERK feedback loop may be differentially regulated in response to various stimuli. For example, tyrosine phosphorylation of FRS2 in NIH3T3/EGFR and A-431 cells occurs to a lesser extent in response to EGF as compared to FGF (Figure 17). Therefore, the balance between serine/threonine and tyrosine phosphorylation of FRS2 is differentially controlled in a stimulus-dependent manner, which might be achieved by, for instance, ERK-mediated phosphorylation of different residues on the docking protein.

Another important result of the present study is that prevention of FRS2 threonine phosphorylation by inhibition of ERK1/2 activation correlates with enhanced FRS2 tyrosine phosphorylation in response to EGF as well as FGF stimulation. This result indicates that *in vivo*, after activation and phosphorylation of ERK1/2, FRS2 tyrosine phosphorylation should be reduced and thereby its signal transmission potential would be curtailed. Since tyrosine phosphorylation is crucial for FRS2-mediated signaling, it is not surprising that tyrosine phosphorylation of the docking protein is under tight control. Growth factor-induced threonine phosphorylation of FRS2 by ERKs provides a molecular fine-tuning approach that adjusts the degree to which FRS2 is tyrosine phosphorylated and associated with downstream effector proteins.

The mechanism underlying this negative feedback fine-tuning remains to be elucidated. One possibility is that FRS2 becomes dephosporylated on tyrosine residues in response to its threonine phosphorylation by ERK. The FRS2 associated protein tyrosine phosphatase SHP2 is therefore the foremost candidate. However, as discussed before (see section 4.1), the attempts to examine the effects of SHP2 on FRS2 phosphorylation gave no clear answer. Alternatively, threonine phosphorylation might induce a structural change in FRS2 resulting in a poorer tyrosine kinase substrate, as exemplified by IRS-1 and Gab2. Phosphorylation of IRS-1 at a serine residue proximal to the PTB domain by JNK1 diminishes the interaction between the insulin receptor and IRS-1, resulting in inhibition of IRS-1 mediated signal transduction (Aguirre *et al.*, 2002). Gab2 constitutively associates with PKB, which phosphorylates the docking protein on a single serine residue leading to inhibition of its tyrosine phosphorylation (Lynch and Daly, 2002). A similar scenario may be deduced for the interaction between the *Xenopus* Src family kinase Laloo and FRS2 (Hama *et al.*, 2001; Kusakabe *et al.*, 2001; Hama *et al.*, 2002). A part of FRS2 PTB domain excluding the PXXP binding

motif for SH3 domain has been shown to bind to the SH4 domain of Laloo (Kusakabe *et al.*, 2001), but an additional requirement of the SH3 domain of Laloo has also been described (Hama *et al.*, 2001). Since the three predicted ERK phosphorylation sites within the N-terminal PTB domain of FRS2 colocalize with the SH3 binding PXXP motif, phosphorylation of these residues might lead to a local structural change and subsequent impairment of the FRS2-Laloo interaction. In addition, FRS2 threonine phosphorylation might trigger its association with phosphoserine/phosphothreonine-binding proteins (Tzivion *et al.*, 2001; Yaffe and Elia, 2001; Yaffe and Smerdon, 2001; Tzivion and Avruch, 2002). The complex formation may interfere with the interaction between the tyrosine kinase and FRS2. Alternatively, if the bound protein is a protein tyrosine phosphatase, it may reduce tyrosine phosphorylation of FRS2 in a phosphothreonine-dependent manner.

4.6 Intracellular Negative Feedback Regulation

As mentioned before, growth factor signaling by RTKs regulates important cellular responses, such as proliferation and differentiation (Hunter, 1998). Inappropriate signaling by RTKs has been implicated in cancer and disorders of developmental processes, implying that the strength and the duration of the signaling must be strictly regulated (Hunter, 2000). A negative feedback loop is one of the fundamental mechanisms that provide an effective way to modulate, limit or terminate RTK signaling (Perrimon and McMahon, 1999; Freeman, 2000). Perhaps the most obvious use of negative feedback is to limit the duration of a signal. In its simplest form, a signal induces its own negative regulator so that when a threshold has been reached, the signal ceases.

The FRS2-ERK feedback loop is not the only negative regulation in which these two proteins are involved. It has been shown that ERKs induce negative feedback on their activation by phosphorylation of upstream components of the kinase cascade including SOS, Raf, and MEK (Waters *et al.*, 1995; Corbalan-Garcia *et al.*, 1996; Lewis *et al.*, 1998; Chen *et al.*, 2001). A recent study suggests that ERK1/2 also cause negative feedback of heterotrimeric G protein signaling (Ogier-Denis *et al.*, 2000).

FRS2 has been found to mediate attenuation of FGFR signaling by forming a complex with Cbl via Grb2, where Cbl functions as an ubiquitin ligase that promotes ubiquitination and consequently degradation of the FGFR and FRS2 (Wong *et al.*, 2002).

A novel negative regulatory pathway for FRS2 mediated signaling has been described recently. Sprouty (Spry) was identified as a negative regulator of FGFR signaling, but was also found to inhibit EGFR signaling (Hacohen *et al.*, 1998; Kramer *et al.*, 1999; Egan *et al.*, 2002). Upon growth factor stimulation, Spry1 and Spry2 translocate to the plasma membrane and become phosphorylated on a conserved tyrosine residue, through which they bind to Grb2. By this means, Spry1-2 impair the recruitment of the Grb2-SOS complex either to FRS2 or to SHP2, and consequently inhibit the FRS2 mediated signaling (Hanafusa *et al.*, 2002).

4.7 FRS2 Involvement in Multiple Signaling Pathways

Docking proteins provide several important features for receptor signaling. Due to their ability of recruiting diverse signaling molecules, docking proteins expand the repertoire of the signaling pathways activated by a single receptor. Moreover, a single docking protein can integrate signals from various receptors by serving as a common substrate to integrate multiple inputs (White, 1998; Hunter, 2000). The fact that most RTKs activate broadly overlapping sets of signaling pathways raised the question of how different RTKs instruct cells to adopt specific fates. Since variations in strength and duration of a given signal are means to convey different instructions to a cell, mechanisms have evolved to ensure that appropriate thresholds of signal are achieved and maintained for the right time (Fiorini *et al.*, 2001).

In this study, it is shown that FRS2 becomes phosphorylated on serine/threonine residues in response to various stimuli, including EGF, FGF, NGF, PDGF, heregulin, IGF-I, insulin as well as GPCR agonists such as carbachol, LPA and thrombin. The major findings of the present study are summarized in Figure 31.

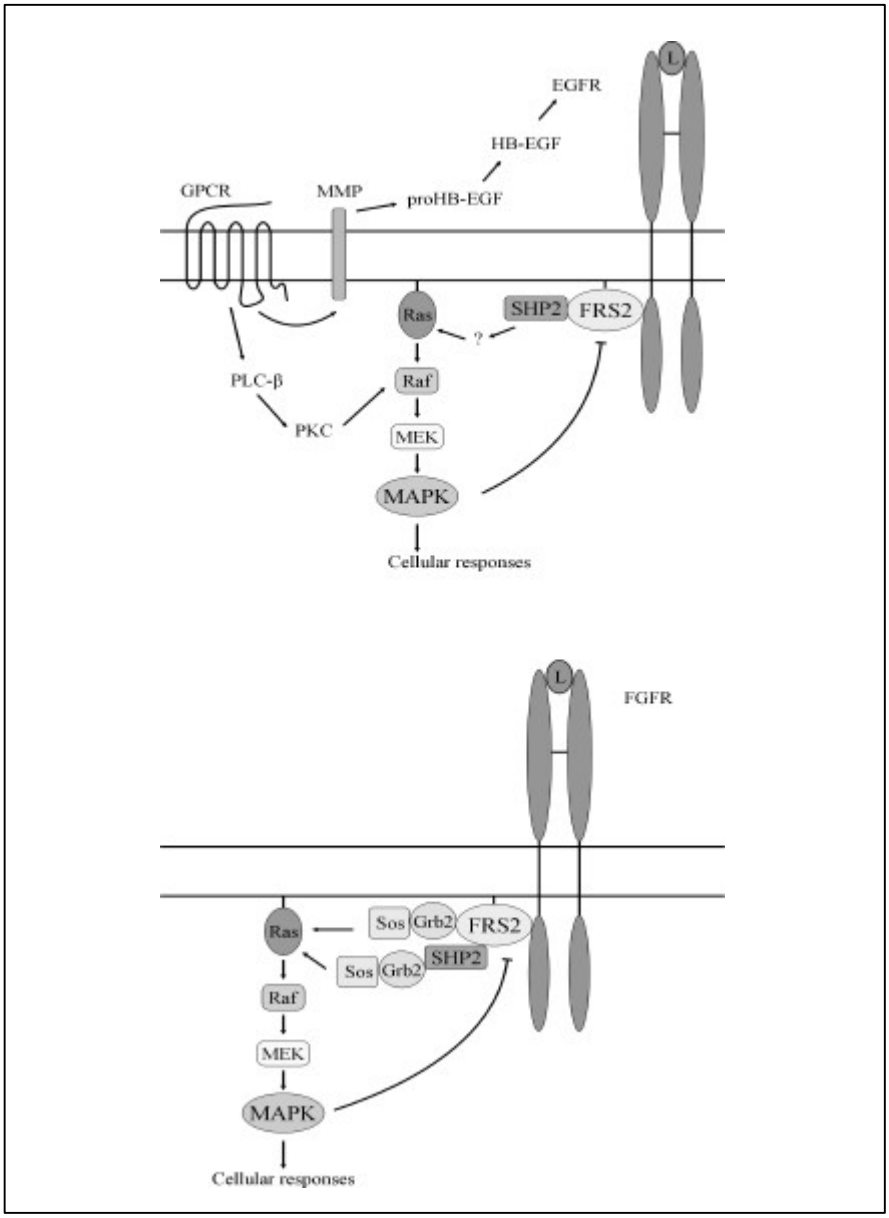


Figure 31: Schematic presentation of FRS2 mediated signaling.
FRS2 undergoes tyrosine as well as serine/threonine phosphorylation upon EGF and FGF stimulation. FRS2 directly associates with the RTKs via its PTB domain and positively regulate the ligand-induced ERK activity. This is mediated by association of FRS2 with SHP2 in EGF-induced signaling, or by direct recruitment of Grb2 or indirectly via SHP2. FRS2 and ERK form a negative regulatory feedback loop in EGF and FGF induced signal transduction pathway, in which ERK phosphorylates FRS2 on threonine residues thereby inhibits the tyrosine phosphorylation of FRS2. The GPCR-induced FRS2 serine/threonine phosphorylation is presumably achieved to some extent through transactivation of the EGFR and partially through other ERK activation pathways.

FRS2 undergoes tyrosine as well as serine/threonine phosphorylation upon EGF and FGF stimulation. Upon EGF stimulation, FRS2 associates directly with the

activated EGFR via its PTB domain and promotes the signal primarily through SHP2 to the MAP kinase, whereas FRS2 propagates the FGF-induced signal both directly through Grb2 and indirectly via SHP2 to the same kinase cascade. FRS2 and ERK form a negative regulatory feedback loop in EGF- and FGF-induced signal transduction pathways, in which ERK phosphorylates FRS2 on threonine residues thereby inhibits the tyrosine phosphorylation of the docking protein.

The GPCR-induced FRS2 serine/threonine phosphorylation is achieved to some extent through transactivation of the EGFR (Daub *et al.*, 1996; Daub *et al.*, 1997) and partially through other ERK activation pathways (Marinissen and Gutkind, 2001). Since FRS2 appears to be targeted in response to activation of a broad range of cell surface receptors, it seems to play a broader role as a central regulatory switch point in intracellular signaling than anticipated so far.

4.8 Outlook

In addition to tyrosine phosphorylation, threonine phosphorylation of FRS2 was established as a result of stimulation with multiple RTK and GPCR ligands. However, further details of FRS2's role in signal transduction downstream the multiple surface receptors remains to be elucidated. As indicated by the variable modification of FRS2 in response to EGF stimulation, it is necessary to analyse FRS2 in individual receptor pathways in different cellular systems, including hematopoietic cells where the FRS2 interaction partner SHP2 seems to play a critical role in modulation of signaling events (Tauchi *et al.*, 1994; Fuhrer *et al.*, 1995; Tauchi *et al.*, 1995). Based on the differential and combinatory effects of EGF and FGF on FRS2 phosphorylation observed in this study, competition and cooperation among RTK and GPCR pathways should also be considered in both physiological and pathophysiological processes.

As suggested by the FRS2-ERK feedback loop, regulation of FRS2 phosphorylation itself might be a main key to address these issues. FRS2 tyrosine phosphorylation was shown to be regulated by the activity of RTKs as well as ERK, which simultaneously regulated the interaction of FRS2 with SHP2 and Grb2 in a stimulus-dependent manner. Thus, the mechanism underlying the ligand- and cell type-

specific differences in tyrosine phosphorylation and protein recruitment of FRS2 is still to be examined. Also the function of basal phosphorylation and induced serine phosphorylation of FRS2 remain to be investigated.

FRS2 was found to be expressed not only in normal tissues (Kouhara *et al.*, 1997), but also in the vast majority of carcinoma cell lines analysed. Although the expression levels in these tumor cells were highly variable, the attempts to generate stable cell lines overexpressing FRS2 failed. This may be due to the toxicity of the overdose of this protein, as injection of high dose xFRS2 mRNA alone caused *Xenopus* embryonic death (Kusakabe *et al.*, 2001). Together with the embryonic lethal phenotype of FRS2 knock out mice (Hadari *et al.*, 2001), these data argue for a strict regulation of FRS2 at the expression level. However, the expression pattern and its potential regulatory function of FRS2 during development, differentiation and proliferation remain to be determined. In this context, the proposed identity of FRS2 with SNT and its potential impact on cell cycle regulation via p34 cdc2 are of specific interest.

Cell fate decision in response to specific stimuli may also be mediated by the FRS2-ERK feedback loop. In PC12 cells, a transient ERK activation by EGF correlates with cell proliferation and a sustained ERK response to FGF or NGF stimulation leads to differentiation (Traverse *et al.*, 1994; Marshall, 1995). FRS2 becomes tyrosine phosphorylated and promotes differentiation upon FGF treatment of PC12 cells (Kouhara *et al.*, 1997). In this study, FRS2 was tyrosine phosphorylated in PC12 cells in response to FGF but not upon stimulation with EGF. Thus, FRS2 is either not in association with the EGFR in these cells or EGF-induced ERK activity abrogates FRS2 tyrosine phosphorylation and SHP2 recruitment. Further experiments will have to elucidate whether FRS2 regulates or is regulated by the strength and duration of ERK activation in FGF- or EGF-stimulated PC12 cells and thus conveys the instructions for differentiation and proliferation, respectively.

SUMMARY

This study provides evidence that the docking protein FRS2 also promotes an EGF signal to the MAP kinase cascade via Grb2 and SHP2, in addition to its known function in FGF, NGF, GDNF, BDNF and VEGF signaling. Upon EGF stimulation, FRS2 undergoes phosphorylation on tyrosine as well as serine/threonine residues. This involves direct interaction of the FRS2 PTB domain with the EGFR and results in a significantly altered mobility of FRS2 in SDS-PAGE that is also observed in FGF stimulated cells. This migration shift of FRS2 is completely abrogated by U0126, a specific MAPK kinase 1 (MEK1) inhibitor, suggesting that ERK1/2 is the serine/threonine kinase upstream of FRS2. Indeed, as the *in vitro* kinase and binding assays demonstrated, the central region of FRS2 constitutively associates with ERK1/2 whereas the FRS2 carboxyl-terminal region serves as substrate for ERK2 phosphorylation in response to both EGF and FGF stimulation. Notably, tyrosine phosphorylation of FRS2 is enhanced when ERK1/2 activation is inhibited after both EGF and FGF stimulation. These results indicate a ligand-stimulated negative regulatory feedback loop in which activated ERK1/2 phosphorylates FRS2 on serine/threonine residues thereby down-regulating its tyrosine phosphorylation. These findings support a broader role of FRS2 in EGFR controlled signaling pathways and provide insight into a molecular mechanism for ligand-stimulated feedback regulation.

Moreover, analysis of a variety of cell lines by immunoprecipitation and Northern analysis revealed that FRS2 is ubiquitously expressed. Caveolae localization of FRS2 implicates possible interactions with other signaling molecules present in this compartment. Immunoprecipitations and *in vitro* binding assays demonstrate that tyrosine phosphorylated FRS2 associates with multiple phosphoproteins, among which the EGFR, SHP2, and ERK1/2 were identified. Furthermore, the serine/threonine phosphorylation of FRS2 is induced in different cell lines by activation of different RTKs as well as by GPCR agonist stimulation. The latter is at least partially mediated by transactivation through the EGFR. The fact that this migration shift phenomenon occurs upon stimulation with a broad range of growth factors and GPCR agonists, implies that FRS2 might be a central regulatory switch point in the cellular signal transduction network.

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ABBREVIATIONS

α	Anti
aa	amino acid
ATCC	American Tissue Culture Catalogue
ATP	Adenosine triphosphate
BCA	Bicinchonic acid
BDNF	Brain-derived neurotrophic factor
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine serum albumin
CaM kinase II	Calmodulin kinase II
cDNA	Complementary DNA
CMV	Cytomegalovirus
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxid
DTT	Dithiothreitol
E	Embryonic day
ECL	Enhanced Chemiluminiscence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescence protein
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
ERK	Extracellular regulated kinase
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FRS2	Fibroblast growth factor receptor substrate 2
Gab1	Grb2-associated binder-1
GDNF	Glial cell-derived neurotrophic factor
GDP	Guanosindiphosphat
GPCR	G protein-coupled receptor
Grb2	Growth factor receptor binding protein 2
GST	Glutathion-S-Transferase
GTP	Guanosintriphosphat
HA	Hemagglutinin
HB-EGF	Heparin-binding epidermal growth factor
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HER	Human EGFR
HRP	Horseradish peroxidase
Ig	Immunglobulin
IGF-I	Insulin-like growth factor I
IPTG	Isopropyl-β-thiogalactopyranoside

IP	Immunoprecipitation
JNK	c-Jun amino (N)-terminal kinase
Kb	Kilobase
kDa	Kilodalton
LPA	Lysophosphatidic acid
M	Molar (mole per liter)
MAP kinase	Mitogen activated protein kinase
MBP	Myelin basic protein
MEK	MAP kinase kinase
min	Minute
ml	Milliliter
mRNA	Messenger RNA
NDF	Neu differentiation factor
NCBI	National Center for Biotechnology Information
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PDGF	Platelet-derived growth factor
PH	Pleckstrin Homology domain
PI3K	Phosphatidylinositol 3 kinase
PLC	Phospholipase C
PKA	Protein kinase A
PKC	Protein kinase C
PMSF	Phenylmethanesulfonyl fluoride
PoV	Sodium pervanadate
PP	Protein phosphatase
PTB	Phosphotyrosine-binding
PTP	Protein tyrosine phosphatase
PTK	Protein tyrosine kinase
PVDF	Polyvinyldifluorid
RNA	Ribonucleic acid
RS/TK	Receptor serine/threonine kinase
RT	Room temperature
RTK	Receptor tyrosine kinase
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamid gel electrophoresis
sec	Second
SH2, 3, 4	Src homolgy domain 2, 3, 4
Shc	SH2 and collagen domain-containing protein
SNT	Suc1-associated neurotrophic factor target
SOS	Son of Sevenless
TEMED	N, N, N', N'-Tetramethylethylendiamine
TGF-α	Transforming growth factor alpha
VEGF	Vascular Endothelial Growth Factor
WT	Wild type

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