FRS2 is a docker protein that recruits signaling proteins to the plasma membrane in fibroblast growth factor signal transduction. We report here that FRS2 was associated with PKCζ when Swiss 3T3 cells were stimulated with basic fibroblast growth factor. PKCζ, the other member of the atypical PKC subfamily, could also bind FRS2. The association between FRS2 and PKCζ is likely to be direct as shown by yeast two-hybrid analysis. The C-terminal fragments of FRS2 (amino acid residues 300–508) and SNT2 (amino acids 281–492), an isoform bearing 50% identity to FRS2, interacted with PKCζ at a region (amino acids 240–562) that encompasses the catalytic domain. In vitro kinase assays revealed neither FRS2 nor SNT2 was a substrate for PKCζ or ζ. Mutation of the alanine residue (Ala-120) to glutamate in the pseudo-substrate region of PKCζ results in a conformational change in PKCζ that exhibited more than 2-fold greater binding to FRS2 in vitro than its "closed" wild-type counterpart. Tyrosine phosphorylation of FRS2 did not affect its binding to the constitutively active PKCζ mutant, suggesting that the activation of PKCζ is necessary and sufficient for its association with FRS2. It is likely that FRS2 serves as an anchoring protein for targeting activated atypical PKCs to the cell plasma membrane in signaling pathways.

Fibroblast growth factor receptors are members of the receptor-tyrosine kinase family (1). In contrast to other growth factor receptors such as those for EGF1 and PDGF, FGF receptors are poorly auto-phosphorylated upon ligand binding. Instead, a 90-kDa protein called SNT1 or FGF receptor substrate-2 (FRS2) (2, 3) is phosphorylated at multiple tyrosine sites. FRS2 has also been reported to be serine/threonine-phosphorylated in FGF-treated cell lysates (4). SNT2, a recently identified isoform of FRS2, has about 50% identity to FRS2 (5) mainly at the N- and C-terminal ends. FRS2 and SNT2 possess a myristoylation site and a PTB domain at the N terminus. The PTB domain is responsible for the direct interaction of FRS2 and SNT1 with the juxtamembrane region of the FGFR in a phosphotyrosine-independent manner. Deletion of the PTB domain of both proteins abrogates the association and tyrosine phosphorylation of FRS2 and SNT2 by FGF receptors (5, 6).

FRS2 and SNT2 substitute for their receptors as docking proteins, a role similar to that of insulin receptor substrate (IRS) in insulin signaling (7). To date, two important signaling proteins, Grb2 and SHP-2, have been reported to bind directly to tyrosine-phosphorylated FRS2 (2, 8). Grb2 is an adapter protein best known for its role in linking receptor tyrosine kinases to the Ras pathway via the guanine nucleotide-releasing factor Sos (9). The binding of Grb2 to FRS2 occurs via the interaction of the SH2 domain of Grb2 with some or all of the potentially phosphorylated tyrosine residues at Tyr-186, Tyr-306, Tyr-349, and Tyr-392 on FRS2. Mutational studies showed that when the tyrosine residues at all 4 sites were changed to phenylalanine, the downstream MAP kinase activation was significantly reduced (2). SHP-2 is a tyrosine phosphatase whose activity has been proposed to be necessary for cell growth and proliferation (10, 11). When cells are stimulated with growth factors such as PDGF, SHP-2 is tyrosine-phosphorylated and binds to the SH2 domain of Grb2 (12). SHP-2 also binds to the activated receptors via its own SH2 domain (12). As a result, SHP-2 functions not only as a phosphatase but also serves as an adapter protein recruiting Grb2 to the receptors. Recently, SHP-2 has been reported to bind directly to tyrosine-phosphorylated FRS2 through its N-terminal SH2 domain (8). The association of SHP-2 with FRS2 and the activation of SHP-2 are essential for a sustained MAP kinase response as well as for the potentiation of FGF-induced neurite outgrowth in PC12 cells (8). Hence, by recruiting Grb2 and SHP-2, FRS2 plays a crucial role in linking the FGF receptors to the Ras/MAP kinase pathway.

Apart from the Grb2 and SHP-2 proteins, the activity of the atypical PKCs (aPKCs) is necessary for mitogenic signaling via the MAP kinase cascade (13, 14). There are two members in the aPKC subfamily, PKCδ and PKCζ, and they share more than 75% identity. PKCs have been subdivided into 3 subfamilies, and they are distinguished by their lipid activation profiles. Conventional PKCs (cPKCs e.g. α, β, and γ) are activated by diacylglycerol and calcium; novel PKCs (nPKCs e.g. δ, ε, η, and θ) do not respond to calcium but require diacylglycerol for their activation; and aPKCs are not activated by either diacylglycerol or calcium. It has been shown that MAP kinase and MEK are activated in vivo by an active mutant of PKCζ, and a kinase-defective dominant negative mutant of PKCζ impairs

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‡ The abbreviations used are: EGF, epidermal growth factor; FRS2, FGF receptor substrate-2; Grb2, growth factor receptor-binding protein-2; SH2 domain, Src homology domain-2; PTB domain, phosphotyrosine binding domain; FGF, fibroblast growth factor; bFGF, basic fibroblast growth factor; FGFR (or Flg), FGF receptor-1; PDGF, platelet-derived growth factor; PDK-1, phosphoinositide-dependent protein kinase 1; MAP, mitogen-activated protein; PTB, Proline, Tyrosine, Basic motif; IRS, insulin receptor substrate; SNT, Suc1-associated tyrosine/threonine kinase; PKA, protein kinase A; SNT1, Suc1-associated tyrosine/threonine kinase; PKC, protein kinase C; cPKC, conventional PKCs; nPKC, novel PKCs; HA, hemagglutinin; MAP, mitogen-activated protein; PDB, protein kinase C, domain chain reaction; PDK-1, phosphoinositide-dependent protein kinase 1; PI 3-kinase, phosphatidylinositol 3-kinase; PTP, protein-tyrosine phosphatase.
the activation of both MEK and MAP kinase by serum and tumor necrosis factor (14). However, whereas Grb2 and SHP-2 lie upstream of Ras, PKCζ can bind to and act as a direct effector of Ras (15). This is consistent with the observation that expression of a dominant negative mutant of Ras (Asn-17) severely impairs the activation of PKCζ by mitogens such as PDGF in mouse fibroblasts (15).

A few groups of proteins that are either regulators or substrates of aPKCs bind to the members in this subfamily. In the first group, a protein called Zeta-Interacting Protein (ZIP) binds specifically to the regulatory domain of PKCζ (16), whereas Lambda-Interacting Protein (LIP) binds specifically to the regulatory domain of PKCλ resulting in an activation of the kinase (17). The Par-4 protein also binds to the regulatory domain of PKCλ and PKCζ but inhibits their activity (18). The second group comprises proteins like heterogeneous nucleoprotein A1 protein that has been found to bind to the kinase domain of PKCζ in yeast two-hybrid screening and is a specific substrate of the aPKCs (19).

We have been studying p75, a phosphotyrosine protein that is dephosphorylated and dissociates from Grb2 upon growth factor stimulation (27). In our attempt to identify p75 by immunoprecipitating phosphotyrosyl proteins that are about 75-kDa in molecular mass, we observed that a 90-kDa tyrosine-phosphorylated protein, p90, associates specifically with members of the aPKC subfamily but not with other PKC family members. In this report, we identified the p90 protein as FRS2. We have also characterized the factors that regulate its association with the aPKCs. We propose that FRS2 plays an important role in the targeting of activated PKC λ or ζ to the plasma membrane. Thus FRS2 may constitute a third group of proteins that bind to the aPKCs and localize them in specific subcellular compartments. The recruitment of aPKCs by FRS2 to the cell-surface membrane may be an important event contributing to the regulation of the aPKC activity.

**EXPERIMENTAL PROCEDURES**

### Reagents—

**Monoctonal antibodies against phosphotyrosine (PY20), Grb2, SNT1, and ZIP** were purchased from Transduction Laboratories (Lexington, KY). Polyclonal antibodies against PKCα, β, and PKCζ/λ were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies against FRS2 (A872) were raised against amino acids (residues 491–506) and produced by Neosystem Laboratoire (Strasbourg, France). Secondary anti-mouse and anti-rabbit antibodies conjugated to horseradish peroxidase were from Sigma, and protein A/G plus agarose was from Santa Cruz Biotechnology. Anti-activation domain and anti-binding domain antibodies are from CLONTECH (Palo Alto, CA). Recombinant human EGF and PDGF were from Sigma, and basic FGF (bFGF) was from Roche Molecular Biochemicals (Mannheim, FRG). PKCζ, PKCα, PKCδ, and PKA purified enzymes were from Life Technologies, Inc.

**Cell Lines.** Cell stimulation, and lysis—Swiss 3T3 fibroblasts (ATCC CCL92, Rockville, MD) were grown and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), 2 mM glutamine, 10 mM HEPES, pH 7.4, and 100 units/ml penicillin and streptomycin. Human 293T kidney epithelial cells were grown in 150-mm culture dishes with RPMI medium supplemented with 10% fetal bovine serum (HyClone Laboratories). 2 mM glutamine, 10 mM HEPES, pH 7.4, and 100 units/ml penicillin and streptomycin. When the cells were about 80–90% confluent, the medium was aspirated, and the cells were washed and maintained for another 18–24 h in serum-free medium. Various growth factors were added to the quiescent cells prior to aspiration of the medium. The cells were then washed rapidly in cold phosphate-buffered saline and lysed in 500 μl of lysis buffer containing 5.5 mM Nonidet P-40, 20 mM Tris-HCl, pH 7.3, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1 mM sodium orthovanadate, and a mixture of protease inhibitors (Roche Molecular Biochemicals) added according to the manufacturer’s instructions. The cell lysates were spun at 11,000 × g for 5 min at 4°C, and the supernatants were used for subsequent analyses. The protein concentrations of all cell lysates were normalized after estimation of their protein content using a BCA protein assay kit from Pierce.

**Construction of Plasmids—** PKCζ cDNA and HA-tagged PKCζ in pC Duchess were kind gifts from Dr. Jorge Moscat (Universidad Autonoma de Madrid, Spain). PKCβII cDNA and PKCζ cDNA were gifts from Dr. Alexandra Newton (University of California, San Diego) and Dr. Li (National Cancer Institute, Bethesda). Tandem SH2 domain containing cDNAs encoding the full-length PKCλ, PKCλ fragment A (amino acids 1–239), PKCζ fragment B (amino acids 240–586), PKCζ fragment B (amino acids 240–592), PKCζ fragment B (amino acids 354–701), and PKCβII fragment B (amino acids 345–673) were obtained by PCR. These inserts were introduced into pGEX4T1 vector for the expression of GST fusion proteins in bacterial cells. FRS2 cDNA was obtained first by reverse transcription from mRNA extracted from Swiss 3T3 cells. PCR was then carried out with the following primers, which were designed based on the published sequence of FRS2 (2), to obtain the full-length cDNA as follows: (forward) 5’ CGG GGA TCC TCG ATG GGT TGT TGC CGG TGC TG 3’ and (reverse) 5’ CGG GCCG CGCG TCA GAT GGT TGG ACT ATT G 3’. The BankIt/Novf insertion was introduced into pGEX4T1 and pXJ40HA for the expression of GST fusion protein in bacteria cells and HA-tagged proteins in mammalian cells, respectively. The expressed proteins were partially microsequenced and shown to be authentic. The FRS2 fragments X (amino acids 1–152), Y (amino acids 153–300), Z (amino acids 301–508), XX (amino acids 1–300), and YY (amino acids 153–508) were obtained by PCR using the full-length FRS2 cDNA as template. These inserts were cloned for the expression of GST fusion proteins. Human SNT2 cDNA was a kind gift from Dr. Mitchell Goldfarb (Mount Sinai School of Medicine, New York). cDNA encoding the fragment Z (amino acids 281–492) of SNT2 was obtained by PCR and cloned into pGEX4T1 for the production of GST fusion proteins. The cDNA for human FGF receptor 1 (Flg) was a kind gift from Dr. Lena Claesson-Welsh (Ludwig Institute for Cancer Research, Uppsala, Sweden). cDNA encoding the cytoplasmic domain (amino acids 398–822) of Flg was obtained by PCR and cloned into pXJ40Flag for the expression of Flag-tagged cytosolic Flg in mammalian cells.

For yeast two-hybrid screening, cDNAs encoding the full-length fragment A (amino acids 1–239) and fragment B (amino acids 240–586) of PKCζ were obtained by PCR as described above and introduced into CAS vector suitable for yeast transformation and expression of Gal4-binding domain fusion protein. SHP-2 cDNA was a kind gift from Dr. Tony Pawson (Mount Sinai Hospital, Ontario, Canada). Full-length FRS2 and SHP-2 were subcloned into pACT vector for yeast expression of Gal4 activation domain fusion proteins. cDNAs for the tandem SH2 domains (amino acids 1–213) and FTP catalytic domain (amino acids 94–603) of SHP-2 were obtained via PCR, and the inserts were also cloned into the pACT vector.

**Mutagenesis—** Mutagenesis of alanine to glutamate A120E in the pseudo-substrate site of PKC λ was carried out using the QuickChange mutagenesis kit from Stratagene (La Jolla, CA) according to the manufacturer’s instructions. The template used was wild-type full-length PKCλ in pGEX4T1 and pXJ40HA. The primers used were as follows: 5’-GG AAG GAC ACC TCG TGG GAG 3’ and 5’-CTC CAC CGG GTG TCT CTC CGG 3’. The products were sequenced and verified to be correct.

**Transfections—** Human 293T kidney epithelial cells were grown in 100-mm culture dishes as described above. Cells that were about 90% confluent were used for transfection. For single or co-transfections, 15 μg of each DNA followed by 4.5 μg/μl DNA of TSK 50 from Promega (Madison, WI) were added to 6 ml of serum-free RPMI and incubated at room temperature for 15 min. The transfection mix was then added to cells prewashed with serum-free medium and left at 37 °C for 1 h. After this, 12 ml of RPMI supplemented with 10% fetal bovine serum was added, and the cells were left to recover for 48 h. The cells were lysed with RIPA buffer (50 mM Tris-HCl, pH 7.3, 150 mM NaCl, 0.25 mM EDTA, 1% sodium deoxycholate, 1% Triton X-100, 1 mM sodium orthovanadate, and a mixture of protease inhibitors from Roche Molecular Biochemicals) and processed as described under “Cell Lines, Cell Stimulation, and Cell Lysis.”

**GST Fusion Proteins—** All the constructs for the production of GST fusion proteins were transformed into DH5α cells. The transformed cells were grown in 1 liter of LB containing ampicillin (50 μg/ml) and incubated at 37 °C with shaking (220 rpm) for 12–20 h. The cells were then induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside at room temperature overnight. The cells were spun down and frozen at −80°C. The cell pellet was then thawed on ice, and 10 ml of lysis buffer (phosphate-buffered saline, 1% Triton X-100, 1 mM dithiothreitol, and a mixture of protease inhibitors from Roche Molecular Biochemicals) was added to the cell. The cell suspension was subse-
quently sonicated for a total of 12 pulses of 15 s with a 30-s pause between each pulse. The lysates were centrifuged and supernatants were incubated with glutathione beads overnight at 4 °C to purify the GST fusion proteins.

**Immunoprecipitations, in Vitro Binding Assays**—Quiescent or activated cells were lysed as described above, and an equal volume of 2× precipitation buffer (20 mM Tris-HCl, pH 7.4, 300 mM NaCl, 2% Triton X-100, 2 mM EDTA, 2 mM EGTA, and 1% Nonidet P-40) was added to the cell lysate. For immunoprecipitation, 2.5 µg of the appropriate antibodies were added to the diluted cell lysate and incubated for 1 h or overnight at 4 °C. 2.5 µg of secondary antibodies conjugated to agarose was added to capture the immunocomplex for 1 h or overnight at 4 °C. In the depletion studies, the immunoprecipitation described above was repeated 5 times, each for 1.5 h. After washing, the immunoprecipitates were pooled together and resolved by SDS-PAGE.

For in vitro binding assays with GST fusion proteins, 10 µg of the GST fusion proteins were incubated with the lysates for 1 h or overnight at 4 °C. All the beads were washed 5 times with 1× precipitation buffer, and the bound proteins were eluted with 2× Laemmli buffer before separation by SDS-PAGE.

**Yeast Two-hybrid Analysis**—The various constructs of PKC λ (full length, fragment A, and fragment B) in pAS were sequenced and verified to be correct before they were introduced into yeast strain 190 using the yeast transformation kit from CLONTECH (Palo Alto, CA). The transformants were grown in selective media SD-Trp at 30 °C, and colonies appeared. Single transformants were then subjected to a second round of transformation with the pACT vectors containing full-length FRS2, full-length SHP-2, SH2 domains, or PTP catalytic domain of PTP. The dual transformants were also identified to be correct before they were introduced into yeast strain 190 using the yeast transformation kit manufacturer's instructions, and the lysates were prepared according to the manufacturer’s instructions, to detect for protein interactions in the yeast. Yeast transformation was carried out as described elsewhere (4).

**Results**

**FRS2 Co-precipitates with PKC λ in Response to FGF Stimulation**—Our laboratory has been characterizing p75 and its association with Grb2. In quiescent cells, p75 is tyrosine-phosphorylated and binds to the SH2 domain of Grb2. Upon stimulation with growth factors including FGF, p75 is dephosphorylated and dissociates from Grb2 (27). We are keen to identify p75 and to test existing phosphotyrosyl antibodies that are about 75 kDa for dephosphorylation upon FGF treatment. One of the candidates that we selected was PKC δ, a member of the nPKC subfamily which is about 78 kDa and is the only PKC member currently known to be tyrosine-phosphorylated (33). We therefore set out to investigate whether PKC δ is tyrosine-phosphorylated in quiescent cells. Representative members, namely PKC α and PKC λ, from the cPKC and aPKC subfamilies, respectively, were also included for comparison.

Preliminary studies in our laboratory have shown that two FGF-responsive cell lines, Swiss 3T3 and PC12 cells, expressed all the three PKCs of interest. Swiss 3T3 cells were chosen for further experiments because they respond better to bFGF than PC12 cells. To examine whether PKC δ, PKC α, or PKC λ could be the p75 that undergoes dephosphorylation upon growth factor stimulation, immunoprecipitation of the various PKCs was carried out on lysates on Swiss 3T3 cells that were either untreated or stimulated with bFGF. The immunoprecipitates were resolved by SDS-PAGE and Western blotted. The membrane was probed with phosphotyrosine antibodies to detect for the presence of tyrosine-phosphorylated PKCs. None of the PKCs was tyrosine-phosphorylated in the lysates of quiescent cells (Fig. 1A, upper panel). The blot was stripped and reprobed either with PKC α, PKC δ, or PKC λ antibodies to show that the individual PKCs had been immunoprecipitated (Fig. 1A, lower panel). We conclude that none of the PKCs tested are likely to be p75.

In these experiments, we noted that a 90-kDa tyrosine-phosphorylated protein, similar to an FGF-specific p90 protein that has been reported to bind Grb2 (34), was co-immunoprecipitated with PKC λ (Fig. 1A, upper panel). Neither PKC δ nor PKC α co-precipitated this tyrosine-phosphorylated protein significantly when compared with PKC λ in the lysates from bFGF-stimulated cells. We therefore decided to investigate this apparently specific association.

It is possible that in the experiments carried out above, differential amounts of the various PKCs were immunoprecipitated by the antibodies due to the different affinities of the individual antibodies for their respective PKCs. The apparently larger amount of p90 co-immunoprecipitated with PKC λ may due to higher amounts of PKC λ immunoprecipitated compared with the other PKCs. Hence, it was necessary to ensure that the majority (>80%) of each PKC was immunoprecipitated. Preliminary optimization showed that five successive rounds of immunoprecipitation were enough to deplete 80% or more of the various PKCs (data not shown). Therefore, five rounds of immunoprecipitation of PKC λ, PKC α, and PKC δ (as described under “Experimental Procedures”) were carried out on lysates from Swiss 3T3 cells that have been stimulated with bFGF. The immunoprecipitates were pooled, resolved by SDS-PAGE, and transferred to a polyvinylidene difluoride membrane. The membrane was then probed with phosphotyrosine antibodies to detect p90. Fig. 1B, top panel, shows that p90 co-immunoprecipitated only with PKC λ. The blot was stripped and cut between lanes and probed for the various PKCs immunoprecipitated (Fig. 1B, middle panel). The amounts of the various PKCs present in the lysates before and after multiple immunoprecipitations were assessed by Western blot analyses. Fig. 1B, bottom panel, shows that more than 80% of PKC λ, PKC α, or PKC δ were immunoprecipitated. Therefore, the co-immunoprecipitation of p90 with PKC λ is not due to a relatively larger proportion of PKC λ being immunoprecipitated compared with PKC α or PKC δ.

The molecular mass and the gel migration pattern of the 90-kDa tyrosine-phosphorylated protein resembled that of FRS2, a protein that our laboratory is currently studying. To determine whether the p90 protein was FRS2, lysates from Swiss 3T3 cells that were untreated or stimulated with bFGF were subjected to immunoprecipitation of PKC λ. The immu-
Association of Atypical PKCs with FRS2

**Fig. 1.** A, co-immunoprecipitation of a tyrosine-phosphorylated 90-kDa protein with PKC λ. Quiescent Swiss 3T3 cells were either not stimulated or stimulated with 10 ng/ml bFGF for 10 min. The cells were lysed and the lysates subjected to immunoprecipitation (IP) of PKC λ, PKC δ, or PKC α as described under “Experimental Procedures.” Upper panel, the immunoprecipitates were separated by SDS-PAGE and immunoblotted (IB) with phosphotyrosine antibodies (PY20). The arrows indicate the positions of p90 and p75. Lower panel, the blot was stripped and re-probed with PKC λ, PKC δ, or PKC α antibodies to reveal the amount of the various PKCs immunoprecipitated. B, multiple immunoprecipitations of PKC λ, PKC α, and PKC δ. Swiss 3T3 cells were stimulated with bFGF (10 ng/ml) for 10 min and lysed. The lysates were subjected to 5 successive rounds of immunoprecipitation of PKC λ, PKC δ, and PKC α as described under “Experimental Procedures” before the immunoprecipitates for each PKC were pooled, resolved by SDS-PAGE, and Western blotted. Top panel, the membrane was probed with phosphotyrosine antibodies. Middle panel, the blot containing the immunoprecipitates was cut into strips and re-probed either with PKC λ, PKC α, or PKC δ antibodies. These individual strips were then re-aligned and the respective PKCs detected using the ECL reagents to reveal the proteins immunoprecipitated. Bottom panel, the immunoprecipitation efficiency for each of the PKCs was assessed by probing the lysates before (Pre) or after (Post) successive rounds of immunoprecipitation with the respective antibodies. The arrows indicate the positions of the immunoprecipitates were processed as described above. The blot was first probed with phosphotyrosine antibodies revealing the 90-kDa tyrosine-phosphorylated protein co-precipitating with PKC λ upon bFGF stimulation (Fig. 1C, top panel). The blots were stripped and re-probed with A872, a polyclonal antibody raised against FRS2. As shown in Fig. 1C, middle panel, FRS2 co-precipitated with PKC λ from lysates of bFGF-stimulated but not non-stimulated cells. It is noted that phosphotyrosine signal for p90 that co-immunoprecipitated with PKC λ (Fig. 1C, top panel) is greater than that of FRS2 co-immunoprecipitated with PKC λ (Fig. 1C, middle panel). This is attributed to the observation that FRS2 is a multiply tyrosine-phosphorylated protein with at least 6 tyrosine phosphorylation sites. By comparing the amount of FRS2 in the total lysate with the amount co-immunoprecipitated with PKC λ in other independent experiments (data not shown), it is estimated that the amount of FRS2 co-immunoprecipitated with PKC λ was approximately 5%. Probing this blot with PKC λ antibodies showed equal amounts of PKC λ being immunoprecipitated from both lysates (Fig. 1C, bottom panel). The experiment in Fig. 1C has also been repeated by probing the blot first with FRS2 antibodies followed by anti-phosphotyrosine antibodies. Similar results were obtained (data not shown). This demonstrated that FRS2 and PKC λ exist in a complex following bFGF stimulation of Swiss 3T3 cells. The association of FRS2 with PKC λ in FGF-stimulated cells might be 1) mediated by other proteins, 2) dependent on the activation of PKC λ, 3) dependent on the tyrosine phosphorylation of FRS2, or 4) a combination of some or all the above factors. We therefore set out to address these possibilities.

**FRS2 Binds to the Catalytic Domain of PKC λ**—Although FRS2 co-immunoprecipitated with PKC λ, it is possible that the association of PKC λ with FRS2 is mediated through other proteins in the immunoprecipitated complex. We have shown previously that SHP-2 associates with FRS2, and it is possible that PKC λ associates directly with SHP-2 and not FRS2. Grb2 also binds to FRS2 but experiments showed that Grb2 was not present in complexes containing FRS2 and PKC λ (data not shown). We employed the yeast two-hybrid technique to investigate whether FRS2 is likely to bind to PKC λ directly. The PKC λ protein exists in a “closed,” catalytically inactive state in non-stimulated cells. In addition to full-length PKC λ, two other fragments that contained the regulatory domain (fragment A) or the catalytic domain (fragment B) of PKC λ were generated. These fragments would theoretically “expose” potential regions in the protein that are normally masked. PKC λ fragment A contains amino acids 1–239 and encompasses the N terminus extension, pseudo-substrate site, zinc finger, and part of the hinge region; fragment B contains amino acids 240–586 and includes part of the hinge, the kinase domain, and carboxyl tail. Plasmids containing these proteins were transformed into yeast as described under “Experimental Procedures.” To study the interaction of FRS2 with the various fragments of PKC λ, yeast expressing the various PKC λ proteins underwent another transformation with an expression vector encoding full-length FRS2. In addition, the SHP-2 full-length, the tandem N- and C-terminal SH2 domains, and the PTP catalytic domain of SHP-2 were also separately introduced to assess their binding to various PKCs.

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**bFGF**

**IB:** anti-PY

**IB:** anti-FRS2

**IB:** anti-PKC λ

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**Pre Post**

**IB:** PKC λ

**IB:** PKC δ

**IB:** PKC α

---

**FGF**

**+**

**-**

**+**

**-**

---

**lysates:**

**Pre Post**

**IB:** PKC λ

**IB:** PKC α

**IB:** PKC δ

---

**C**

---

**IB:** anti-PY

**IB:** anti-FRS2

**IB:** anti-PKC λ

---

**IP:** PKC λ

**IP:** PKC δ

**IP:** PKC α
3T3 cells were incubated with the fragment B of PKC\(\lambda\) and \(\xi\) but not PKC\(\beta\) II. A, quiescent Swiss 3T3 were stimulated with 10 ng/ml bFGF for 10 min and the cells lysed. The lysates were incubated with 10 \(\mu g\) of GST fragment A or fragment B (containing the catalytic domain) of PKC\(\lambda\) as described under “Experimental Procedures.” The precipitates were washed, eluted, and separated on SDS-PAGE followed by immunoblotting (IB) using phosphotyrosine antibodies. B, lysates from bFGF-stimulated cells were incubated with the fragment B of PKC\(\lambda\), PKC\(\xi\), or PKC \(\beta\) II as described above. The precipitates were resolved by SDS-PAGE, Western blotted, and the membrane probed with phosphotyrosine antibodies (upper panel). The amounts of the various GST fusion proteins used for this precipitation experiment are shown by Coomassie Blue staining (lower panel). PD, pull-down.

Table I

| Yeast two-hybrid | LacZ assay |
|------------------|------------|
| pAS PKC\(\lambda\)/pACT FRS2 | White |
| pAS PKC\(\lambda\)/pACT SHP-2 | White |
| pAS PKC\(\lambda\)/pACT SH2 SHP-2 | White |
| pAS PKC\(\lambda\)/pACT PTP SHP-2 | White |
| pAS fragment A PKC\(\lambda\)/pACT FRS2 | White |
| pAS fragment A PKC\(\lambda\)/pACT SHP-2 | White |
| pAS fragment A PKC\(\lambda\)/pACT SH2 SHP-2 | White |
| pAS fragment A PKC\(\lambda\)/pACT PTP SHP-2 | White |
| pAS fragment B PKC\(\lambda\)/pACT FRS2 | Blue |
| pAS fragment B PKC\(\lambda\)/pACT SHP-2 | White |
| pAS fragment B PKC\(\lambda\)/pACT SH2 SHP-2 | White |
| pAS fragment B PKC\(\lambda\)/pACT PTP SHP-2 | White |

Following co-transformation of yeast strain Y190 with a combination of plasmids shown in the table, dual transformants were selected on SD-Trp/Leu-selective media. Colonies that grew were subjected to LacZ assay as described under “Experimental Procedures” to test for the induction of \(\beta\)-galactosidase activity that results from interaction between two proteins expressed in the yeast. Blue colonies indicate a positive interaction, and white indicates a negative interaction.

to PKC\(\lambda\). In all cases, colonies expressing all combination of proteins were obtained (data not shown) and were subjected to LacZ assays. Yeast transformed with pCL1 expressing functional Gal4 protein turned blue between 0.5 and 1 h. This serves as a positive control for the LacZ assay. Colonies turning blue after 8 h were considered negatives according to the manufacturer’s instruction. In the above assays, only yeast expressing FRS2 and PKC\(\lambda\) fragment B (encompassing the kinase domain) turned blue (at 2.5 h), indicating a strong interaction (Table I). Of particular note was the observation that full-length PKC\(\lambda\) and fragment A containing the PKC\(\lambda\) regulatory domain did not interact with FRS2. Thus, FRS2 interacts with PKC\(\lambda\) through a region (amino acids 240–586) that is predominantly the catalytic domain. This region is probably masked in the non-activated, full-length molecule since the latter cannot interact with FRS2. The observation that the full-length SHP-2, the tandem SH2 domains, and the PTP catalytic domains of SHP-2 did not interact with any of the PKC\(\lambda\) polypeptides indicates that the interaction between FRS2 and PKC\(\lambda\) is both specific and most likely direct. FRS2 is not likely to be an activator of PKC\(\lambda\) since the well-characterized activators of the kinase bind to the regulatory region of the kinase (16, 17).

To verify the yeast two-hybrid results, GST-PKC\(\lambda\) fragment A and GST-PKC\(\lambda\) fragment B were tested for their ability to bind to FRS2. Since FRS2 co-immunoprecipitated with PKC\(\lambda\) only upon FGF stimulation, Swiss 3T3 cells were stimulated with bFGF and the lysates incubated with either GST-PKC\(\lambda\) fragment A or fragment B. An aliquot of the lysate was also incubated with GST as a control. The precipitates were resolved by SDS-PAGE and Western blotted. The presence of FRS2 in the precipitates was detected by probing the membrane with phosphotyrosine antibodies rather than FRS2 antibodies because of the ease of detection as well as the avoidance of the high nonspecific background signal encountered when using FRS2 antibodies. Consistent with the yeast two-hybrid results, Fig. 2A shows that fragment B of PKC\(\lambda\) is responsible for binding to FRS2. The amounts of tyrosine-phosphorylated FRS2 seen to bind to PKC\(\lambda\) fragment A and GST represent background signal.

To determine whether the binding of FRS2 to PKC\(\lambda\) fragment B is specific, various GST fusion proteins were prepared for in vitro binding assays. Lysates of bFGF-stimulated Swiss 3T3 cells were incubated with the fragment B of PKC\(\lambda\) or, for comparison, fragment B of PKC \(\beta\) II (a member of the cPKC family) and PKC \(\xi\) (the other member of the aPKC). The precipitates were resolved by SDS-PAGE and immunoblotted with phosphotyrosine antibodies to detect for tyrosine-phosphorylated FRS2. Fig. 2B, top panel, shows that the fragment B of PKC\(\lambda\) but not that of PKC \(\beta\) II precipitated tyrosine-phosphorylated FRS2. Fragment B of PKC\(\xi\), the other member of the atypical PKCs (aPKCs), also bound tyrosine-phosphorylated FRS2. This is not surprising given the observation that the fragment B containing the kinase domains of PKC\(\lambda\) have more than 85% identity with PKC\(\xi\). On the other hand, the kinase domain of PKC \(\beta\) II did not bind significant amounts of FRS2 compared with the aPKCs. The fragment B of PKC \(\delta\) also did not bind FRS2 significantly (data not shown). This reflects a lack of affinity in the fragment B of cPKCs and nPKCs for FRS2. Together, the above results demonstrate that only the members of the atypical PKC subfamily interact with FRS2 through a region that encompasses the catalytic domain, and this interaction is most likely to be direct.

PKC\(\lambda\) Binds to a Region in the C Terminus of FRS2—To define the region on FRS2 that binds to PKC\(\lambda\), GST fusion proteins containing various fragments of FRS2 were produced (Fig. 3A). Lysates of human 293T cells grown in 10% serum were incubated with each of the GST-FRS2 polypeptides overnight at 4°C. The precipitates were washed and resolved by SDS-PAGE and immunoblotted with PKC\(\lambda\) monoclonal antibodies (Fig. 3B). Fragment Z (amino acids 301–508) of FRS2 precipitated a comparatively larger amount of PKC\(\lambda\) compared with fragments X and Y. Consistent with this observation, full-length FRS2 and fragment YZ but not fragment XY could also bind PKC\(\lambda\). However, full-length FRS2 and FRS2 fragment YZ bind slightly lesser PKC\(\lambda\) than fragment Z, suggest-
binding assays similar to those described above were performed. PKC λ showed essentially equal binding to each of the subfragments of fragment Z (data not shown), indicating that binding occurs at multiple points within this peptide sequence.

Next, we investigated whether SNT2, an isoform that has 50% identity to FRS2, can bind to PKC λ. A GST fusion protein of SNT2 fragment Z (amino acids 281–492), whose sequence was aligned with that of FRS2 fragment Z, was produced and assessed for its binding to PKC λ. The fragment Z from FRS2 and SNT2 share about 50% identity. GST full-length FRS2 and GST fragment Z of FRS2 were included for comparison, and a similar experiment to that described above was carried out. Fig. 3C shows that fragment Z of SNT2 could bind endogenous PKC λ as well as fragment Z from FRS2.

“Activated” PKC λ Binds to FRS2—By having demonstrated that the fragment B of PKC λ binds to the carboxyl portion of FRS2, we proceeded to investigate whether activation of PKC λ was necessary for its association with FRS2. First, we examined the activation status of PKC λ by FGF. Swiss 3T3 cells were either not stimulated or stimulated with bFGF. The cells were lysed, and immunoprecipitation of PKC λ was performed on these lysates. Subsequently, the immunoprecipitated PKC λ was tested for in vitro kinase activity toward hnRNPA. hnRNPA1 is the only convincing aPKC substrate identified so far (19). Fig. 4A showed that bFGF stimulated the activity of PKC λ by about 3-fold. This level of activation is comparable to that obtained by stimulating NIH3T3 cells with PDGF (19).

The opening of the otherwise closed PKC λ protein as a result of activation by FGF may expose sites that are necessary for its association with FRS2. This is consistent with the yeast two-hybrid results (Table I) where only fragment B of PKC λ binds to FRS2. Apparently, the masking of this fragment in the inactive, full-length PKC λ contributed to its inability to bind FRS2.

We postulated that if the inactive PKC λ could be “artificially” opened up, thus exposing the sites represented on fragment B, full-length PKC λ might acquire the ability to bind FRS2. It has been shown previously that mutation of the alanine residue in the pseudo-substrate site of PKC λ to glutamate would switch the kinase to a constitutively active form (21, 22). We therefore set out to investigate the relative affinities of the constitutively active PKC λ A120E mutant and the wild-type PKC λ for tyrosine-phosphorylated FRS2. Although the data from yeast two-hybrid analysis showed that non-tyrosine-phosphorylated FRS2 can bind to fragment B of PKC λ, tyrosine-phosphorylated FRS2 was used in the assay because tyrosine phosphorylation may enhance the binding to PKC λ (refer to Fig. 1C). To eliminate any potential contribution of SNT2, we transfected 293T cells with HA-tagged FRS2 (HA-FRS2). We have previously noted that the overexpression of the cytosolic fragment of FGFR1 (Flg) leads to the activation of the endogenous tyrosine kinase activity without the addition of exogenous bFGF (data not shown). Hence, to obtain tyrosine-phosphorylated HA-FRS2, 293T cells were co-transfected with the cytosolic fragment of Flg (Flg-cyto) and HA-FRS2. Total cell lysates were separated on SDS-PAGE and Western blotted. Probing the blot with FRS2 (A872) antibodies showed that the level of HA-FRS2 was much higher than the endogenous FRS2, which was present in very low abundance (data not shown). Immunoprecipitation with HA antibodies showed that the expressed HA-FRS2 was tyrosine-phosphorylated (data not shown).

To assess the binding of the constitutively active PKC λ mutant or wild-type PKC λ to tyrosine-phosphorylated FRS2, 293T cells were co-transfected with HA-FRS2 and the cytosolic fragment of Flg. The cell lysates containing tyrosine-phosphorylated HA-FRS2 were then incubated with equal amounts of GST-PKC λ A120E mutant or GST-wild-type PKC λ. The GST

![Diagram showing the various GST fusion proteins of FRS2 produced.](image_url)
Figure 4. A, PKC λ is activated by bFGF in Swiss 3T3 cells. Swiss 3T3 cells were either non-stimulated or stimulated with bFGF at 20 ng/ml for 10 min before the cells were lysed. The lysates were subjected to immunoprecipitation using PKC λ antibodies. After the immunoprecipitates were washed, they were used for in vitro kinase assays using eluted hnNPA1 as substrate as described under “Experimental Procedures.” After the reaction, the proteins were resolved by SDS-PAGE. The gel was dried and exposed to x-ray film (Fuji). The phosphorylated protein bands obtained were quantitated using a densitometer (Bio-Rad), and the relative activity of PKC λ is shown. The values shown represent the average ± half the range. B, constitutively active PKC λ A120E mutant binds more HA-tagged FRS2 than wild-type inactive PKC λ. 293T cells were co-transfected with HA-tagged FRS2 (HA-FRS2), and the cytosolic domain of Flg (Flg-cyto). The cells were lysed after 48 h of recovery and the lysates incubated with 10 μg of GST fusion proteins of PKC λ A120E mutant (mut), wild-type (wt) PKC λ, PKC βII fragment B, and PKC λ fragment B as described under “Experimental Procedures.” The precipitates were washed, eluted, and separated on SDS-PAGE. After Western blotting the blot was probed with phosphorytose antibodies to reveal the amounts of tyrosine-phosphorylated FRS2 precipitated by the various fusion proteins. C, GST-FRS2 fragment Z binds with a higher affinity to HA-tagged PKC λ fusion proteins containing the fragment B of PKC λ or PKC βII were also included for comparison. The precipitates were separated by SDS-PAGE, Western blotted, and the membrane probed with phosphorytose antibodies. Fig. 4B revealed that the constitutively active PKC λ mutant binds much more tyrosine-phosphorylated FRS2 protein than its wild-type counterpart. The amount of FRS2 bound by wild-type PKC λ was about the same as that bound by PKC βII and is likely to represent the background level of binding as we have shown previously that FRS2 does not associate with this PKC isoform (Fig. 2B). In addition, the PKC λ fragment B is able to bind as much FRS2 as PKC λ A120E mutant indicating that fragment B of the PKC λ molecule is sufficient for binding FRS2 without the cooperation of other parts of the molecule. A similar experiment was also carried out using lysates from Swiss 3T3 cells that were stimulated with FGF. The lysates were incubated with GST-PKC λ A120E mutant or GST-wild-type PKC λ, and the precipitates were probed with phosphorytose antibody to detect endogenous tyrosine-phosphorylated FRS2. The results (data not shown) were essentially the same as Fig. 4B.

The in vitro binding of mutant and wild-type PKC λ to FRS2 was also assessed using GST-FRS2 fragment Z, and the lysates of 293T cells were transfected with HA-tagged wild-type or constitutively active mutant PKC λ. The transfected cells expressed equivalent amounts of HA-tagged mutant and wild-type PKC λ (Fig. 4C, top panel). Following incubation of the cell lysates with GST-FRS2 fragment Z, the precipitated proteins were separated on SDS-PAGE, Western blotted, and probed with HA antibodies. As shown in Fig. 4C (bottom panel), more mutant compared with the wild-type PKC λ was bound to GST-FRS2 fragment Z. This strengthens the observation that constitutively active PKC λ A120E mutant has a stronger affinity for FRS2 than the inactive wild-type PKC λ and indicates that activation of PKC λ is required to bind to FRS2.

Tyrosine Phosphorylation of FRS2 Is Not Required for Association with aPKC—Although the activation of PKC λ can account for the induced association of the two proteins, we cannot exclude the possibility that tyrosine phosphorylation of FRS2 is also a factor regulating this association. Therefore, we set out to investigate the role of tyrosine phosphorylation in the FRS2/PKC λ interaction. 293T cells were transfected with either HA-FRS2 alone or with cytosolic Flg, allowing expression of nonphosphorylated or tyrosine-phosphorylated HA-FRS2. The cells were lysed and the lysates incubated with equal amounts of GST fusion proteins containing the constitutively active PKC λ mutant or wild-type PKC λ. An equivalent amount of agarose-conjugated GST protein was included as a control. The precipitates were separated by SDS-PAGE and immunoblotted with phosphorytose antibodies to detect FRS2. As expected, tyrosine-phosphorylated HA-FRS2 was precipitated by mutant PKC λ. Very low levels of tyrosine-phosphorylated FRS2 were seen with wild-type PKC λ and GST alone, and these represented background signals (Fig. 5, top panel).
Association of Atypical PKCs with FRS2

When the blot was stripped and re-probed with FRS2 (A872) antibodies (Fig. 5, bottom panel), equivalent amounts of FRS2 were shown to be precipitated by the PKC λ A120E mutant regardless of whether FRS2 was tyrosine-phosphorylated or not. Very low and equal amounts of FRS2 signal were detected in all the lanes containing wild-type PKC λ or GST only after prolonged exposure of the blot (data not shown) and is unlikely to be significant. The association of PKC λ with FRS2 is therefore independent of tyrosine phosphorylation.

FRS2 Is Not an in Vitro Substrate of aPKCs—FRS2 has been reported to be serine/threonine-phosphorylated (4). We have also shown here that stimulation of Swiss 3T3 cells with FGF leads to the activation of PKC λ and the binding of FRS2 or SNT2 to regions in PKC γ and PKC λ that contain the catalytic domain. All the above evidence suggests that FRS2 is a likely substrate of PKC λ. We therefore addressed the enzyme-substrate relationship between PKC λ/γ and FRS2. We used hnRNPA1 and/or myelin basic protein (a general substrate for PKC) as positive controls. GST full-length FRS2 or FRS2 fragment Z were tested as substrates for the aPKCs. In vitro kinase assays, as described under “Experimental Procedures,” were carried out using purified PKC λ or HA-tagged PKC λ fragment B (containing the kinase domain) or HA-tagged PKC λ A120E constitutively active mutant as a source of kinase activities. Fig. 6A (top left panel) revealed that hnRNPA1 and MBP are phosphorylated by purified PKC λ, but full-length FRS2 is not. In addition, although the HA-tagged PKC λ kinase domain and PKC λ A120E constitutively active mutant were active against MBP, they did not phosphorylate full-length FRS2 or FRS2 fragment Z, which is known to bind more strongly to PKC λ than the full-length FRS2 (Fig. 6A, lower panels). To investigate whether post-translational modification of FRS2 was necessary for it to be a substrate of the aPKCs, FRS2 was immunoprecipitated from Swiss 3T3 cell lysates that had been either non-stimulated or stimulated with bFGF. In vitro kinase assays with PKC λ enzyme or with enzymatically active HA-tagged PKC λ proteins were carried out with the immunoprecipitated tyrosine-phosphorylated or non-phosphorylated FRS2. Again, the aPKC enzymes failed to phosphorylate either form of FRS2 (data not shown).

To show that the GST-FRS2 proteins could be an authentic substrate in vitro, GST full-length FRS2 was tested as a substrate for protein kinase A (PKA). FRS2 contains potential PKA phosphorylation sites (Ser-366 and Ser-429), and preliminary studies had shown that it was a likely substrate of that kinase. The hnRNPA1 protein was added as a positive control.
as it can also be phosphorylated by PKA (20). Myelin basic protein was also included in the assay. Fig. 6A (top right panel) shows that full-length GST-FRS2 could be phosphorylated by PKA to a greater degree than hnRNPA1. We have also performed in vitro kinase assays with purified PKC \( \alpha \) and PKC \( \delta \) to determine whether FRS2 might be a substrate for PKCs from other subfamilies. Fig. 6B shows that whereas both PKC \( \alpha \) (left panel) and PKC \( \delta \) (right panel) were able to phosphorylate MBP, they either failed to phosphorylate FRS2 (PKC \( \alpha \)) or they phosphorylated FRS2 only very weakly (PKC \( \delta \)). Taken together, these results demonstrate that whereas FRS2 was phosphorylated by PKA, it is not an in vitro substrate and hence is an unlikely in vivo substrate for the PKCs.

**DISCUSSION**

The concept of non-receptor, tyrosine-phosphorylated proteins serving as dockers was conceived when the IRS family of proteins was discovered and characterized. Gradually, more proteins were identified that fall under this description. Like IRS proteins, Gab-1 and Dos possess modules such as pleckstrin homology or phosphotyrosine-binding (PTB) domains that help in their membrane localization (7). More recently, another docker protein named FRS2 has been identified and cloned (2). It has a myristoylation site for membrane association, an N-terminal phosphotyrosine-binding domain (PTB) for protein-protein interactions, and multiple tyrosine residues that are targets for phosphorylation by the FGFRs upon ligand binding. FRS2 has been reported to bind to the SH2 domains of Grb2 (2) and SHP-2 in a tyrosine phosphorylation-dependent manner (8), and these associations exert a significant influence on the MAP kinase cascade. Docker proteins, including FRS2, have been shown to play important roles as initiation centers for diverse signaling pathways. Identification of proteins that bind to FRS2 will therefore contribute to the understanding of the signal transduction of FGF in cells.

In our studies, we found that about 5% of total FRS2 co-immunoprecipitated specifically with PKC \( \lambda \), a member of a subfamily of the family of PKC kinases called the atypical PKCs (aPKCs). The interaction between FRS2 and PKC \( \lambda \) was shown to be mediated by a region (fragment B) in the aPKCs that encompasses the catalytic domain. We have also shown that activation of the aPKCs is necessary for its association with FRS2. It can be construed that the absence of co-immunoprecipitation of FRS2 with the cPKCs or nPKCs may be due to the inability of FGF to stimulate the various cPKCs and nPKCs. However, the B fragments of cPK (e.g. PKC \( \beta II \)) and nPK (e.g. PKC \( \delta \)) did not have the affinity to bind FRS2 and are unlikely to bind FRS2 even if they were activated. The sequence identities between the fragment B of PKC \( \lambda \) and those of other members were 86% for PKC \( \zeta \) and 44–55% for other PKC members. The greater amino acid sequence homology between the aPKCs is sufficient to provide specificity for their binding to FRS2 but not for the more distantly related members of the PKC family.

The association between aPKCs and FRS2 is not that of an enzyme-substrate relationship. Only one strong in vitro substrate of the aPKCs has so far been identified. hnRNPA1 protein was identified in a yeast two-hybrid screen using the PKC \( \zeta \) kinase domain as bait (19). The optimal peptide sequence, determined by peptide library screening, for phosphorylation by aPKC is \( RFFKRQGS(S)PFFYFF \) (where boldface indicates the motif required for phosphorylation and boldface italic indicates phosphorylation at serine) (23). This is similar to the motif on hnRNPA1 that surrounds the phosphorylated serine residue SQGRRSGS(P)GNFGG. It is crucial to have the basic and hydrophobic amino acid residues to the N and C terminus of the core sequence RXGS, respectively. Such a sequence was not found in FRS2, and this validates our experimental data. Although SNT2 seems to possess one such potential motif PL-TRG(S/P)PRVFNFD, it is only very weakly phosphorylated by the aPKCs (data not shown).

FRS2 is located at the plasma membrane of cells and associates with FGF receptors in a tyrosine phosphorylation-independent manner (5, 6). It is possible that FRS2 may recruit the aPKCs to substrates in the vicinity of the FRS2-receptor complex at the cell-surface membrane. Proteins that are associated with FRS2 would therefore be potential substrates. SHP-2 has a potential PKC \( \lambda \) phosphorylation site, AGIRRTGT(P)?TFFIV (where \( P? \) indicates a potential threonine phosphorylation site). In vitro phosphorylation studies, however, showed that SHP-2 is not a substrate of PKC \( \lambda \). Furthermore, yeast two-hybrid assays did not show any association between PKC \( \lambda \) and SHP-2. The FGF receptor was excluded as a plausible substrate because it does not possess any potential aPKC phosphorylation sites in the cytosolic region. The identification and characterization of additional FRS2-associated proteins may lead to the identification of novel PKC \( \lambda \) substrates.

The association of aPKCs or SHP-2 with FRS2 has a common feature. Both the aPKCs and SHP-2 are enzymes but their binding partner, FRS2, is not a substrate for either the kinase or the phosphatase. On the other hand, the aPKCs and SHP-2 show differences in their manner of binding to FRS2. The binding of SHP-2, via its N-terminal SH2 domain, to FRS2 is dependent on tyrosine-phosphorylated residues in FRS2, and this interaction is necessary for the activation of the phosphatase (24). In contrast, the binding of PKC \( \lambda \) to FRS2 is not dependent on tyrosine phosphorylation of FRS2 but dependent on the activation of PKC \( \lambda \). Hence, although FRS2 is both an activator and locat or protein for SHP-2, it is likely to be only a locat or protein for the activated aPKCs. However, we cannot rule out other possible functions FRS2 plays in the regulation of aPKCs such as post-translational modification.

Preliminary experiments suggested that FRS2 is unlikely to be an inhibitor of aPKC activity since incubation of a reaction mixture containing PKC \( \zeta \) and hnRNPA1 with FRS2 did not block phosphorylation of hnRNPA1 (data not shown). Although we cannot totally exclude the possibility that FRS2 is an inhibitor for aPKCs, we suspect that the role of FRS2 in binding the aPKCs is similar to that of a group of proteins called RACKs (Receptors for Activated C Kinase). The RACKs have been proposed to anchor PKC at specific locations in the cell (25, 26). Like FRS2, these proteins have been shown to bind to the active conformation of the PKC but are themselves not substrates for the PKC.

All PKCs appear to be activated at the plasma membrane with phosphatidylinositol being an important co-activator for all members of the greater family. In addition, the aPKCs are activated by particular inositol phospholipids (29). PKC \( \zeta \) has also received considerable recent attention as a target for PI-3 kinase (31, 32). Two reports show that the phosphoinositide-dependent protein kinase 1 (PDK-1), which binds with high affinity to the PI-3 kinase lipid product phosphatidylinositol 3,4,5-trisphosphate, phosphorylates and potently activates PKC \( \zeta \) along with two other substrates, also kinases, Akt/PKB and p70S6K. PKC \( \zeta \) and PDK-1 are associated in vivo, and membrane targeting of PKC \( \zeta \) renders it constitutively active in cells. The association between PKC \( \zeta \) and PDK-1 reveals extensive cross-talk between enzymes in the PI-3 kinase pathway (28, 30). Evidence has been presented previously indicating that the IRS protein associates with enzymes involved in the PI-3 kinase pathway (7). It is possible that the strategic membrane locations of the IRS and FRS2 docker proteins may see them playing a central role in the PI-3-kinase pathway as well.
as the MAP kinase pathways and the likely interactions between the two pathways.

In conclusion, the association of activated aPKCs to FRS2 may serve to target the aPKCs to specific sites on the plasma membrane where their substrates are located or where their activities are regulated. Interestingly, the three proteins Grb2, SHP-2, and PKC θ that are recruited by FRS2 to the membrane are implicated in the MAP kinase pathway at different positions (either upstream or downstream of Ras). The association may be part of a large multimeric signaling complex where signals can be integrated and “fine-tuned,” resulting in their propagation from the cell surface to the nucleus.

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REFERENCES

1. Jaye, M., Schlessinger, J., and Diane, C. A. (1992) Biochim. Biophys. Acta 1135, 185–199
2. Kouhara, H., Hadari, Y. R., Spivak, T., Schilling, J., Bar-Sagi, D., Lax, I., and Schlessinger, J. (1997) Cell 89, 693–702
3. Rahin, S. J., Clemo, V., and Kaplan, D. R. (1993) Mol. Cell. Biol. 13, 2203–2213
4. Wang, J. K., Xu, H., Li, H. C., and Goldfarb, M. (1996) Oncogene 13, 721–729
5. Xu, H., Lee, K. W., and Goldfarb, M. (1998) J. Biol. Chem. 273, 17887–17990
6. Lin, Y. P., Xu, J. S., Ieschken, I., Ornitz, D. M., Hagleoua, S., and Hayman, M. (1998) Mol. Cell. Biol. 18, 3762–3770
7. Yenush, L., and White, M. F. (1997) BioEssays 19, 491–500
8. Hadari, Y. R., Koubara, H., Lax, I., and Schlessinger, J. (1998) Mol. Cell. Biol. 18, 3966–3973
9. Schlessinger, J. (1994) Curr. Opin. Genet. & Dev. 4, 25–30
10. Bennett, A. M., Hausdorff, S. F., O'Reilly, A. M., Freeman, R. M., and Neel, B. G. (1996) Mol. Cell. Biol. 16, 1189–1202
11. Herbst, R., Carroll, P. M., Allard, J. D., Schilling, J., Raabe, T., and Simon, M. A. (1996) Cell 85, 899–909
12. Li, W., Nishimura, R., Kashishian, A., Batzer, A. G., Kim, W. J. H., Cooper, J. A., and Schlessinger, J. (1994) Mol. Cell. Biol. 14, 509–517
13. Edurne, B., Diaz-Meco, M. T., Dominguez, I., Munifico, M. M., Sanz, L., Chapkin, S., and Moscat, J. (1993) Cell 74, 555–563
14. Edurne, B., Diaz-Meco, M. T., Lozano, J., Frutos, S., Munifico, M. M., Sanchez, P., Sanz, L., and Moscat, J. (1995) EMBO J. 14, 6157–6163
15. Diaz-Meco, M. T., Lozano, J., Munifico, M. M., Berra, E., Frutos, S., Sanz, L., and Moscat, J. (1994) J. Biol. Chem. 269, 31706–31710
16. Plus, A., Schmidtf, S., Grawe, F., and Stabel, S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6191–6196
17. Diaz-Meco, M. T., Munifico, M. M., Sanchez, P., Lozano, J., and Moscat, J. (1996) Mol. Cell. Biol. 16, 105–114
18. Diaz-Meco, M. T., Munifico, M. M., Frutos, S., Sanchez, P., Lozano, J., Sanz, L., and Moscat, J. (1996) Cell 86, 777–786
19. Munifico, M. M., Lozano, J., Sanchez, P., Munifico, M. T. (1995) J. Biol. Chem. 270, 15884–15891
20. Cobianchi, F., Calvio, C., Stoppini, M., Buvoli, M., and Riva, S. (1993) Nucleic Acids Res. 21, 949–955
21. Pears, C. J., Kour, G., House, C., Kemp, B. E., and Parker, P. J. (1990) Eur. J. Biochem. 194, 89–94
22. Kampfer, S., Hellbert, K., Villunger, A., Doppler, W., Baier, G., Gruenicke, H., and Uberall, F. (1998) EMBO J. 17, 4046–4055
23. Nishikawa, K., Toker, A., Johannes, F.-J., Zhou, S., and Cantley, L. C. (1997) J. Biol. Chem. 272, 952–960
24. Hof, P., Pluskey, S., Dhe-Paganon, S., Eck, M. J., and Shoelson, S. E. (1998) Cell 92, 441–459
25. Mochly Rosen, D., Khaner, H., and Lopez, J. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3997–4000
26. Ron, D., Chen, C. H., Caldwell, J., Jamieson, L., Orr, E., and Mochly Rosen, D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 839–843
27. Lim, Y. P., Low, B. C., Ong, S. H., and Guy, G. R. (1997) J. Biol. Chem. 272, 29892–29898
28. Chou, M. M., Hou, W. M., Johnson, J., Graham, L. K., Lee, M. H., Chen, C. S., Newton, A. C., Schaffhausen, B. S., and Toker, A. (1998) Curr. Biol. 8, 1069–1077
29. Nakamichi, H., Brewer, K. A., and Exton, J. H. (1993) J. Biol. Chem. 268, 13–16
30. Le Good, J. A., Ziegler, W. H., Parekh, D. B., Alessi, D. R., Cohen, P., and Parker, P. J. (1998) Science 281, 2042–2045
31. Herrera-Velit, P., Knutson, K. L., and Reiner, N. E. (1997) J. Biol. Chem. 272, 16445–16452
32. Standaert, M. L., Galloway, L., Karmann, P., Bandhyopadhyay, G., Moscat, J., and Farese, R. V. (1997) J. Biol. Chem. 272, 30075–30082
33. Le Good, J. A., Ziegler, W. H., Parekh, D. B., Alessi, D. R., Cohen, P., and Parker, P. J. (1998) Science 281, 2042–2045
34. Klintz, P., Randa, S., and Claesson-Welsh, L. (1995) J. Biol. Chem. 270, 23337–23344