The Signaling Adapter FRS-2 Competes with Shc for Binding to the Nerve Growth Factor Receptor TrkA

A MODEL FOR DISCRIMINATING PROLIFERATION AND DIFFERENTIATION*

(Received for publication, November 12, 1998, and in revised form, December 23, 1998)

Susan O. Meakin‡§, James I. S. MacDonald‡, Ela A. Gryz‡§**, Christopher J. Kubu‡,
and Joseph M. Verdi†‡§§§§

From the ‡Neurodegeneration Research Group, The John P. Robarts Research Institute, London,
Ontario N6A 5K8, Canada and the §Department of Biochemistry, the ¶Graduate Program in Neuroscience, and the 
††Department of Physiology, University of Western Ontario, London, Ontario N6A 5C1, Canada

We have isolated a human cDNA for the signaling adapter molecule FRS-2/suc1-associated neurotrophic factor target and shown that it is tyrosine-phosphorylated in response to nerve growth factor (NGF) stimulation. Importantly, we demonstrate that the phosphorylation of FRS-2 is a direct target of the Trk receptors at the same phosphotyrosine residue that binds the Trk receptors in the signaling adapter FRS-2, suggesting a model in which competitive binding between FRS-2 and Shc regulates differentiation versus proliferation. Consistent with this model, FRS-2 binds Grb-2, Crk, the Src homology domain containing tyrosine phosphatase SH-PTP-2, the cyclin-dependent kinase substrate p13*uc1, and the Src homology 3 (SH3) domain of Src, providing a functional link between TrkA, cell cycle, and multiple NGF signaling effectors. Importantly, overexpression of FRS-2 in cells expressing an NGF nonresponsive Trk receptor mutant reconstitutes the ability of NGF to stop cell cycle progression and to stimulate neuronal differentiation.

The molecular mechanisms regulating proliferation versus differentiation in the developing nervous system depend, in part, on the availability of the neurotrophins (nerve growth factor (NGF)), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5)) (1). Importantly, it is apparent that the neurotrophins not only act as target-derived survival factors but can also regulate mitotic activity and promote differentiation of neuronal progenitors in both the peripheral (2, 3) and central (4–6) nervous systems. The biological effects of the neurotrophins are mediated by two classes of cell surface receptors: a low affinity p75NTTR receptor and a high affinity tyrosine kinase Trk receptor. As a low affinity receptor, p75NTTR modulates ligand binding (7, 8) and some signaling pathways stimulated by TrkA (9, 10) and, as a member of the Fas antigen family, stimulates sphingomyelin hydrolysis and ceramide synthesis leading to apoptosis in some cellular contexts (11–13).

In contrast, the Trk receptors are essential to proliferation, differentiation, and cell survival (14). TrkA is specifically activated by NGF, whereas TrkB and TrkC are the primary receptors for BDNF and NT-3, respectively (15, 16). Upon stimulation, the Trk receptors activate numerous intracellular signaling molecules, including the Shc adapter proteins, Grb-2/Sos, Ras, Map kinase (MAPK), phosphatidylinositol 3-kinase, Src, phospholipase Cγ, SH-PTP-1, SH-PTP-2, and the suc1-associated neurotrophic factor target protein (SNT) (14). Phosphorylation of the Shc adapter proteins, and the concomitant recruitment of Grb-2/Sos, results in the Ras-dependent transient activation of MAPK (17), which is correlated with mitogenic and proliferative cell signaling. In contrast, phosphorylation of SNT and prolonged activation of MAPK correlate with neurotrophin-dependent cell cycle arrest and differentiation (18, 19). Interestingly, prolonged activation of MAPK has recently been shown to be regulated by a parallel pathway involving Crk/C3G-dependent activation of a Ras-like GTPase, Rap-1 (17).

SNT has been considered a likely candidate to regulate a decision between cell cycle progression and cell cycle arrest based on its tyrosine phosphorylation by factors that induce differentiation (NGF, BDNF, NT-3, and fibroblast growth factor (FGF)) and by its ability to bind to the cyclin-dependent kinase substrate p13*uc1. The role of SNT in neurotrophin signaling is underscored by the observation that two independent Trk receptor mutants that do not affect either mitogenic signaling and/or cell survival responses, do selectively affect the tyrosine phosphorylation of SNT and NGF-dependent cell cycle arrest/neuronal differentiation (20, 21). The identity of the SNT proteins(s) has remained elusive until recently, when a murine FGFR signaling molecule, mFRS-2, was cloned and found to share properties comparable to SNT (22).

To better understand the role of FRS-2/SNT in neurotrophin signaling and cell cycle arrest, we have cloned and characterized human FRS-2. Amino acid sequence comparisons between murine and human FRS-2 indicate that FRS-2 has been evolutionarily conserved between mouse and human (96%). FRS-2
contains an amino-terminal myristylation site to facilitate tight association with the membrane, an amino-terminal phospho-
inosine binding (PTB) domain, four putative binding sites for
Grb-2, two putative binding sites for the SH2 domain-
containing tyrosine phosphatase SH-PTP-2, and numerous po-
tential binding sites (PXXP) for SH3 domain-containing pro-
teins. We demonstrate that FRS-2 binds p13
1
2
1
 and the SH3 domain of Src constitutively but shows NGF-dependent tyro-
sin phosphorylation and association with the SH2 domains of
Grb2, Crk, and SH-PTP-2. Importantly, unlike signaling by the
FGFR (22, 23), we show that FRS-2 binds directly to neurotro-
phin-activated Trk receptors in a phosphorynosine-dependent
manner at a site that also regulates the binding of Shc. In vitro
binding assays indicate that Shc and FRS-2 compete for bind-
ing to TrkA, suggesting a model in which competition between
signaling proteins may regulate neurophin-dependent pro-
liferation and/or differentiation. The role of FRS-2 expression
in regulating cell cycle arrest and neuronal differentiation is
further supported by the observation that FRS-2 overexpress-
ion in nnr5 cells expressing a differentiation minus TrkA
receptor mutant (TrkAS3 cells) (21) reproduces NGF-dependent
cell cycle arrest and neuronal differentiation.

MATERIALS AND METHODS

Antibodies and Growth Factors—Anti-phosphotyrosine antibody (PY20) and the horseradish peroxidase (HRP)-conjugated derivative of
PY20 (RC20) were from Transduction Laboratories. Hybridoma 12CA5
cells (anti-HA) (24) and 9E10 (anti-c-Myc) (25) were grown as ascites
in 4–6-week-old female Balb/c mice. Rabbit anti-SH-PTP-2 antibody
was from Santa Cruz. HRP-goat anti-rabbit and HRP-goat
anti-mouse secondary antibodies were from The Jackson Laboratory.
p13
4
4
 1
3
 1
4
1
 was from Upstate Biotechnology or was the gift of D. Litchfield (University of Western Ontario). β-NFG was from Harlan
Bioproducts for Science. BDNF and NT-3 were the gifts of A. W. Welcher
(Amgen Inc.).

Cell Lines—The nnr5 cell line expressing hemaggulutinin (HA)-tagged
rat TrkA (B5 cells) and TrkAS3 mutant receptors (Δ94IMENP696;
S3A3) have been previously described (21). B5 and S3A3 cells express
approximately 100-fold higher levels of TrkA than PC12 cells. B5 cells
were transfected with FRS-2/c-Myc by electroporation (26), and 3–4
days later, cells were transfected with pEGFP-FRS-2/Myc and cells were selected in
0.05 mg/ml G418 and 0.2 mg/ml hygromycin (Boehringer Mannheim).

B5 cells were used in binding assays containing 10
3
 of expressed Trk receptors or lysates prepared from hFRS-2-transfected
B5 cells were used in binding assays containing 10 μg of Sepharose-
bound GST proteins. Samples were assayed by Western blotting with
RC20 (1:2500), anti-HA (1 μg/ml), anti-c-Myc (2 μg/ml), or anti-SH-
PTP-2 (1:2000). HRP-goat anti-rabbit and HRP-goat anti-mouse were
used at a dilution of 1:10,000. The chemiluminescence detection kit was
from NEN Life Science Products.

For competition experiments, GST-hFRS-2/PTB- and GST-PLC-
1
2
 (SH2)-bound Sepharose was digested with 10 units of thrombin for
30 min at room temperature. The digests were washed twice with PBS
containing protease inhibitors and 1 μg EDTA and pooled, and the
eluate was used directly.

Neurite Response and 5-Bromo-2-deoxyuridine (BrdUrd) Assays—TrkA
neurite response assay was performed on poly n-lysine-coated
24-well clusters. Fresh neuronal minishin was added every other day. On day
5, cells were assayed for changes in proliferation by measuring the incorpo-
ration of the thymidine analogue BrdUrd as described previ-
ously (21). The percentage of BrdUrd-positive cells was scored from
10–12 independent frames. The S3-FRS-2 sample represents as aver-
age of two clones expressing FRS-2 (clones 34 and 40) and is thus an
average of 20 independent clones.

RESULTS

Isolation of a Human FRS-2 cDNA and Northern Blot Anal-
ysis—An hFRS-2 cDNA (1.5 kb) was isolated from fetal brain
dNA by PCR techniques with degenerate primers based on the
protein sequence of mFRS-2 (22). The cDNA sequence
predicts a protein with a primary sequence of 508 amino acids
and a molecular mass of approximately 57 kDa. Amino acid
sequence comparison between hFRS-2 and mFRS-2 (Fig. 1A)
indicates that FRS-2 has been evolutionarily conserved between mouse
and human (96% identity). hFRS-2, like mFRS-2, contains an
amino-terminal myristylation signal (MGXXXS) and PTB domain, 15 potential sites of tyrosine phosphorylation, 4 Grb-2 binding sites, 2 potential binding sites for the tyrosine phosphatase SH-PTP-2, and putative SH3 domain-containing proteins (PXXP). Northern blot analysis of FRS-2 expression indicates low levels of approximately 7.2 and 7.5 kb in all tissues examined with slightly higher levels (2–5-fold) detected in brain, testes, and lung (Fig. 1B). The ratio of the two transcripts is comparable between tissues with slightly higher levels of the upper transcript present in lung. The difference between our 1.5-kb cDNA and the native transcripts represent untranslated regions of approximately 1.5 and 4.2 kb, respectively (Fig. 1B).

**FRS-2 Is Tyrosine-phosphorylated by NGF and Binds p13suc1 Constitutively**—To facilitate transfection studies, hFRS-2 was tagged at the carboxyl terminus (c-Myc) without interfering with NGF-dependent tyrosine phosphorylation and/or its signaling properties. As shown in Fig. 2A, following transfection into NGF-responsive cells, hFRS-2/c-Myc (80–90 kDa) was tyrosine-phosphorylated in response to NGF stimulation and was immunoprecipitated with the anti-Myc antibody (9E10) (top panel, compare lanes 2 and 4). Because hFRS-2/c-Myc predicts a protein of approximately 57 kDa, this suggests that FRS-2 is posttranslationally modified by mechanisms that include myristylation (22) and both serine/threonine and tyrosine phosphorylation. Similar to endogenous FRS-2, FRS-2/c-Myc bound p13suc1, although the transfected gene product migrated more slowly than the endogenous protein consistent with the addition of the c-Myc epitope (approximately 3 kDa) to the carboxyl terminus (Fig. 2A, top panel, compare lanes 6 and 8). Stripping and re-Probing the blot with anti-Myc antibodies indicated that hFRS-2/Myc can bind p13suc1 constitutively and that the binding is not regulated by tyrosine phosphorylation (Fig. 2A, bottom panel, lanes 7 and 8). Moreover, more than one form of

---

**FIG. 1. Sequence alignment between mouse (22) and human FRS-2.** A, myristylation signal at the amino terminus is shown in **lowercase** letters, and the PTB domain is **boxed**. Tyrosine residues are shown in **boldface type**. Four putative Grb-2 binding sites are **underlined**, and two putative SH-PTP-2 binding sites are **underlined** in **boldface type** (53). Putative SH3 domain binding sites (PXXP) are shown in italic. Subsequent to our isolation of hFRS-2, the sequence was deposited in GenBank™ (accession number AF036718). B, Northern blot analysis. A rat multiple tissue Northern blot (CLONTECH) was probed sequentially with hFRS-2 and actin. Arrows indicate the position of FRS-2 and actin transcripts.
FRS-2 was consistently detected, indicating multiple forms of posttranslational modifications. Typically, only the highest molecular mass form of FRS-2 was tyrosine-phosphorylated in response to NGF.

Analysis of FRS-2-binding Proteins—As outlined above, the primary sequence of FRS-2 predicts that phosphorylation of specific tyrosines will generate binding sites for the SH2 domains of the adapter protein Grb-2 and the tyrosine phosphatase SH-PTP-2. To assay FRS-2/Grb-2 interactions, FRS-2/c-Myc was transfected into TrkA overexpressing nnr5 cells, termed B5 (26). As shown in Fig. 2B, GST fusion proteins corresponding to both full-length Grb-2 and the central SH2 domain bound NGF-induced, tyrosine-phosphorylated hFRS-2/c-Myc. Neither the amino-terminal nor the carboxyl-terminal SH3 domains of Grb-2 bound hFRS-2. Stripping and reprobing the blot with anti-Myc antibodies confirmed that the 80–90 kDa tyrosine-phosphorylated protein bound by Grb-2(SH2) corresponds to the fully processed hFRS-2/c-Myc and co-migrates with FRS-2/c-Myc immunoprecipitated with 9E10 (Fig. 2B, bottom panel).

As stated above, FRS-2 is also predicted to contain binding sites for SH-PTP-2 (also known as Shp-2, syt, PTP1D, and PTP2C (31)). As shown in Fig. 2C (left panel), immunoprecipitation of hFRS-2/c-Myc with anti-Myc antibodies, from hFRS-2/c-Myc-transfected B5 cells, indicates that SH-PTP-2 also co-immunoprecipitates with FRS-2 following NGF stimulation (compare lanes 2, 4, and 5). Because SH-PTP-2 has been shown to be transiently associated with TrkA (32), we next examined the kinetics of association between FRS-2 and SH-PTP-2 in response to NGF stimulation. As shown in Fig. 2C (right panel), both p13suc1 and anti-SH-PTP-2 immunoprecipitations indicate tyrosine phosphorylation of FRS-2 within 2 min of NGF stimulation (lanes 7 and 10). The tyrosine phosphorylation of SH-PTP-2 was maximally observed 2 min following NGF stim-
FRS-2/SNT and She Compete for Binding to TrkA

9865

ulation (lane 10). Interestingly, tyrosine phosphorylation is only required to initiate FRS-2/SH-PTP-2 binding because SH-PTP-2 was rapidly de-phosphorylated by 5 min (lane 11) without altering FRS-2 binding. Moreover, the anti-SH-PTP-2 immunoprecipitations identified an approximately 120-kDa protein that is tyrosine-phosphorylated in response to NGF stimulation. The identity of this protein is presently unknown, but it may correspond to a member of the signal-regulatory protein (SIRP) family of SH-PTP-2-binding proteins that are both substrates of receptor tyrosine kinases and negative regulators of receptor tyrosine kinase signaling (33). Importantly, stripping the blots and reprobing with anti-SH-PTP-2 antibodies indicates that SH-PTP-2 co-precipitates with p13<sub>suc1</sub>-<sup>−</sup> from NGF-stimulated cells (Fig. 2C, lanes 7 and 8) comparable to the SH-PTP-2 co-immunoprecipitation observed with the transfected hFRS-2/c-myc gene (lanes 4 and 5).

From the primary sequence, it is also predicted that SH3 domain-containing proteins can interact with FRS-2 and facilitate alternate signaling pathways. To this end, we screened a panel of SH3 domains (Grb-2, Nck, Crk, Ras-Gap, c-Abl, PLCγ-1, and v-Src), as well as full-length Crk, for interaction with hFRS-2. As shown in Fig. 2D, the only SH3 domain-mediated interaction observed was with v-Src. As predicted for SH3 domain interactions, the v-Src interaction occurred constitutively and was independent of NGF stimulation (lanes 19 and 20). The constitutive FRS-2/Src interaction is not surprising and may help explain the previous observation that v-Src expression in PC12 cells generates NGF-independent neurite outgrowth (34). Full-length Crk also interacts with hFRS-2, but because the binding is not SH3 domain-mediated (lanes 11, 12), and the interaction is NGF-dependent, it likely involves the Crk SH2 domain.

FRS-2 Directly Binds the Neurotrophin Trk Receptors—As described above, hFRS-2 is modular in nature and contains an amino-terminal PTB domain that in many molecules mediates protein-protein interactions involving phosphotyrosine. Whereas FRS-2 does not readily co-immunoprecipitate with either the FGFR (22) or TrkA (data not shown), FRS-2 has been shown to interact with the FGFR in vitro and in yeast (23). These observations suggest that FRS-2/receptor interactions are labile and/or weak in cells but that direct binding can be observed by alternative methods. These observations are reminiscent of weak interactions between the insulin receptor β-chain and its major signaling component IRS-1 in cells relative to readily detectable interactions detected in yeast (35).

Accordingly, we used in vitro binding assays and yeast interaction-trap assays to determine whether hFRS-2 could interact with TrkA and if so, where the site of interaction might be. HA-tagged TrkA, TrkB and TrkC were expressed in baculovirus-infected insect cells and lysates containing equivalent amounts of expressed Trk protein were used in in vitro binding experiments with purified GST fusion proteins. As shown in Fig. 3A, GST-hFRS-2(PTB), like the Shc(PTB) domain and the PLCγ-1(SH2) domain, effectively precipitates TrkA as well as TrkB and TrkC, indicating that hFRS-2 is a signaling component common to neurotrophic signaling by NGF, BDNF and NT-3. To assay direct interaction between TrkA and hFRS-2 in vivo, we utilized the yeast two-hybrid assay. Specifically, hFRS-2 was expressed as a fusion protein with the DNA binding domain of Gal4 (pAS2-FRS-2; Trp<sup>+</sup>) and cells were transfected and tested for interaction with either p13<sub>suc1</sub>-<sup>−</sup> (pGAD-p13; Leu<sup>+</sup>) or the intracellular domain of either kinase-active TrkA or kinase-inactive TrkA547A (pGAD-TrkA; Leu<sup>+</sup>) in a standard two-hybrid assay (36). Untransfected yeast, yeast expressing pAS-FRS-2, and yeast co-expressing pAS and pGAD vectors were plated on nonselective medium (plate 1), sc medium minus Trp (plate 2), sc medium minus Leu and Trp (plate 3), and sc medium minus leucine, tryptophan, histidine, and adenine plus 20 mM 3-amino triazole (AT) (plate 4).

![Fig. 3. FRS-2 binds Trk receptors.](image)

A. HA-tagged Trk receptors were assayed for binding to GST, GST-hFRS-2(PTB), GST-Shc(PTB), and GST-PLCγ-1(SH2). Arrows indicate the position of the Trks. B. yeast two-hybrid interaction between hFRS-2, p13<sub>suc1</sub>-<sup>−</sup>, and the intracellular domain of TrkA. Yeast (PJ694A(lane 1)) expressing pAS-FRS-2 (Trp<sup>+</sup> (lane 2)) was re-transfected with pGAD-p13 (Leu<sup>+</sup> (lane 3)), pGAD-TrkA kinase (Leu<sup>+</sup> (lane 4)), and pGAD-TrkA kinase dead (K547A) (Leu<sup>+</sup> (lane 5)). Cells were plated on nonselective sc medium (plate 1), sc medium minus Trp (plate 2), sc medium minus Leu and Trp (plate 3), and sc medium minus leucine, tryptophan, histidine, and adenine plus 20 mM 3-amino triazole (AT) (plate 4).
indicated that the PTB domain of FRS-2 interacts with the kinase active TrkA, as does GST-Shc(PTB), and not the catalytically inactive form (Fig. 4B). Thus, as we observed in yeast (Fig. 3), FRS-2 interaction with TrkA is phosphotyrosine-dependent.

**FRS-2 Binds TrkA at the Shc Binding Site, Tyr**<sup>499</sup>—Thus far, we have demonstrated that hFRS-2 interacts with the Trk receptors through an amino-terminal PTB domain in a tyrosine phosphorylation-dependent manner. To map the binding site(s) on TrkA for hFRS-2(PTB) interaction, we took advantage of a series of rat TrkA receptor mutants that selectively affect specific signaling pathways. In this respect, NGF induces receptor dimerization and tyrosine phosphorylation at five intracellular tyrosine residues in rat TrkA (Tyr<sup>499</sup>, Tyr<sup>679</sup>, Tyr<sup>683</sup>, Tyr<sup>684</sup>, and Tyr<sup>794</sup>), Shc and PLC<sub>γ</sub>-1 interact at phosphotyrosine residues Tyr<sup>499</sup> and Tyr<sup>794</sup>, respectively (Tyr<sup>499</sup> and Tyr<sup>794</sup> in human TrkA) through intrinsic PTB and SH2 domains. A total of four independent TrkA signaling mutants have been previously described and were used in the present study (see Table I and Fig. 5A). Trk receptors were HA-tagged at the amino terminus and expressed in baculovirus-infected insect cells. Lysates containing comparable levels of expressed Trk receptors were used in *in vitro* binding assays with purified GST fusion proteins. As shown in Fig. 5B, GST fusion proteins corresponding to Shc(PTB), PLC<sub>γ</sub>-1(SH2), and hFRS-2(PTB) effectively precipitate wild type TrkA to levels significantly greater than GST alone. As predicted, the TrkAS9 mutant showed a loss of PLC<sub>γ</sub>-1 binding (Fig. 5B, lane 20) but retained both Shc(PTB) and hFRS-2(PTB) domain binding, indicating that tyrosine 794 is not involved in hFRS-2-TrkA binding. Analysis of the KFG minus TrkAS17 mutant indicates that it retained PLC<sub>γ</sub>-1 and Shc(PTB) binding but showed a reduction in hFRS-2(PTB) domain binding. This suggests that the KFG deletion reduced hFRS-2 binding but did not completely abolish the interaction. In contrast, both the TrkAS9 deletion mutant (Δ<sup>493</sup>IMENP<sup>497</sup>) and the Shc binding site mutant (Y499F) retained PLC<sub>γ</sub>-1 binding but showed a complete loss of both Shc(PTB) and hFRS-2(PTB) binding. Because Tyr<sup>499</sup> is still phosphorylated in the TrkAS3 mutant (21), these data indicate that both tyrosine 499 and the amino acids 5′ of Tyr<sup>499</sup> are critical for hFRS-2(PTB)/TrkA binding and are consistent with the observation that residues 5′ of the phosphotyrosine residue are also critical for high affinity Trk-Shc PTB domain binding (38). Importantly, we also found that a low, but detectable, amount of TrkA-FRS-2 binding could be recovered when very high concentrations of either TrkAS3 or TrkAS8 were used (data not shown), indicating that overexpression of either TrkA mutant can facilitate some protein-protein interaction.

**FRS-2 and She Compete for Binding to TrkA at Tyr**<sup>499</sup>—The observation that both Shc and hFRS-2 bind to the same phosphotyrosine residue on TrkA suggests a model in which Shc and FRS-2 may compete for binding to TrkA *in vitro*. Because Shc/Grb-2/Ras/Map kinase activation facilitates mitogenic signaling (39) and a decrease in FRS-2 phosphorylation correlates with an inability of TrkA to support NGF-dependent neurite outgrowth (20, 21), this suggests that competition between Shc and FRS-2 for binding to Tyr<sup>499</sup> may, in part, regulate a cellular switch between cell cycle progression/mitogenesis and cell cycle arrest/differentiation.

To this end, competition between Shc and hFRS-2 binding to TrkA was assayed *in vitro*. GST-Shc (5 μg) was used to precipitate baculovirus expressed HA-TrkA in the presence of increasing concentrations of soluble hFRS-2(PTB). As shown in Fig. 5C, hFRS-2 competes for binding to TrkA with an EC<sub>50</sub> requiring a 2–5-fold excess (between 10 and 25 μg). Complete competition was obtained with a 20-fold excess (100 μg). In comparison, increasing concentrations of the SH2 domain of PLC<sub>γ</sub>-1 or bovine serum albumin (up to 100 μg) did not compete, indicating that the hFRS-2 competition both is specific and involves tyrosine 499.

**FRS-2 Overexpression Reconstitutes NGF-dependent Neurite Outgrowth by a Differentiation Minus TrkA Receptor Mutant**—As shown above, FRS-2 showed a dramatic reduction in binding to both the Y499A TrkAS8 mutant and the Δ<sup>493</sup>IMENP<sup>497</sup> TrkAS3 deletion mutant, consistent with the decrease in stoichiometry of FRS-2/SNT tyrosine phosphorylation observed in TrkAS3 expressing nnr5 cells (21). To determine whether the reduced FRS-2/SNT tyrosine phosphorylation and receptor binding ability in TrkAS3 expressing nnr5 cells underscores the NGF nonresponsive phenotype, FRS-2 was ectopically expressed in TrkAS3A3 cells (21) on a vector that co-expresses EGFP from an internal ribosome entry site. Cells expressing EGFP were selected and assayed for NGF-dependent tyrosine phosphorylation of FRS-2, as well as an NGF-dependent decrease in proliferation and a concomitant increase in neuronal differentiation. As shown in Fig. 6A, two independent clonal lines demonstrate high levels of NGF-induced tyrosine-phosphorylated FRS-2/Myc in comparison to either the parental S3A3 cells or cells expressing EGFP alone. To determine whether ectopic expression of FRS-2/Myc can stop cell cycle progression and facilitate neuronal differentiation, cells were grown for 5 days in the absence or presence of NGF and scored for neurite outgrowth. As shown in Fig. 6B, S3-FRS-2 expressing cells (clone 40 is shown, but a similar response was also found in clone 34; data not shown) demonstrated significant neurite outgrowth 5 days poststimulation with 100 ng/ml NGF (approximately 80–90% of the culture) relative to the parental S3A3 cells or cells expressing EGFP (approximately 2%). Thus, FRS-2 overexpression in S3 cells reconstitutes NGF-dependent differentiation that is morphologically similar to wild type TrkA-expressing nnr5 cells (B5). To address whether the rescue of process outgrowth was accompanied by a corresponding decrease in the mitotic index, S3A3 cells, S3 cells
expressing FRS-2 or EGFP, and wild type TrkA-expressing B5 cells were assayed for changes in BrdUrd incorporation. As shown in Fig. 6C, B5 cells showed an NGF-dependent decrease in BrdUrd incorporation of approximately 70%, whereas the levels of BrdUrd incorporation in S3A3 cells and cells expressing EGFP were unaffected by NGF. In contrast, S3 cells co-expressing FRS-2/Myc showed an NGF-dependent decrease in BrdUrd incorporation of approximately 50%.

**DISCUSSION**

In summary, we have cloned the human FRS-2 adapter protein and begun to characterize its role in NGF-dependent signaling through TrkA. Although TrkA can stimulate both a mitogenic response and differentiation and cessation of cell cycle progression in nonneuronal and neuronal cells, respectively, the molecular mechanism(s) underlying this differential response is not well understood. Here, we provide evidence of a novel mechanism by which competitive binding between FRS-2 and Shc may regulate a cellular switch between cell cycle progression and cell cycle arrest/differentiation. Moreover, we have begun to address the mechanisms by which FRS-2 may regulate these processes. hFRS-2 is tyrosine-phosphorylated in response to NGF-stimulation and binds directly to TrkA through its amino-terminal PTB domain in a phosphotyrosine-dependent manner.

### TABLE I

Summary of TrkA receptor mutants

| TrkA mutant | Mutation | Signaling defect | Phenotype |
|-------------|---------|-----------------|-----------|
| TrkAS17     | Δ450KFG452 | SNT/FRS-2       | Differentiation (−) (20) |
| TrkAS9      | Δ493IMENP497 | SNT/FRS-2       | Differentiation (−) (20) |
|             |          |                 | Proliferation (+) (21) |
| TrkAS8      | Y499F    | SNT/FRS-2       | Differentiation (−/+)(Refs. 21, 37, 52 and data not shown) |
| TrkAS9      | Y794F    | PLCγ-1          | Differentiation (+)(37, 52) |

**Fig. 5.** FRS-2 competes with Shc for binding to rat TrkA at Tyr499. A, schematic of TrkA mutants: S17 (Δ450KFG452) (20), S3 (Δ493IMENP497) (21), S8 (Y499F) (37, 52), and S9 (Y794F) (37). Shc binds Tyr499 and PLCγ-1 binds Tyr794. Arrows indicate the signaling molecules affected (decreased activation and/or receptor binding) by each mutant. B, HA-tagged rat TrkA (lanes 1–4), TrkAS17 (lanes 5–8), TrkAS3 (lanes 9–12), TrkAS8 (lanes 13–16), and TrkAS9 (lanes 17–20) were assayed for binding to GST, GST-hFRS2(PTB), GST-Shc(PTB), and GST-PLCγ-1(SH2). Western blotting was performed with anti-HA antibodies. Arrows indicate the positions of the Trks. C, HA-tagged rat TrkA was assayed for binding to GST-Shc(PTB) in the presence of increasing concentrations of soluble hFRS-2(PTB), PLCγ-1(SH2), or bovine serum albumin (BSA). Western blotting was performed with anti-HA antibodies. Arrows indicate the position of TrkA (gp140).
dependent manner. hFRS-2 also binds to neurotrophin-stimulated TrkB and TrkC receptors, indicating that it is probably involved in an analogous role in BDNF and NT-3 signaling as well. Recently, the PTB domain of FRS-2 has also been shown to bind the juxtamembrane region of the FGFR (23) by both yeast two-hybrid assays and in vitro binding assays using recombinant fusion proteins. In these assays, FRS-2 binding to the FGFR is independent of kinase activation and has been localized to a juxtamembrane region that lacks both asparagine and tyrosine residues (23). Although these data indicate that FRS-2 binding to the FGFR is constitutive, the possibility that FGF may regulate FRS-2 recruitment in vivo through an alternative mechanism has not yet been determined. Moreover, the precise residues within the FGFR juxtamembrane region that are essential to FRS-2 recruitment and activation have not yet been determined.

Thus, FRS-2 recruitment by the FGFR appears to be phosphotyrosine-independent, whereas we have shown that FRS-2 binding to TrkA requires both an active kinase and is phosphotyrosine-dependent. Tyrosine-phosphorylated FRS-2 binds the SH2 domains of Grb-2 and the tyrosine phosphatase SH-PTP-2. SH-PTP-2 is transiently tyrosine-phosphorylated in response to NGF stimulation, which correlates with an initial ability to bind FRS-2. Because SH-PTP-2 also binds Grb-2 through carboxyl-terminal phosphotyrosines (22, 40), FRS-2 facilitates the Shc-independent recruitment of Grb-2 into the NGF-Trk signaling complex both directly and indirectly.

FRS-2 also binds the SH2 and SH3 domain-containing adapter protein Crk, in an NGF and phosphotyrosine-dependent manner. The relevance of this pathway to neuronal differentiation is exemplified by the fact that Crk/C3G/Rap1, but not Grb-2/Sos/Ras, appears to regulate prolonged MAPK activation (17) and by the fact that Crk overexpression induces constitutive PC12 cell differentiation (41). Consistent with these data, the Grb-2 binding sites on FRS-2 are dispensable for FGF-induced differentiation of PC12 cells (40). Importantly, the recent observation that prolonged MAPK activation stimulates

---

**Fig. 6—continued**

**A**. FRS-2 Expression in nrr5 cells expressing the TrkA-S3 deletion (S3A3 cells). A, cells unstimulated and β-NGF (100 ng/ml)-stimulated, 5 min were analyzed for NGF-dependent tyrosine phosphorylation of stably expressed FRS-2/c-Myc by precipitation with anti-Myc antibodies 9E10. Samples were analyzed by Western blotting with anti-Tyr(P) antibodies (RC20). Arrows indicate the positions of FRS-2/c-Myc. Two independent S3A3 lines express FRS-2/c-Myc (clones 34 and 40) and cells expressing EGFP were analyzed. B, neurite response assay of mutant S3A3 cells, S3 cells expressing FRS-2/c-Myc or EGFP, and nrr5 cells expressing wild type TrkA, B5 cells. Cells were cultured for 5 days in the absence and presence of NGF (100 ng/ml). C, BrdUrd assay. S3A3 cells, S3 cells expressing FRS-2/c-Myc or EGFP, and B5 cells were assayed for proliferation in the absence and presence of NGF (100 ng/ml, 5 days). Proliferation was assayed by determining the percentage of cells incorporating BrdUrd. Values represent the mean of 10–12 independent frames. The S3-FRS-2 samples reflect a pool of two independent clones (clones 34 and 40), and the values were calculated from counting 10 independent frames from each clone.
expression of the cell cycle inhibitor p21CIP/Waf1 (42) provides an important functional link between FRS-2, Crk, cell cycle arrest, and neuronal differentiation. Collectively, these data highlight the functional differences between Shc and FRS-2-dependent mechanisms to activate MAPK (Grb-2/Sos/Ras and Crk/C3G/Rap1) and emphasize the need to assay site-directed FRS-2 mutants incapable of binding Crk on NGF-dependent cell cycle arrest and neuronal differentiation.

By comparison, recent studies indicate that SH-PTP-2 activation and binding to FRS-2 are essential to FGF-induced differentiation of PC12 cells (40). Although the mechanism is not yet fully understood, given the arguments outlined above that Crk is essential to prolonged MAPK activation and neuronal differentiation, it is possible that SH-PTP-2 may inactivate an antagonist of MAPK activation, such as Ras-Gap or a MAPK phosphatase, and thereby positively regulate neuronal differentiation. Although overexpression of SH-PTP-2 in PC12 cells does not enhance NGF-dependent differentiation (43), as observed with FGF (40), overexpression of a catalytically inactive SH-PTP-2 decreases NGF-dependent differentiation (43), consistent with the model suggested above that inactivation of a MAPK antagonist, rather than recruitment of Grb-2, is essential to the role of SH-PTP-2 in neuronal differentiation.

FRS-2 also contains several potential binding sites for SH3 domain-containing proteins (PXXP). Of the several proteins that we tested, only the SH3 domain of Src bound FRS-2 constitutively in our in vitro binding assays. It has long been known that constitutive activation of Src generates NGF-independent neuronal differentiation (34) and that the activation of Src precedes the activation of Ras in NGF-dependent TrkA signaling (44). Our demonstration that FRS-2 binds Src in vitro provides the first biochemical evidence of how the Trk receptors recruit Src into their signaling cascades in vivo and further supports the importance of Trk-dependent binding of FRS-2 to the process of NGF-dependent neuronal differentiation.

By taking advantage of a series of specific TrkA signaling mutants, we demonstrate that the FRS-2 binding site on TrkA involves tyrosine 499, as well as the amino acids just 5’ of this residue. Interestingly, these same residues (around the NPXY motif) also bind the PTB domain of Shc (37, 38, 45). The possibility that more than one protein could bind to TrkA at this site was previously suggested (21) and is consistent with the observation that Shc, IRS-1, and IRS-2 compete for binding to the NPXY motif of the β-chain of the insulin and insulin-like growth factor-1 receptors (46–48). Overlapping binding sites have also been demonstrated for Nck and PI-3 kinase (p85) binding to the platelet-derived growth factor receptor (49). Thus, the possibility that Shc and FRS-2 can compete for binding to the Trk receptors is not unprecedented. Importantly, we provide direct evidence that Shc and FRS-2 do, in fact, compete for binding to TrkA in vitro. Using a constant amount of GST-Shc(PTB), we found that soluble FRS-2 was able to compete with Shc for binding to TrkA with an EC50 requiring an approximately 2–5-fold excess of FRS-2. Collectively, these data provide a novel model by which competition between Shc and FRS-2 may occur in vivo and, possibly, regulate a switch between cell cycle progression/mitogenesis and cell cycle arrest/differentiation.

Although overexpression of many components of the Shc/ MAPK pathway (Shc, MAPK kinase (MEK), and MAPK) (50, 51) is sufficient to stimulate constitutive neurite outgrowth, it does not imply that Shc-dependent activation of MAPK is the physiologically relevant pathway correlated with prolonged MAPK activation and neuronal differentiation in vivo. Rather, overexpression studies simply saturate one pathway at the expense of another, and if driving prolonged MAPK stimulation is required for neuronal differentiation, then it is perhaps not surprising that overstimulation of Shc-dependent MAPK can drive constitutive neurite outgrowth. Importantly, however, FRS-2 overexpression in nnr5 cells expressing mutant TrkA S3 (499IMEPN497) receptors (S3A3 cells) (21) reconstitutes NGF-dependent differentiation and cessation of cell cycle progression. TrkAS3 receptors retain the ability to phosphorylate Shc, as well as mitogenic signaling in transfected fibroblasts, yet show a dramatic reduction in the stoichiometric phosphorylation of FRS-2/SNT, as well as the ability to support neurite outgrowth in S3A3 cells (21). Because high concentrations of mutant TrkAS3 receptors can stimulate low levels of FRS-2 binding in vitro (discussed above), it is likely that stable overexpression of FRS-2 in TrkAS3 cells either reconstitutes sufficient TrkA binding or permits sufficiently low levels of FRS-2 tyrosine phosphorylation to facilitate NGF-dependent cell cycle arrest and differentiation. The role of FRS-2 in NGF-dependent differentiation is also supported by mutant Trk receptors in which Tyr499 is replaced with phenylalanine (TrkAS8). Cells expressing TRK Y499F do not stimulate NGF-dependent Shc tyrosine phosphorylation and/or Shc/Grb-2 binding (21, 37). Moreover, Trk Y499F receptors show a reduction in the stoichiometry of NGF-dependent FRS-2 phosphorylation (data not shown). However, as described above for TrkAS3, we have found that overexpression of Trk Y499F receptors can stimulate both FRS-2 phosphorylation and low levels of neuronal differentiation, which may account for the observations by Obermeier et al. (52) and Stephens et al. (37) in which cells expressing high levels of Y499F Trk receptors retain NGF-dependent SNT phosphorylation, as well as varying degrees of neurite outgrowth. The important relevance of the FRS-2 overexpression studies described here are that they reconstitute NGF-dependent neuronal differentiation, suggesting that FRS-2 may be the physiologically relevant substrate essential to NGF-dependent mitotic arrest. The competition between Shc and FRS-2 for binding to NGF-activated TrkA suggests a novel mechanism by which proliferation and differentiation may be regulated in response to neurotrophin stimulation.

Acknowledgments—We acknowledge T. Pawson (Samuel Lunenfeld Research Institute, Toronto, Ontario, Canada) and P. P. Di Fiore (European Institute of Oncology, Milan, Italy) for the gifts of numerous GST constructs, J. McGlade (The Hospital for Sick Children, Toronto, Ontario, Canada) for GST-Shc, and P. James (University of Wisconsin) for the yeast strain PJ694A.

REFERENCES

1. Snider, W. D. (1994) Cell 77, 627–638
2. Sieber-Blum, M. (1991) Neuron 6, 949–955
3. Verdi, J. M., Groves, A. K., Farinaz, I., Jones, K., Marchionni, M. A., and Reichardt, L. F. (1996) Neuron 16, 515–527
4. Cattaneo, E., and McKay, R. K. (1990) Nature 347, 762–765
5. Collazo, D., Takahashi, H., and McKay, R. D. (1992) Neuron 9, 643–656
6. Ghosh, A., and Greenberg, M. E. (1995) Neuron 15, 89–103
7. Barker, P. A., and Shooter, E. M. (1994) Neuron 13, 203–215
8. Chan, M. V., and Hemstead, B. L. (1995) Trends Neurosci. 18, 321–326
9. Veldman, J. M., Birren, S. J., Ibanez, C. F., Persson, H., Kaplan, D. R., Benedetti, M., Chao, M. V., and Anderson, D. J. (1994) Neuron 12, 733–745
10. MacPhee, I. A., and Barker, P. A. (1997) J. Biol. Chem. 272, 23547–23551
11. Dukowitz, R. T., Werner, T., Ellis, C. L., Linino, A. M., Chao, M. V., and Hanssouy, Y. A. (1994) Science 265, 1596–1599
12. Frade, J. M., Rodriguez-Tebar, A., and Barde, Y.-A. (1996) Nature 383, 168–168
13. Majdan, M., Lachance, C., Gloster, A., Aloyz, R., Zeindler, C., Bamji, S., Bhakar, A., Belliveau, D., Fawcett, J., Miller, F. D., and Barker, P. A. (1997) J. Neurosci. 17, 6988–6998
FRS-2/SNT and She Compete for Binding to TrkA