Multiple Effector Domains within SNT1 Coordinate ERK Activation and Neuronal Differentiation of PC12 Cells*

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Differentiation of neuronal precursor cells in response to neurotrophic differentiation factors is accompanied by the activation of membrane-anchored SNT signaling adaptor proteins. Two classes of differentiation factors, the neurotrophins and fibroblast growth factors, induce rapid tyrosine phosphorylation of SNT1(FRS2α), which in turn enables SNT1 to recruit Shp2 tyrosine phosphatase and Grb2 adaptor protein in complex with the Ras GDP/GTP exchange factor Sos. To determine effector functions of SNT that promote neuronal differentiation of PC12 pheochromocytoma cells, we engineered a chimeric protein, SNT1(IRS)/CX, bearing the effector region of SNT1 and the insulin receptor recognition domains of IRS2. Insulin promoted tyrosine phosphorylation of SNT1(IRS)/CX in transfected PC12 cells accompanied by sustained activation of ERK1/2 mitogen-activated protein kinases and neuronal differentiation. The SNT1(IRS)/CX-mediated response was dependent on endogenous Ras, MEK, and Shp2 activities. Mutagenesis of SNT1(IRS)/CX identified three classes of effector motifs within SNT critical for both sustained ERK activation and neuronal differentiation: 1) four phosphotyrosine motifs that mediate recruitment of Grb2, 2) two phosphotyrosine motifs that mediate recruitment of Shp2, and 3) a C-terminal motif that functions by helping to recruit Sos. We discuss possible mechanisms by which three functionally distinct SNT effector motifs collaborate to promote a downstream biochemical and biological response.

Within the developing vertebrate nervous system, proliferating progenitor cells differentiate into post-mitotic neurons under the control of specific secreted polypeptide growth factors. Neurotrophins and fibroblast growth factors (FGFs),¹ which promote neuronal differentiation, signal through the activation of the Trk and FGF receptor classes of receptor tyrosine-specific protein kinases (RTKs), respectively. Other growth factors and protein hormones, such as epidermal growth factor and insulin, also signal through RTKs present on neuronal progenitor cells, but these factors promote proliferative or anti-apoptotic responses without favoring differentiation. Elucidating the RTK signaling pathways specific to the neuronal differentiation response has been the subject of extensive research over the past decade (1–3).

Following progenitor cell stimulation with differentiation factors, the earliest marker of neuronal differentiation is tyrosine phosphorylation of SNTs (suc1-binding neurotrophic targets), which occurs within 15 s of cell stimulation (4). SNT1 (also termed FRS2α) and SNT2 (FRS2β) are related membrane-anchored docking proteins (5, 6) that are tyrosine-phosphorylated following specific interaction between activated Trks or FGFRs and the phosphotyrosine binding (PTB) domain of SNTs (6–8). Phosphorylated SNTs recruit the Grb2 adaptor protein in complex with the Ras activators Sos1 and Sos2 (5, 9) and also recruit the SH2 domain-regulated protein tyrosine phosphatase, Shp2 (10, 11). These interactions have suggested the importance of SNTs in the differentiation response, because experiments with dominant-negative protein inhibitors have shown that Ras and Shp2 are required for neurotrophin- and FGF-induced differentiation of PC12 neuronal progenitor cells (12, 13). Furthermore, overexpression of SNT1 in PC12 cells potentiates the length of FGF-induced neurites in a Ras- and Shp2-dependent manner (5, 11).

Although activation of Ras and Shp2 are critical events in neuronal differentiation, these proteins are also activated by cell stimulation with the nondifferentiating growth factors epidermal growth factor and insulin (14–17). Induction of neuronal differentiation must require activation of additional signaling pathways or be dependent on the magnitude or duration of Ras and Shp2 stimulation. A downstream target of both Ras and Shp2, the ERK, mitogen-activated protein kinase, undergoes prolonged activation in response to neuronal differentiation factors but only transient activation in response to other growth factors (3). It has been proposed that sustained ERK activation is necessary and sufficient to drive neuronal differentiation, because only sustained activation allows for substantial ERK nuclear translocation and potential phosphorylation and activation of key transcription factors (3).
We have developed a robust methodology to corroborate whether SNT tyrosine phosphorylation can drive sustained ERK activation and the neuronal differentiation response, and to analyze SNT effector domains required for SNT's functional attributes. Using this methodology, we show that SNT activation drives sustained ERK activity and neuronal differentiation of PC12 cells. We further identify three effector functions on SNT1 that act coordinately to mediate downstream responses.

**EXPERIMENTAL PROCEDURES**

*Immunological Reagents*—Mouse monoclonal antibodies were used 4G10 anti-phosphotyrosine (pY) (Upstate Biotechnology Inc.), anti-Ras and anti-Shp2 (PTP1D) (Transduction Laboratories), 12CA5 anti-HA tag and 9E10 anti-Myc tag (courtesy of T. Moran), and E10 anti-phospho-ERK (p-ERK1/2) (New England BioLabs). Rabbit polyclonal antibodies included anti-Grb2 and anti-Sos1 (Santa Cruz Biotechnology) and anti-ERK (ERK1/2) (New England BioLabs). Conjugated secondary antibodies were from CALTAG Laboratories: Horseradish peroxydase-conjugated anti-rabbit IgG, mouse IgG1, and mouse IgG2b, alkaline phosphatase (AP)-conjugated anti-mouse IgG1, FITC-conjugated anti-mouse IgG1, and biotin-conjugated anti-mouse IgG2b. Texas Red-conjugated streptavidin was from CALTAG, unconjugated goat anti-mouse IgG was from Jackson Immunoresearch Labs, and protein G-Sepharose was from Amersham Pharmacia Biotech.

Nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate was obtained from Roche Molecular Biochemicals, and ECL was from Amersham Pharmacia Biotech.

*Cells and Cell Culture Reagents*—Mouse NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% bovine calf serum, rat PC12 cells (variant from M. Chao's laboratory, kindly provided by R. Krauss) were cultured in DMEM with 10% fetal bovine serum. Both cells were grown in an atmosphere of 5% CO2 at 37 °C. PD98059 was from New England BioLabs. Acidic FGF was purified from Escherichia coli (18), recombinant β-NGF was from R&D Systems, and recombinant human insulin was from Sigma.

*Cell Lines, Immunoprecipitation, and Immunoblotting*—Cells were starved for 2 h in serum-free medium and subsequently treated with 100 ng/ml FGF plus 5 μg/ml heparin, 5 μg/ml insulin, or 50 ng/ml NGF for times as indicated in the figures. Cells were lysed with 150 mM NaCl, 20 mM Tris, pH 7.6, 50 mM NaF, 1 mM Na3VO4, 5 mM benzamidine, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotonin, 10 μg/ml leupeptin, and 1% v/v Nonidet P-40, and nuclei were removed by centrifugation. For immunoprecipitations, antibodies were incubated with lysates overnight at 4 °C. Immunoprecipitates from polyclonal antibodies were captured directly with protein G-Sepharose for 1 h, whereas immunoprecipitates from monoclonal antibodies were captured with protein G-Sepharose preincubated with goat anti-mouse IgG (100 μg/ml gel). Beads were pelleted and washed twice with lysis buffer before eluting proteins by boiling in sample buffer containing 2% SDS and 5% β-mercaptoethanol.

Proteins were electrophoresed through SDS-polyacrylamide gels (SDS-polyacrylamide gel electrophoresis) (7.5% or 10% polyacrylamide), electroblotted to polyvinylidene difluoride membranes (Immobilon-P, Millipore), and probed with primary antibodies as indicated in the figures, followed by incubation with corresponding horseradish peroxidase-conjugated secondary antibodies (typically 110,000 dilution) and detection by ECL. Apparent molecular weights of proteins were estimated based on migration of prestained molecular weight standards (Amersham Pharmacia Biotech).

*Expression of Myc-tagged Proteins in NIH3T3 Cells*—Myc-tagged SNT1 expression vector was derived by inserting full-length human SNT1 Domains Coordinate ERK Activation and Differentiation into the K-red(B) C-terminal membrane-targeting sequence (CAAX motif: KGDKKKKKKRRITRCTVM) into the position C-terminal to the Myc epitope sequence of pSRa-SNT1(IRS)/MDM (19) expressing Myc-tagged full-length SNT1 or SNT/IRS chimera proteins were treated with different growth factors (GF) for 10 min: none (-), 50 ng/ml insulin (I), and 5 μg/ml insulin (I, ΔT). SNT1(IRS)CX mutants (2YF, 4YF, 6YF, and ΔT) were engineered by replacing restriction enzyme segments of pSRa-SNT1(IRS)/CX with corresponding mutant segments of SNT1(IRS)CX mutants (2YF, 4YF, 6YF, and ΔT).

NIH3T3 cells (3 × 10⁴ per 10-cm dish) were cotransfected as calcium phosphate precipitates (20) with 5 μg of SNT1 expression plasmid, 1 μg of pLRNeo, and 25 μg of human placental carrier DNA and selected with G418 (400 μg/ml, Life Technologies, Inc.) to establish pools of stably transfected cells.

*Expression of Proteins in PC12 Cells*—PC12 cells were transiently transfected with pSRα-Myc-tagged SNT/IRS constructs or with corresponding HA-epitope-tagged constructs in which the triple Myc tag was replaced with annealed oligonucleotides encoding two tandem copies of the HA-epitope sequence (YDYDPYAS). For transient transfection of single plasmids, ~1.2 × 10⁶ cells per 60-mm dish were transfected with 3 μg of expression plasmids and 16 μl of LipofectAMINE Reagent (Life Technologies, Inc.) in a total of 3 ml.

For transient cotransfection, 0.5 μg of Myc-tagged pSRα-SNT1(IRS)/CX was mixed with 2.5 μg of pCEV29-Ras11T (kindly provided by A. Chan), pCMV-HA-Shig22KΔAT (kindly provided by B. Neel), or corresponding empty vectors and transfected with LipofectAMINE, as above.

For PC12-stable transfections, each Myc-tagged SNT1(IRS)/CX open reading frame was first shuttled into the expression vector pMir18R (21) (kindly provided by D. Ornitz) for expression driven by Moloney murine leukemia virus LTR as a bicistronic mRNA bearing a downstream IRES-neo-cassette. ~1.5 × 10⁶ PC12 cells were electroporated (250
V/500 microfarads) in a Gene Pulser (Bio-Rad) in 1 ml of DMEM containing 40 μg of linearized pMIRB vectors, and plated onto two 10-cm dishes. Three days after transfection, 800 μg/ml G418 was added and maintained for 3 weeks. G418-resistant clones were picked, expanded, maintained in 200 μg/ml G418, and screened for similar level of protein expression by both immunoblot and Myc-immunostaining.

**Northern Blot Analysis**—Total cellular RNA was isolated using the TRIzol reagent (Life Technologies, Inc.). RNA samples (10 μg per lane) were electrophoresed through 1.1% agarose gels containing 2.2 M formaldehyde in MOPS buffer (20 mM MOPS, pH 7.0, 5 mM sodium acetate, and 1 mM EDTA), and then transferred overnight to HyBond N+ nylon membrane (Amersham Pharmacia Biotech). Full-length VGF cDNA (kindly provided by S. Halegoua) was radiolabeled with 32P-dNTPs (PerkinElmer Life Sciences) by DNA polymerase and random hexamer primers and hybridized to the blot using ExpressHyb hybridization solution (CLONTECH) at 68 °C for 2 h, washed to a final stringency of 50 °C, 0.1× SSC, 0.1% SDS, and autoradiographed. To confirm equivalent loading and transfer of RNA samples, the blot was reprobed under the same conditions with 32P-labeled rat glyceraldehyde phosphate dehydrogenase cDNA.

**Ras Activation Assay**—A GST-RafRBD (Ras-binding domain) fusion protein, which can bind Ras GTP (kindly provided by S. Taylor), was expressed in E. coli and purified on glutathione-agarose at 2 mg of fusion protein per 1 ml of beads, as previously described (22). PC12 cells were lysed in Mg2+-containing lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 0.25% sodium deoxycholate, 10% glycerol, 10 mM MgCl2, 1 mM Na4VO4, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1% Nonidet P-40). 250 μg of

![Fig. 2](image1.png) SNT1(IRS)/CX enables PC12 cells to terminally differentiate in response to insulin. A, B, and C, PC12 cells were transiently transfected with Myc-tagged wild-type SNT1 or SNT1/IRS chimeras. After 24 h, the cells were treated with different growth factors for 48 h before fixation; NGF (50 ng/ml), FGF (100 ng/ml), and INS (insulin, 5 μg/ml). A, detection of transfected cells with anti-Myc and AP-conjugated secondary antibodies, and stained with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (purple). Representative stained cells are shown. B, quantitation of neurite outgrowth. Neurite outgrowth was quantitated by scoring the percentage of stained cells with neurites longer than the size of two cell bodies. The data shown are from one of two experiments, both of which yielded similar results. C, subcellular localization of overexpressed proteins. Transfected cells were incubated with anti-Myc and FITC-conjugated secondary antibodies. Green fluorescence in representative confocal microscope images are shown. D, induction of VGF expression. PC12 cells stably transfected with Myc-tagged SNT1/IRS/CX and parental PC12 cells were treated with different growth factors (GF) for 5 h: None (–), 50 ng/ml NGF (N), 5 μg/ml insulin (I). Total RNA was isolated and subjected to analysis on a Northern blot. The blot was probed with 32P-labeled VGF full-length cDNA, and later reprobed with 32P-labeled rat glyceraldehyde phosphate dehydrogenase cDNA.

![Fig. 3](image2.png) Ras, Shp2, and MEK are required for SNT1(IRS)/CX-mediated PC12 cell neurite outgrowth. Effect of (A) RasN17 and (B) Shp2C459S on PC12 cell neurite outgrowth. Myc-tagged SNT1(IRS)/CX was transiently cotransfected with indicated constructs or corresponding empty vectors (as controls) into PC12 cells. After 24 h, the cells were treated with NGF (50 ng/ml) or INS (insulin, 5 μg/ml) for 72 h, then fixed and immunostained for Myc-tagged proteins. The number of transfected cells displaying neurite outgrowth was quantitated, and the data were expressed relative to a maximum of 100%. The data shown are from one of two experiments, both of which yielded similar results. C, effect of PD98059 on PC12 cell neurite outgrowth. PC12/SNT1(IRS)/CX cells were pretreated with 20 μM PD98059 or 0.1% Me2SO (–, as control) for 60 min prior to the addition of NGF or insulin for 48 h. Neurite outgrowth was scored as described above.
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RESULTS

An SNT1/IRS Chimera Activated by Insulin Drives PC12 Neuronal Differentiation—FGFs and neurotrophins induce SNT tyrosine phosphorylation, whereas insulin induces tyrosine phosphorylation of multifunctional insulin receptor substrate (IRS) docking proteins. We reasoned that a chimeric SNT1/IRS protein bearing the SNT backbone, but containing the insulin receptor recognition domains of IRS, would undergo tyrosine phosphorylation in response to insulin. We constructed SNT1(IRS), a Myc epitope-tagged SNT1 protein in which the N-terminal 137 amino acid residues of SNT1, bearing a myristoylation motif and the FGFR/Trk receptor-specific PTB domain, were replaced with the N-terminal pleckstrin homology (PH) and PTB domains of IRS2 (residues 1–307) (Fig. 1A). When SNT1(IRS) was stably transfected into NIH3T3 fibroblasts, insulin, but not FGF, could induce SNT1(IRS) tyrosine phosphorylation (Fig. 1B). The behavior of SNT1(IRS) stood in contrast to transfected SNT1-Myc, which was phosphorylated in response to FGF, but not insulin (Fig. 1B).

To test whether SNT1(IRS) could promote neuronal differentiation, Myc-tagged SNT1(IRS) or SNT1 were transiently transfected into PC12 cells, which were subsequently challenged for 2 days with the neurotrophin nerve growth factor (NGF), FGF1, or insulin. Subsequently, transfected cells, as identified by anti-Myc-tag immunostaining (Fig. 2A), were scored for neuronal differentiation using neurite outgrowth as the morphological criterion (23). Although PC12 cells transfected with SNT1 or SNT1(IRS) underwent neuronal differentiation in response to NGF or FGF (Fig. 2B), analogous to untransfected cells (data not shown), insulin failed to induce differentiation of SNT1(IRS)-transfected cells (Fig. 2B).

Biochemical and biological activities of SNT1 require its plasma membrane attachment mediated by N-terminal myristoylation (5). The failure of SNT1(IRS) to promote neuronal differentiation may have resulted from reduced membrane association of the chimeric protein lacking the myristoylation motif. Indeed, when SNT1(IRS) subcellular distribution was compared with SNT1 by confocal immunofluorescence analysis of transfected PC12 cells, SNT1(IRS) was evenly distributed throughout the cytoplasm as opposed to the exclusively plasma membrane localization of SNT1 (Fig. 2C).

To relocalize a chimeric SNT1(IRS) protein back to the plasma membrane, we engineered the variant SNT1(IRS)CX (Fig. 1A) by fusing the CAAX farnesylation motif of K-ras to the C terminus (19). As anticipated, SNT1(IRS)CX was largely associated with the plasma membrane in transfected PC12 cells (Fig. 2C) and retained the ability to undergo insulin-induced tyrosine phosphorylation in transfected NIH3T3 cells (Fig. 1B). When SNT1(IRS)CX was transiently transfected into PC12 cells, it could effectively mediate insulin-induced neuronal differentiation (Fig. 2, A and B) and up-regulation of the VGF differentiation-specific gene transcript (Fig. 2D). This result demonstrates that artificial stimulation of SNT effector functions is sufficient to recapitulate the biological response to neuronal differentiation factors.

SNT1(IRS)CX-mediated Neuronal Differentiation Requires Endogenous Ras, MEK, and Shp2 Activities and Is Accompanied by Sustained Ras and ERK1/2 Activation—Differentiation factor-induced PC12 neuronal differentiation requires Ras, MEK, and Shp2 activities (12, 13) and is accompanied by sustained ERK activation. We sought to determine whether SNT1(IRS)CX promotes neuronal differentiation through the same biochemical mechanisms. To determine whether Ras and Shp2 are required for SNT1(IRS)CX-mediated differentiation, Myc-tagged SNT1(IRS)CX was transiently cotransfected with empty vector controls or with dominant-negative mutant forms of Ras (RasN17) or Shp2 (Shp2C450S), and neurite outgrowth was quantitated by scoring Myc-positive cells following 3-day insulin stimulation. As shown in Fig. 3, either RasN17 (Fig. 3A) or Shp2C450S (Fig. 3B) could significantly reduce SNT1(IRS)CX-mediated differentiation induced by insulin.

Additional analyses made use of PC12 cells stably transfected with SNT1(IRS)CX (see “Experimental Procedures”). PC12/SNT1(IRS)CX cells were also capable of undergoing insulin-induced neurite outgrowth (Fig. 3C). Pretreatment of

Fig. 4. SNT1(IRS)CX-mediated PC12 cell neurite outgrowth is accompanied by sustained Ras/ERK activity. Parental PC12 and PC12/SNT1(IRS)CX cells were treated with different growth factors (GF) for the indicated times: None (--), 50 ng/mL NGF (N), 5 μg/mL insulin (I), or 50 ng/mL NGF (NGF), FGF1, or insulin. Subsequently, transfected cells, as identified by anti-Myc-tag immunostaining (Fig. 2A), were scored for neuronal differentiation using neurite outgrowth as the morphological criterion (23). Although PC12 cells transfected with SNT1 or SNT1(IRS) underwent neuronal differentiation in response to NGF or FGF (Fig. 2B), analogous to untransfected cells (data not shown), insulin failed to induce differentiation of SNT1(IRS)-transfected cells (Fig. 2B).
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PC12/SNT1(IRS)CX with the MEK-specific kinase inhibitor PD98059 dramatically reduced insulin-induced differentiation (Fig. 3C).

To determine whether SNT1(IRS)CX stimulation promotes sustained ERK activation, parental PC12 and PC12/SNT1(IRS)CX cells were treated with either NGF or insulin for 0.2 or 3 h, and total cell lysates were analyzed for ERK activation by probing immunoblots with monoclonal antibody specific for tyrosine/threonine dually phosphorylated ERKs. As shown in Fig. 4, NGF-induced ERK1 and ERK2 activation in transfected or untransfected cells persists for 3 h, whereas insulin-induced ERK activation in parental PC12 cells is transient and disappears by 4 h post-stimulation. By contrast, ERK activity is strongly persistent in PC12/SNT1(IRS)CX cells stimulated with insulin. The mechanism by which SNT1(IRS)CX mediates sustained ERK activity may be through its ability to mediate sustained Ras activation (Fig. 4).

The above results demonstrate that SNT1(IRS)CX promotes PC12 neuronal differentiation through a repertoire of signaling intermediates indistinguishable from those employed by neuronal differentiation factors.
We then tested all SNT1(IRS)CX mutants for their ability to promote neuronal differentiation following transient transfection of PC12 cells. As shown in Table I, the 2YF, 4YF, 6YF, and ΔT mutations dramatically reduced or abolished the ability of SNT1(IRS)CX to mediate neurite outgrowth in response to insulin.

Because neuronal differentiation correlates with sustained ERK activation, we also tested whether each of the mutant SNT1(IRS)CX constructs would fail to sustain ERK activity. Transiently transfected cells were stimulated with NGF, insulin, or no growth factors for 3 h, then fixed and analyzed by double immunofluorescence with anti-phospho-ERK (p-ERK) (green) and anti-HA tag (red). Representative confocal microscopic images of each sample are shown.

Table II

Quantitation of sustained ERK activation in PC12 cells expressing wild-type and mutant SNT1(IRS)CX

<table>
<thead>
<tr>
<th>Transfected plasmid</th>
<th>% Phospho-ERK-positive cells treated with NGF</th>
<th>% Phospho-ERK-positive cells treated with Insulin</th>
<th>Ratio Insulin/NGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNT1(IRS)CX-WT</td>
<td>63.5</td>
<td>48.5</td>
<td>0.76</td>
</tr>
<tr>
<td>SNT1(IRS)CX-2YF</td>
<td>57.0</td>
<td>9.5</td>
<td>0.17</td>
</tr>
<tr>
<td>SNT1(IRS)CX-4YF</td>
<td>50.5</td>
<td>40.5</td>
<td>0.80</td>
</tr>
<tr>
<td>SNT1(IRS)CX-6YF</td>
<td>51.0</td>
<td>6.0</td>
<td>0.12</td>
</tr>
<tr>
<td>SNT1(IRS)CX-ΔT</td>
<td>48.5</td>
<td>15.0</td>
<td>0.31</td>
</tr>
<tr>
<td>Shp2&lt;sup&gt;C459S&lt;/sup&gt;</td>
<td>12.0</td>
<td>NA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NA</td>
</tr>
</tbody>
</table>

<sup>a</sup> NA, not applicable.

Fig. 6, and the quantified data are shown in Table II. Although wild-type SNT1(IRS)CX promoted sustained ERK activation, SNT1(IRS)CX-ΔT had a significantly reduced sustained ERK activation potential. The behavior of SNT1(IRS)CX-ΔT establishes a previously unappreciated role for the conserved SNT C-terminal domain in mediating biochemical and biological responses in PC12 cells. SNT1(IRS)CX-ΔT has been subjected to further biochemical analysis (see below).

SNT1(IRS)CX-2YF failed to promote ERK activity (Fig. 6 and Table II), strongly suggesting that Shp2 recruitment to SNT is essential for sustained ERK activation in PC12 cells. To directly test the importance of Shp2 for ERK activation, we transiently transfected PC12 cells with epitope-tagged dominant-negative Shp2 (Shp2<sup>C459S</sup>) and assayed the effect on NGF-induced sustained ERK activity. As shown in Fig. 6 and Table II, inhibition of Shp2 activity dramatically impaired NGF-induced sustained ERK activation. These findings are consistent with data reported for other types of cells (25, 26).

Surprisingly, SNT1(IRS)CX-4YF, which lacks the major Grb2-Sos recruitment sites, still induced sustained ERK activation in a substantial percentage of transfected cells. However, the levels of active ERK appeared consistently lower in insulin-stimulated SNT1(IRS)CX-4YF transfectants when compared with wild-type SNT1(IRS)CX-transfected cells (see representative cells in Fig. 6). Residual Grb2-Sos recruitment to SNT1(IRS)CX-4YF, which may be mediated indirectly through tyrosine-phosphorylated Shp2 (11), may be sufficient to mediate reduced, but readily detectable, ERK activation. The more dramatic impairment of the 4YF mutation on neurite outgrowth (Table I) may reflect the mutant’s failure to achieve a critical threshold of ERK activation. Alternatively, the 4YF mutation may substantially impair ERK activation beyond the 3-h time window analyzed here.

SNT1(IRS)CX-ΔT Is Defective for Sos Recruitment in PC12 Cells—To better understand the role of the SNT C terminus in mediating sustained ERK activity and neuronal differentiation, we established a stably transfected PC12 cell line expressing...
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Fig. 7. Biochemical analysis of PC12 cells stably expressing SNT1(IRS)CX-ΔT mutant. A, effect of SNT1(IRS)CX-ΔT mutation on Grb2, Sos, and Shp2 binding. Parental PC12 cells and cells from PC12/SNT1(IRS)CX cloned lines (WT and ΔT) were treated with 5 μg/ml insulin (INS) for the indicated times. Lysates were immunoprecipitated (IP) with anti-Myc, anti-Grb2, and anti-Sos1, electrophoresed, and immunoblotted (IB) with anti-pY, anti-Myc, anti-Sos1, or anti-Grb2 as indicated. NIH3T3 cells stably transfected with SNT1(IRS)CX-WT or ΔT were treated with insulin, and lysates were subjected to immunoprecipitations and immunoblot detection. Sos recruitment to SNT(IRS)CX is impaired by the ΔT mutant on Ras/ERK activation. Parental PC12 cells and cells from PC12/SNT1(IRS)CX cloned lines were treated with 50 ng/ml insulin (INS) for the indicated times: None ( ), 0.2 ( ), 0.3 ( ), and cells from PC12/SNT1(IRS)CX cloned lines were treated with different growth factors (GF) for the indicated times: None ( ), 50 ng/ml insulin (INS), 5 μg/ml insulin (INS), and 2 ng/ml FGF (FGF). Lysates were immunoprecipitated with GST-RafRBD agarose and electrophoresed, and immunoblotted with anti-pY, anti-Myc, anti-Grb2, and anti-Sos1, electrophoresed, and immunoblotted with anti-Grb2, anti-Sos1, and anti-Shp2 as indicated.

B, effect of SNT1(IRS)CX-ΔT mutant on Ras ERK activation. Parental PC12 cells and cells from PC12/SNT1(IRS)CX cloned lines were treated with different growth factors (GF) for the indicated times: None ( ), 50 ng/ml insulin (INS), 5 μg/ml insulin (INS), and 2 ng/ml FGF (FGF). Lysates were immunoprecipitated with GST-RafRBD agarose and electrophoresed, and immunoblotted with anti-pY, anti-Myc, anti-Grb2, and anti-Sos1, electrophoresed, and immunoblotted with anti-Grb2, anti-Sos1, and anti-Shp2 as indicated.

Insulin and FGF stimulation of SNT1(IRS)CX-ΔT was comparable to wild-type SNT1(IRS)CX in terms of levels of insulin-induced tyrosine phosphorylation and association with both Grb2 and Shp2. However, association of Sos with insulin-activated SNT1(IRS)CX-ΔT was significantly reduced. We interpret these results to indicate that Sos is recruited to SNT by multiple contacts, including the well-documented Grb2 SH3 domain interaction with a proline-rich domain on Sos (14, 27–29) as well as SNT C-terminal domain interaction with Sos by either direct or indirect mechanisms. Impaired association of SNT1(IRS)CX-ΔT with Sos is cell type-specific, because SNT1(IRS)CX-ΔT and wild-type SNT1(IRS)CX show comparable association with Sos in NIH3T3 cells (Fig. 7A).

Reduced SNT1(IRS)CX-ΔT association with Sos in PC12 cells impairs signaling through the Ras/ERK pathway. As shown in Fig. 7B, insulin stimulation of SNT1(IRS)CX-ΔT failed to induce sustained Ras activity above basal levels and reduced sustained ERK1/2 activation. Commensurate with these findings, the PC12/SNT1(IRS)CX-ΔT cells failed to undergo insulin-induced neuronal differentiation (data not shown).

DISCUSSION

Neurotrophins and FGFs promote neuronal differentiation by coordinately activating Ras and Shp2 with resultant sustained activation of ERK mitogen-activated protein kinases. SNTs were suspected of playing a key role in the differentiation response, because neurotrophins and FGFs specifically induce SNT tyrosine phosphorylation, which is followed by SNT recruitment of Ras activators and Shp2. We have shown here that artificial activation of SNT1 in PC12 cells recapitulates the differentiation response, which is preceded by and is dependent on the same signaling pathways that are mediated by differentiation factors. These findings suggest that SNT is a principle mediator of growth factor-induced neuronal differentiation. We should note that differentiation factors likely induce additional signaling pathways, which act in concert with SNT. For example, although NGF and FGF activate SNT to comparable extents in PC12 cells (4, 5), NGF more potently induces their differentiation (30) (and Fig. 2). Reciprocally, FGF is more potent than NGF at promoting early phase neuronal differentiation of MAH sympathoadrenal progenitor cells (31, 32), whereas both factors activate SNTs comparably.

Although our data demonstrate that SNT phosphorylation leads to sustained ERK activity through the activation of both Ras and Shp2, the mechanism by which Ras and Shp2 coordinate sustained ERK activation is unclear. Candidate targets for Shp2 may include Src-family nonreceptor tyrosine kinases, because these kinases are activated by tyrosine dephosphorylation and play essential roles in FGF-mediated biological responses (33–36). It has been reported that NGF-induced sustained ERK activation may also require and be more dependent on the activation of the G protein Rap1 than on Ras; activated Rap1 was shown to form complexes with RafB in NGF-treated cells, promoting the RafB → MEK → ERK kinase cascade (37). However, the sublineage of PC12 cells used in our studies showed no evidence of NGF-induced or SNT1(IRS)CX-induced Rap1 activation.

Previous studies have shown that FGF induces tyrosine phosphorylation of SNT and She, both of which recruit Grb2; nonetheless, virtually all of the Sos in FGF-treated cells is recruited to SNT-Grb2 complexes at the expense of She-Grb2 complexes (9). Our data offer a possible mechanism for efficient SNT-Sos complex formation. In PC12 cells, recruitment of Sos is dependent on the C-terminal domain of SNT in addition to the phosphotyrosyl motifs that recruit Grb2 (Fig. 7A). Deletion of the SNT1 C-terminal motif in SNT1(IRS)CX did not affect Grb2 or Shp2 recruitment but reduced Sos recruitment with insulin-induced tyrosine phosphorylation and association with both Grb2 and Shp2. However, association of Sos with insulin-activated SNT1(IRS)CX-ΔT was significantly reduced. We interpret these results to indicate that Sos is recruited to SNT by multiple contacts, including the well-documented Grb2 SH3 domain interaction with a proline-rich domain on Sos (14, 27–29) as well as SNT C-terminal domain interaction with Sos by either direct or indirect mechanisms. Impaired association of SNT1(IRS)CX-ΔT with Sos is cell type-specific, because SNT1(IRS)CX-ΔT and wild-type SNT1(IRS)CX show comparable association with Sos in NIH3T3 cells (Fig. 7A).

Reduced SNT1(IRS)CX-ΔT association with Sos in PC12 cells impairs signaling through the Ras/ERK pathway. As shown in Fig. 7B, insulin stimulation of SNT1(IRS)CX-ΔT failed to induce sustained Ras activity above basal levels and reduced sustained ERK1/2 activation. Commensurate with these findings, the PC12/SNT1(IRS)CX-ΔT cells failed to undergo insulin-induced neuronal differentiation (data not shown).

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Hong Xu and Mitchell Goldfarb

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