Neurotrophin-dependent Tyrosine Phosphorylation of Ras Guanine-releasing Factor 1 and Associated Neurite Outgrowth Is Dependent on the HIKE Domain of TrkA*

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Ras guanine-releasing factor 1 (RasGrf1), a guanine nucleotide exchange factor for members of the Ras and Rho family of GTPases, is highly expressed in the brain. It is regulated by two separate mechanisms, calcium regulation through interaction with its calcium/calmodulin-binding IQ domain and serine and tyrosine phosphorylation. RasGrf1 is activated downstream of G-protein-coupled receptors and the non-receptor tyrosine kinases, Src and Ack1. Previously, we demonstrated a novel interaction between the intracellular domain of the nerve growth factor-regulated TrkA receptor tyrosine kinase and an N-terminal fragment of RasGrf1. We now show that RasGrf1 is phosphorylated and interacts with TrkA, -B, and -C in co-transfection studies. This interaction and phosphorylation of RasGrf1 is dependent on the HIKE domain of TrkA (a region shown to interact with pleckstrin homology domains) but not on any of the phosphotyrosine residues that act as docking sites for intracellular signaling molecules such as Shc and FRS-2. The PH1 domain alone of RasGrf1 is sufficient for phosphorylation by the TrkA receptor. A potential role for Trk activation of RasGrf1 is suggested through transfection studies in PC12 cells in which RasGrf1 significantly increases neurite outgrowth at low doses of neurotrophin stimulation. Notably, this neurite outgrowth is dependent on an intact HIKE domain, as nnr5-S10 cells expressing a TrkA HIKE domain mutant do not exhibit potentiated neurite outgrowth in the presence of RasGrf1. These studies identify RasGrf1 as a novel target of neurotrophin activation and suggest an additional pathway whereby neurotrophin-stimulated neurite outgrowth may be regulated.

The neurotrophins, including nerve growth factor (NGF),1 brain-derived neurotrophic factor (BDNF), neurotrophin-3, and neurotrophin-4/5, are a family of growth factors that mediate diverse cellular responses such as mitogenesis, apoptosis, and neuritogenesis in the developing nervous system (1). Additionally, in the brain, the neurotrophins have been shown to be involved in the regulation of axonal growth (2), memory and long term potentiation (3), calcium signaling (4), and synaptogenesis (5). The receptors for the neurotrophins are the low affinity p75

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1 The abbreviations used are: NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; GEF, guanine nucleotide exchange factor; PH, pleckstrin homology; DH, Dbl homology; HA, hemagglutinin; EGF, enhanced green fluorescent protein; LTP, long term potentiation; RasGrf1, Ras guanine-releasing factor 1; Trk, tropomyosin-related kinase; GST, glutathione S-transferase; CC, coiled-coil motif; FRS, fibroblast growth factor receptor substrate.
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as Ras and Rac by facilitating the release of GDP (13). The structural domains of RasGrf1 include two pleckstrin homology (PH) domains, a coiled-coil motif (CC), an IQ motif, a Dbl homology domain (DH), a Ras exchange motif/PEST motif and a C-terminal CDC25 homology domain that is required for guanine exchange toward Ras (Fig. 1A). Pleckstrin homology domains interact with phosphoinositides in the lipid membrane (14), can act as phosphotyrosine-binding domains (15, 16), and have been shown to interact with other proteins, such as the Gβγ subunit (17). Mutation studies, using a mutant of RasGrf1 in which the N-terminal PH domain has been deleted, have indicated that this domain is necessary for the localization of RasGrf1 to the plasma membrane (18). IQ domains bind the calcium-regulated protein calmodulin (19), whereas the Ras-Grf DH domain binds to Rac, a member of the Rho family of GTPases facilitating its activation (20).

Several studies have shown RasGrf1 interaction downstream of G-protein-coupled receptors (21, 22) and Rac family members (23). Studies have also indicated that receptor tyrosine kinases are not involved in RasGrf1 activation (21, 22). There does, however, appear to be a role for non-receptor tyrosine kinases in RasGrf1 activation, namely Src (20) and Ack1 (24). Specifically, c-Src phosphorylates multiple sites on RasGrf1 in activation, namely Src (20) and Ack1 (24).

In the following study we have examined the ability of the Trk receptors to tyrosine phosphorylate and to interact with RasGrf1 in transfected cells and have sought to determine which site(s) or domains are required for this interaction and phosphorylation. A potential role for Trk-dependent tyrosine phosphorylation of RasGrf1 in facilitating neurotrophin-dependent neurite outgrowth is suggested through transfection studies in PC12 cells.

MATERIALS AND METHODS

Reagents—The anti-HA antibody (3F10) was from Roche Applied Science. The anti-RasGrf1 (C-20) and anti-Trk (C-14) antibodies were provided by Santa Cruz Biotechnology. Anti-ShcB has been described previously (25). Horseradish peroxidase-coupled anti-phosphotyrosine antibody (p-Tyr-100) was from Cell Signaling (Figs. 5–7). Anti-rabbit antibodies were from The Jackson Laboratories. The anti-rabbit antibodies were from The Jackson Laboratories. Anti-Grf1 (C-20) and anti-Trk (C-14) antibodies were prepared using standard techniques. The original antibody (J203) was used in Figs. 1–4, whereas the second batch of antibody (1478) was used in Figs. 5–7. Horseradish peroxidase-coupled goat anti-mouse and anti-rabbit antibodies were from Santa Cruz Biotechnology. Anti-ShcB has been described previously (25). Horseradish peroxidase-coupled anti-phosphotyrosine antibody (p-Tyr-100) was from Cell Signaling (Figs. 5–7). Anti-rabbit antibodies were from The Jackson Laboratories. The anti-rabbit antibodies were from The Jackson Laboratories. Anti-Grf1 (C-20) and anti-Trk (C-14) antibodies were prepared using standard techniques. The original antibody (J203) was used in Figs. 1–4, whereas the second batch of antibody (1478) was used in Figs. 5–7. Horseradish peroxidase-coupled anti-mouse and anti-rabbit antibodies were from The Jackson Laboratories.
were stimulated with either NGF, BDNF, or neurotrophin-3 (100 ng/ml, 5 min), and lysates were assayed by Western blotting for the ability of glutathione-Sepharose to precipitate Trk.

As shown in Fig. 1B, GST-RasGrf1 (full-length) but not GST alone precipitated all three kinase-active Trk receptors suggesting that RasGrf1 interacts with TrkA, TrkB, and TrkC in an activity-dependent manner. Next, we cotransfected mouse RasGrf1 and HA-tagged wild-type TrkA, TrkB, TrkC, or a kinase-inactive TrkA receptor mutant (K547A) and assayed neurotrophin-stimulated lysates for tyrosine phosphorylation of both immunoprecipitated Trks and/or RasGrf1. As shown in Fig. 1C (left panel), TrkA, TrkB, and TrkC but not kinase-dead TrkA are tyrosine-phosphorylated. Similarly, although RasGrf1 shows a basal level of phosphorylation in the presence of kinase-inactive TrkA, this level is significantly increased when cotransfected with wild-type TrkA, -B, and -C (Fig. 1C, right panel). As the structure of all three Trk receptors (TrkA, -B, and -C) is very similar and because of greater expression levels of TrkA and the availability of TrkA-specific mutants, we primarily used the TrkA receptor to further evaluate RasGrf1 tyrosine phosphorylation by Trk.

Mutations in the N-terminal PH Domain and the IQ Domain of RasGrf1 Decrease Trk-dependent Tyrosine Phosphorylation—As stated earlier, mutations in RasGrf1 have been used to investigate the differential properties of individual domains in relation to the overall function of RasGrf1. In this respect, based on its ability to bind phosphoinositides (18), the N-terminal PH domain is important for membrane localization, and the IQ domain is essential for Ca\(^{2+}\)-dependent binding of calmodulin (19) and Ca\(^{2+}\)-dependent RasGrf1 activation of Ras (reviewed in Ref. 35). Because the PH and phospho-tyrosine binding domains express a similar three-dimensional structure (a 7-stranded \(\beta\)-sandwich followed by a C-terminal \(\alpha\)-helix) (15, 16, 36) and, in some cases, can bind similar substrates, we predicted that one of the PH domains, in particular the N-terminal domain, might be essential to Trk-dependent binding and phosphorylation. Consequently, we assayed three PH-domain deletion mutants of RasGrf1 (Fig. 2A). The first two contained deletions in the N-terminal PH domain (N59, which is a partial deletion of the PH1 domain, and N158, which is missing the entire PH domain). A third mutant, nPH(-), contains alanine substitutions at the conserved tryptophan residue (position 121) and the semiconserved isoleucine (position 125) residue in the C-terminal \(\alpha\)-helix of PH1 (18). All mutants are stably expressed in transfection studies, but they interfere with RasGrf1 activation in response to the calcium ionophore ionomycin (18). Although the N59 and the nPH(-) mutants are retained at the cell membrane, the N158 mutant partitions equally between soluble and particulate fractions indicating that the deletion has impaired membrane localization probably through decreased ability to bind phosphoinositides (18).

As shown in Fig. 2B, co-transfection of the PH domain mutants with TrkA in COS cells followed by immunoprecipitation and Western blot analysis indicates that neither the N59 deletion nor the nPH(-) mutation affects RasGrf1 tyrosine phosphorylation by TrkA. By comparison, however, deletion of the
entire PH domain (N158) significantly decreased the level of Trk-dependent tyrosine phosphorylation (compare lane 3 (wild-type RasGrf1) and lane 5 (N158)). Importantly, the decrease in tyrosine phosphorylation of N158 does not reflect lower amounts of kinase-active TrkA co-expressed in the cell lysates (Fig. 2B, lower left panel) or lower levels of N158 expression as shown by stripping and reprobing with anti-RasGrf1 antibodies (Fig. 2B, lower right panel). In addition, we found that a triple point mutant in the IQ domain (R214Q/R219Q/K223Q) (19), which decreases both Ca\textsuperscript{2+}/calmodulin binding and Ca\textsuperscript{2+}-dependent activation of mitogen-activated protein kinase (18), also decreased TrkA-dependent phosphorylation of RasGrf1 in COS cell co-transfection assays (Fig. 2B, right panel, compare lanes 3 and 7). As before, this decreased phosphorylation is not a result of decreased expression of either IQ(-) RasGrf1 or active TrkA. Collectively, these results suggest that there may be two independent processes regulating Trk-dependent phosphorylation of RasGrf1. First, the N-terminal PH domain of RasGrf1 is essential to its tyrosine phosphorylation by TrkA by either directly recruiting RasGrf1 to TrkA or by indirectly targeting RasGrf1 to the membrane in the proximity of the activated receptor. Second, the decreased phosphorylation of the IQ(-) mutant could indicate an additional means of RasGrf1 regulation by TrkA. This might occur indirectly through conformational changes that do not affect membrane localization of RasGrf1 (18) but may affect Trk interaction and/or phosphorylation. Alternatively, because Trk activation increases Ca\textsuperscript{2+} mobilization, either through the influx of Ca\textsuperscript{2+} or through the release of Ca\textsuperscript{2+} from intracellular stores (37), it raises the possibility that Trk-dependent increases in intracellular Ca\textsuperscript{2+} and the binding of Ca\textsuperscript{2+}/calmodulin to RasGrf1 may be important to its subsequent interaction and/or phosphorylation by TrkA.

To further investigate the role of membrane localization on Trk-dependent RasGrf1 phosphorylation, we performed two additional experiments. First, we utilized another RasGrf1 mutant containing the N-terminal PH domain of RasGAP in place of the PH1 domain of RasGrf1 (Fig. 3A, Gap PH) (18). Although RasGAP does not bind TrkA directly, it is recruited, via interaction with Shc, in response to NGF stimulation (38). Interestingly, as shown in Fig. 3B (right panel), the Gap PH domain-containing RasGrf1 mutant is tyrosine-phosphorylated in Trk cotransfected COS cells at levels comparable with wild-type RasGrf1 (lanes 3 and 4). Although these data suggest that the membrane localization of RasGrf1 may be sufficient for tyrosine phosphorylation by TrkA, it is also possible that the RasGap PH domain, via its ability to interact with ShcA, recruits RasGrf1 into an active signaling complex that facilitates its subsequent phosphorylation. As a second experiment, we anchored the N158 mutant in the lipid bilayer by the addition of the c-Src myristoylation signal (MGSSKS) at the N terminus (Fig. 3A) and then assayed the ability of this mutant (N158-Myr) to be phosphorylated by TrkA.
Interestingly, as shown in the fraction studies in Fig. 3C, although the myristoylation signal reconstituted the membrane localization of the N158 RasGrf1 mutant, it was insufficient to reconstitute Trk-dependent tyrosine phosphorylation (Fig. 3D, right panel). These data indicate that membrane localization of RasGrf1 is not itself sufficient to support phosphorylation by TrkA, raising the possibility that RasGrf1 interacts directly with TrkA through its N-terminal PH1 domain. It could be argued, however, that this mutant may have suffered a conformational change because of the loss of the PH1 domain that prevents another region of RasGrf1 from interacting with Trk. Another possibility is that the PH1 domain itself contains the sites of tyrosine phosphorylation.

To confirm the importance of the PH1 domain for tyrosine phosphorylation by Trk, we assayed three internal RasGrf1 deletions (Fig. 4A) in co-transfection assays with wild-type and kinase-inactive TrkA. Specifically, we assayed PCQ-Cat (containing the PH1, CC, and IQ domains fused to the CDC25 (or Cat) domain), which retains membrane localization, as well as PC-Cat (PH1-CC-Cat) and P-Cat (containing PH1-Cat), both of which have significantly decreased membrane localization (18). As shown in Fig. 4B (right panel), PCQ-Cat, but not P-Cat, retained Trk-dependent tyrosine phosphorylation (lanes 7 and 9); in fact, PCQ-Cat showed higher levels of tyrosine phosphorylation compared with full-length RasGrf1, given similar levels of protein expression (lower blot), suggesting a possible
negative regulatory role for the internal region containing the DH and PH2 domains. Although the PC-Cat retains some degree of tyrosine phosphorylation (above that observed for the P-Cat mutant), there is still a significant loss of phosphorylation as compared with PCQ-Cat. As the coiled-coil and IQ motifs seem to contribute to localization of RasGrf1 to the membrane, the low phosphorylation levels of PC-Cat might represent a higher degree of membrane localization as compared with the P-Cat mutant.

Additionally, a mutant consisting of only the PH1 domain (PH1), which contains the first five N-terminal tyrosine residues, and a mutant consisting of the PH1 and IQ domains (PH1IQ), which contains two additional tyrosine residues, were evaluated for phosphorylation by TrkA. Importantly, these two mutants have previously been shown to be membrane-localized (39). As shown in Fig. 5B, both the PH1 domain and PH1IQ domain mutants are tyrosine-phosphorylated by wild-type TrkA but not by the kinase-dead receptor. This indicates that the PH1 domain alone is sufficient for tyrosine phosphorylation by TrkA. It is not surprising that the PH1 domain mutant is phosphorylated by TrkA, whereas the P-Cat mutant (which also contains the PH1 domain) is not (Fig. 4B), as membrane localization of the P-Cat mutant is significantly reduced (18). Fig. 5B (lower panels) confirms expression levels of TrkA and the PH1 and PH1IQ mutants.

The HIKE Domain of TrkA Is Essential for Interaction with and Phosphorylation of RasGrf1—To map the potential binding site(s) of RasGrf1 to TrkA, we assayed a series of TrkA mutants that do not affect kinase activation. Specifically, we analyzed TrkAS8 (which contains a Y499F mutation at the Shc/FRS-2 binding site (9, 32, 38)); TrkAS3 (which has a 5-amino acid deletion at 493IMENP497 also affecting interaction with Shc and FRS-2 (9, 32)); TrkAS9 (which contains a point mutation at the PLC/H9253 binding site (Y794F) (9, 38)); TrkAS17 (which contains the juxtamembrane deletion 450KFG452 decreasing FRS-2 phosphorylation/binding (9, 40)); TrkA-S13b Y683E/Y684D (a constitutively active mutant, which contains acidic amino acid (Glu → Asp) substitution of the activation loop tyrosines (41) affecting Grb2 binding (42)); a double S8S9 mutant (which removes both Tyr499 and Tyr794); and a C-terminal truncation mutant (TrkA-T1) (which removes the C-terminal residues Leu787–Gly799 (Fig. 6A)). In all cases, TrkA kinase activity is retained. Lysates from mutant Trk and RasGrf1 cotransfected COS cells were assayed by immunoprecipitation with anti-Trk or anti-RasGrf1 antibodies and analyzed by Western blotting for phosphotyrosine. Interestingly, all of the single site-directed (S8, S9, S13b) and internal deletion TrkA mutants (S3, S17) retained the ability to tyrosine-phosphorylate RasGrf1 (data not shown).

Because it is possible that TrkA and TrkB interact differen-
tially with some target proteins and TrkB is co-expressed with RasGrf1 and PHI and PHI1IQ mutants by TrkA. Cells were cotransfected with Myc-tagged mutant RasGrf1 and HA-tagged TrkA vectors. Lysates were immunoprecipitated with anti-Trk 1478 or anti-Myc and Western blotted with anti-phosphotyrosine (Anti-pTyr) antibody p-Tyr-100. Blots were stripped and reprobed with anti-Trk C-14 or anti-Myc to confirm the level of expression of each transfected plasmid. IP, immunoprecipitate; B, blot.

Fig. 5. The PH1 and PH1IQ domain mutants are phosphorylated by TrkA. A, schematic of wild-type and mutant RasGrf1 (PH1 and PHI1IQ). DB, cyclin destruction box. B, tyrosine phosphorylation of RasGrf1 and PHI and PHI1IQ mutants by TrkA. Cells were cotransfected with Myc-tagged mutant RasGrf1 and HA-tagged TrkA vectors. Lysates were immunoprecipitated with anti-Trk 1478 or anti-Myc and Western blotted with anti-phosphotyrosine (Anti-pTyr) antibody p-Tyr-100. Blots were stripped and reprobed with anti-Trk C-14 or anti-Myc to confirm the level of expression of each transfected plasmid. IP, immunoprecipitate; B, blot.
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Fig. 6. Mutations in the HIKE domain of TrkA prevent RasGrf1 tyrosine phosphorylation and interaction with TrkA. A, schematic of TrkA mutants including the TrkA-S10 HIKE domain mutation. B, tyrosine phosphorylation of RasGrf1 by wild-type TrkA but not by kinase-dead TrkA or the TrkA-S10 mutant. 293T cells were cotransfected with RasGrf1 and HA-tagged TrkA, kinase-dead TrkA (S11), or the HIKE domain mutant TrkA (S10). Lysates were immunoprecipitated with anti-Trk 1478 or anti-RasGrf1 and Western blotted with anti-phosphotyrosine (Anti-pTyr). Blots were stripped and reprobed with anti-Trk C14 or anti-RasGrf1 to confirm the level of expression of each transfected plasmid. IP, immunoprecipitate; B, blot.

C, a significant loss of interaction with RasGrf1 is observed with the TrkA-S10 mutant as compared with TrkA, HA-TrkA, HA-TrkA-S10, or HA-TrkA-S11 and a mammalian expression vector encoding GST or GST fused to full-length RasGrf1 were transfected into 293T cells, and lysates were precipitated with glutathione-agarose. Trk interaction was assayed by Western blotting with the anti-Trk C14 antibody. D, 293T cells were cotransfected with wild-type TrkA, kinase-dead TrkA, or TrkA-S10 and ShcB. Lysates were immunoprecipitated with anti-Trk 1478 or anti-ShcB antibodies and Western blotted with anti-phosphotyrosine (Anti-pTyr) antibody p-Tyr-100. Blots were stripped and reprobed with anti-Trk C14 or anti-ShcB to confirm the level of expression of each transfected plasmid.

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Mutations in the HIKE domain of TrkA prevent RasGrf1 tyrosine phosphorylation and interaction with TrkA. A, schematic of TrkA mutants including the TrkA-S10 HIKE domain mutation. B, tyrosine phosphorylation of RasGrf1 by wild-type TrkA but not by kinase-dead TrkA or the TrkA-S10 mutant. 293T cells were cotransfected with RasGrf1 and HA-tagged TrkA, kinase-dead TrkA (S11), or the HIKE domain mutant TrkA (S10). Lysates were immunoprecipitated with anti-Trk 1478 or anti-RasGrf1 and Western blotted with anti-phosphotyrosine (Anti-pTyr). Blots were stripped and reprobed with anti-Trk C14 or anti-RasGrf1 to confirm the level of expression of each transfected plasmid. IP, immunoprecipitate; B, blot.

C, a significant loss of interaction with RasGrf1 is observed with the TrkA-S10 mutant as compared with TrkA, HA-TrkA, HA-TrkA-S10, or HA-TrkA-S11 and a mammalian expression vector encoding GST or GST fused to full-length RasGrf1 were transfected into 293T cells, and lysates were precipitated with glutathione-agarose. Trk interaction was assayed by Western blotting with the anti-Trk C14 antibody. D, 293T cells were cotransfected with wild-type TrkA, kinase-dead TrkA, or TrkA-S10 and ShcB. Lysates were immunoprecipitated with anti-Trk 1478 or anti-ShcB antibodies and Western blotted with anti-phosphotyrosine (Anti-pTyr) antibody p-Tyr-100. Blots were stripped and reprobed with anti-Trk C14 or anti-ShcB to confirm the level of expression of each transfected plasmid.
The recruitment of GEFs to many activated receptor tyrosine kinases is mediated through the Src homology-2 domain binding of adapters such as Grb2 and Crk to sites of tyrosine phosphorylation on the receptors themselves (direct binding) or to sites of tyrosine phosphorylation on receptor-bound adapters (indirect binding). This results in the recruitment of GEFs, such as Sos1 or C3G, to the membrane where they gain access to their GTP-binding proteins such as Ras, Rac, Rap1, CDC42, or Rho. The brain-specific guanine nucleotide exchange factor RasGrf1, however, unlike Sos1, is unable to bind to adapter proteins such as Grb2, both in vitro and in vivo, and is not tyrosine-phosphorylated in response to epidermal growth factor stimulation in transfected cells (21). Rather, RasGrf1 has been shown to be phosphorylated and activated in response to G-protein-coupled receptor activity (21, 22) and by the non-receptor tyrosine kinases Src and Ack (20, 24). These data, together with the observation that serum-induced phosphorylation and activation of RasGrf1 is prevented by pertussis toxin, an inhibitor of G-protein-coupled receptors, but not by the tyrosine kinase inhibitor genistein (21), suggested that RasGrf1 may not be activated downstream of receptor tyrosine kinases.

On the contrary, however, we reported previously a novel interaction between Trk and RasGrf1 in an activity-dependent manner in yeast two-hybrid studies raising the alternative postulate that RasGrf1 may be a target of some receptor tyrosine kinases, such as the neurotrophin Trk receptors, in transfected cells and in primary neurons (10). In the present study, we extended these observations and demonstrated that full-length RasGrf1 is tyrosine-phosphorylated by TrkA, -B, and -C in an activity-dependent manner in transfected cells. Although the N-terminal PH1 domain of RasGrf1 is essential for binding and is tyrosine-phosphorylated by TrkA, our current data do not exclude the possibility of additional sites of tyrosine phosphorylation also being contained within other C-terminal regions of the protein.

Although TrkA mutants containing substitutions at each of the phosphotyrosine docking sites (Tyr499 or Tyr794) and the activation loop tyrosines (Tyr683 and Tyr684) retain the ability to tyrosine-phosphorylate RasGrf1 at levels comparable with wild-type TrkA, a mutation in the more recently described HIKE domain (K509A/R510A) of TrkA results in both a loss of tyrosine phosphorylation and interaction between TrkA and RasGrf1. This suggests a novel role for the HIKE domain of the Trk receptors as the site of interaction with the guanine exchange factor RasGrf1 in an activity-dependent manner. As HIKE domains have been shown to interact with PH domains (45), this further supports the model that the N-terminal PH domain of RasGrf1 is essential to interaction with the Trk receptors. A functional consequence of TrkA activation of RasGrf1 is suggested by the NGF-induced tyrosine phosphorylation and potentiated neurite outgrowth studies in PC12 cells expressing wild-type TrkA and RasGrf1 but not in cells expressing the TrkA HIKE domain mutant S10.

The HIKE domain was originally identified as a candidate binding site for PH domain-containing proteins through sequence homology analyses between three of the strongest PH...
domain-binding proteins, Gβ, Akt, and PKC (43). These proteins were found to share a similar sequence pattern (LXnRX3,4GXRXnRX4(D,E)), and the core of this region in Akt (HIKXE) gave the HIKE motif its name. HIKE domains have also been identified in various protein kinases (45, 46), neuremodulin (47), and fibroblast growth factor receptors (48), to name a few. The HIKE domain consists of specific charged residues and spacing with a high conservation of a histidine residue N-terminal to the highly conserved lysine. There is also a highly conserved acidic residue (Asp or Glu) eight amino acids downstream from the same conserved lysine. Although the structure of the proteins containing the HIKE domain is diverse, the structure of the core region consists of a highly conserved β strand-loop-β strand structure. As these two strands are oriented at largely differing angles in the various proteins, it has been suggested that this might be a means of contributing to binding specificity (49, 50). Interestingly, the PH domain has a similar structure to phosphotyrosine binding domains, and the HIKE domain is similar in structure to phosphotyrosine binding peptides (16, 43). The HIKE region also contains a consensus sequence for phosphoinositol binding (43), and binding of phosphoinositols to HIKE has been confirmed for neuromodulin (45) and Bruton tyrosine kinase (52) and potentially occurs in Gα1–4-tertiary proteins were found to share a similar sequence pattern (LXnRX3,4GXRXnRX4(D,E)), and the core of this region in Akt (HIKXE) gave the HIKE motif its name. HIKE domains have also been identified in various protein kinases (45, 46), neuromodulin (47), and fibroblast growth factor receptors (48), to name a few. The HIKE domain consists of specific charged residues and spacing with a high conservation of a histidine residue N-terminal to the highly conserved lysine. There is also a highly conserved acidic residue (Asp or Glu) eight amino acids downstream from the same conserved lysine. Although the structure of the proteins containing the HIKE domain is diverse, the structure of the core region consists of a highly conserved β strand-loop-β strand structure. As these two strands are oriented at largely differing angles in the various proteins, it has been suggested that this might be a means of contributing to binding specificity (49, 50). Interestingly, the PH domain has a similar structure to phosphotyrosine binding domains, and the HIKE domain is similar in structure to phosphotyrosine binding peptides (16, 43). The HIKE region also contains a consensus sequence for phosphoinositol binding (43), and binding of phosphoinositols to HIKE has been confirmed for neuromodulin (51) and Bruton tyrosine kinase (52) and potentially occurs in Gβ. The HIKE domain of Gβγ has been shown to be a site of interaction with numerous proteins including calmodulin (53), Ga (54), and β-adrenergic receptor kinase (46), and it has been suggested that the structure of this domain might assist in modulating multiprotein interactions. HIKE mutations in the receptor tyrosine kinase fibroblast growth factor receptor-3 have been linked to human diseases (55), and one mutation has been identified as leading to constitutive activation of the kinase (K650E) (56). Additionally, residues in the HIKE domain of fibroblast growth factor receptor 1 (Asn645 and Asp647) are essential for mitogenic activity (48). The demonstration of an interaction between the HIKE domain of TrkA and a downstream signaling protein such as RasGrf1 is novel. Notably, TrkB and -C also possess HIKE domains and most likely also interact with RasGrf1 in a similar fashion to TrkA. Whether the HIKE domain of Trk is involved in regulating the binding of other PH domain-containing proteins in an activity-dependent manner, as well as the nature of its interaction with the PH1 domain of RasGrf1, remains to be clarified. Although these studies do not absolutely determine whether Trk phosphorylates RasGrf1 directly or whether an intracellular tyrosine kinase phosphorylates RasGrf1 following Trk activation, studies using the Src family inhibitor PP2 do not interfere with Trk phosphorylation of RasGrf1 (data not shown). This provides an argument that Src family kinases are not involved and that the mechanism may be direct. Moreover, the fact that the TrkA-S10 mutation does not interfere with Trk kinase activation and/or recruitment/phosphorylation of Shc at Tyr695, and yet it does interfere with RasGrf1 phosphorylation, provides an additional argument that RasGrf1 is directly phosphorylated by Trk following its binding to the HIKE domain of Trk.

Although the majority of these studies were performed with TrkA, it is expected that RasGrf1 would bind all three Trk receptors in a transfected cellular system. Structurally the three receptors are very similar, but they do differ in expression patterns of receptor and neurotrophin, both temporally and spatially, during development (57). Expression differences, in addition to modulation by the low affinity p75 receptor and differences in ligand binding specificities, are the primary means of regulation between the three Trk receptors. Differential regulation may also be reflected in the increased selectivity/specificity of one Trk receptor over another for a particular substrate. There is overlap in expression patterns between TrkB/C and RasGrf1 in vivo (58–60). Although TrkA is expressed in dorsal root and sympathetic ganglia, in trigeminal neurons and neurons of the basal forebrain and striatum, TrkB and -C are widely expressed in the central and peripheral nervous systems including the hippocampus and cerebral cortex (57). Similarly, RasGrf1 has been reported previously to be primarily expressed in neurons of the central nervous system including the hippocampus and cerebral cortex (58). Both RasGrf1 and TrkB are present at synaptic junctions with enhanced expression in synaptosomes and postsynaptic densities (59, 60).

It is interesting that there is also some functional overlap between activated Trk and RasGrf1. Long term potentiation (LTP), a cellular form of synaptic plasticity associated with learning and memory in the hippocampus, requires activation of several receptors including the BDNF-activated TrkB receptor (3). Although considerable progress has been made in delineating which protein pathways are activated downstream of these receptors (reviewed in Ref. 61), our understanding of the molecular mechanisms underlying LTP in the hippocampus and other areas of the brain remains incomplete. Targeted TrkB/BDNF knock-out mice have reduced induction and maintenance of LTP and reduced expression of proteins involved in synaptogenesis (62). RasGrf1 knock-out mice have also been shown to be deficient in long term memory development (11, 12, 63). RasGrf1 mediation of TrkB-dependent LTP in the hippocampus remains an exciting possibility that warrants further study.

One important question to consider is why Trk might activate different guanine exchange factors, RasGrf1 and Sos, in primary neurons. There may be some degree of redundancy between RasGrf1, Sos, and other GEF family members. Another possibility is that Sos and RasGrf1, which are regulated by different mechanisms, activate separate pathways downstream to bring about different cellular effects. Although Sos is regulated by recruitment to the plasma membrane (64), RasGrf1 is primarily localized to the plasma membrane in transfected cells and is regulated by intracellular calcium and phosphorylation (18). There may also be a temporal difference in the mechanisms of activation of Sos and RasGrf1. A recent study has indicated that Sos is activated downstream of N-methyl-D-aspartate receptors in neonatal cortical neurons, whereas RasGrf1 activation by the same receptors might be more important in mature adult neurons (65).

RasGrf1 activation by Trk might lead to specific activation of Rac family members through its DH domain, leading to changes in actin polymerization and axonal growth (66, 67), or might alternatively activate Ras, which is involved in neurite outgrowth, memory formation, and LTP (68, 69). It is not yet known whether RasGrf1 can interact simultaneously with Ras and Rac, but RasGrf1 might act to couple the activation of both GTPases to bring about coordinated cellular activity involved in the formation and outgrowth of neurites. In this study, we found a significant increase in neurite outgrowth (55%) in NGF-stimulated PC12 cells transfected with RasGrf1. Although a previous study has shown that RasGrf1 serine phosphorylation at Ser916 is important for Ras activation and neurite outgrowth in unstimulated PC12 cells cotransfected with RasGrf1 and Ras (70), the site(s) of tyrosine phosphorylation induced by Src (20) and Trk (this study) are unknown. Thus, it is not presently known whether tyrosine phosphorylation plays a regulatory role or acts to modulate RasGrf1 function. Although NGF stimulation increases phosphorylation of Ser916, it remains unknown whether this modification is essential to NGF-stimulated, RasGrf1-mediated neurite outgrowth (70). It is possible that there are multiple sites of tyrosine and serine phosphorylation on RasGrf1 mediated by different tyrosine or

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serine kinases. These phosphorylation events could act to regulate one another or act in a complementary fashion to bring about coordinated cellular responses. It will be important in future studies to determine whether the enhancement of NGF-dependent neurite outgrowth by RasGrf1 is mediated through Ras or Rac pathways and to determine the specific roles tyrosine and serine phosphorylation of RasGrf1 play in regulating neurotrophin-dependent neurite outgrowth.

Collectively, our results indicate that RasGrf1 is a potential downstream signaling molecule of the Trk receptors in the central nervous system. The specific tyrosine(s) that are phosphorylated on RasGrf1 by Trk remain to be determined, but at least one site is contained within the PH domain. A novel role for the HIKE domain is demonstrated as the site of interaction between the receptor and the exchange factor. Further studies are necessary to determine which proteins are activated downstream of RasGrf1 to mediate neurite outgrowth and to identify the role that tyrosine phosphorylation might play in this response. The clarification of the signaling pathways activated downstream of the interaction between Trk and RasGrf1 and the subsequent response of the cell will assist in the goal of clarifying the signaling pathways that mediate the diverse cellular effects induced by the neurotrophins.

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Neurotrophin-dependent Tyrosine Phosphorylation of Ras Guanine-releasing Factor 1 and Associated Neurite Outgrowth Is Dependent on the HIKE Domain of TrkA
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