Analysis of SHP-1-mediated Down-regulation of the TRK-T3 Oncoprotein Identifies Trk-fused Gene (TFG) as a Novel SHP-1-interacting Protein*

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SHP-1 is a cytoplasmic SH2 domain containing protein-tyrosine phosphatase (PTP) involved in the negative regulation of multiple signaling pathways in hematopoietic, nervous, and epithelial cells. The thyroid TRK-T3 oncogene consists of the NTRK1 tyrosine kinase domain fused in-frame with sequences of the TFG (TRK-fused gene), encoding a protein of unknown function. TFG contains a coiled-coil domain responsible for TRK-T3 oligomerization. In addition, recent analysis of the sequences outside of the coiled-coil domain suggested possible interactions with other proteins. Based on the presence of a putative SHP-1 SH2-binding site within the TFG sequences, we have investigated the role of the SHP-1 phosphatase in TRK-T3 oncoprotein signaling. In this study we show that SHP-1 interacts with and down-regulates TRK-T3. We provide evidence that SHP-1 SH2 and catalytic domains, respectively, associate with the TFG- and NTRK1-derived portions of TRK-T3. Our data contribute to the definition of cellular mechanisms involved in thyroid tumorigenesis. Moreover, it reveals TFG as a novel protein able to modulate SHP-1 activity.

Phosphotyrosine-specific phosphatases attenuate the phosphorylation level of proteins with tyrosine kinase activity and contribute in keeping it at a dynamic equilibrium within biological systems. The SH2 domain-containing PTPs (SHPs) belong to a protein family, which include two vertebrate SHPs (SHP-1 and SHP-2) and invertebrate SHP orthologs, Corkscrew and Ptp-2, in Drosophila and Caenorhabditis elegans, respectively (1). SHP-1 is expressed in the hematopoietic system, the nervous system, epithelial cells, and the NGF-responsive PC12 cell line (2). Based on its function as an antagonist of the growth promoting and oncogenic potential of tyrosine kinases, SHP-1 has been proposed as a candidate tumor suppressor gene in lymphoma, leukemia, and other cancers (3). The first evidence of SHP-1 as a negative regulator of cell proliferation was provided by moth-eaten mice, which lack SHP-1 expression. These mice displayed an array of hematopoietic abnormalities caused by the overproduction of the hematopoietic cell lineage (4). SHP-1 is involved in many hematopoietic signaling pathways insofar as it modulates the activity of the cytokine receptors, c-kit (5), CSF-1R (6), and EpoR (7). In addition, several SHP-1 targets in non-hematopoietic cells have also been identified, including EGFR, IGF-1R, PDGFR (2), ROS (8), and the RET MEN2A oncogene (9). Recently, Marsh et al. (10) have shown that SHP-1 negatively regulates the activity of the NGF receptor (NTRK1 or TRKA), thus modulating neuron number during development. SHP-1 is a 68-kDa protein composed of two SH2 domains, a catalytic domain, and a flexible C terminus. SHP-1 is dormant in the cytoplasm, and its phosphatase activity is inhibited by both SH2 domains and the C-terminal tail (Fig. 1A). Recent studies based on the crystal structure of SHP-1 have demonstrated that the N-terminal SH2 domain, rather than C-terminal SH2, is responsible for autoinhibition. In response to activation signals, tyrosine-phosphorylated proteins bind to SHP-1 SH2 domains. This causes structural rearrangements, which expose the active site and renders the SHP-1 catalytic domain capable of interacting with downstream substrates (11).

TRK-T3 is a chimeric cytoplasmic oncoprotein isolated from a papillary thyroid tumor. This oncoprotein is the consequence of a t(1, 3) translocation juxtaposing the tyrosine kinase domain of the NTRK1 receptor to the N-terminal portion of a protein encoded by TFG (TRK-fused gene), a novel gene first discovered in the rearranged form (12). TFG is also involved in the generation of a class of ALK oncogenes in anaplastic large cell lymphoma (13, 14). Moreover, TFG has been recently detected as a novel fusion partner for NOR1 in extraskeletal myxoid chondrosarcoma (15). Studies performed on the normal counterpart have shown that the TFG gene is ubiquitously expressed in human adult tissues and that it is conserved among several species, including C. elegans (16). The major characteristic of TFG is the presence of a coiled-coil domain (12), which exerts a crucial role in TRK-T3 oncogenic activation by mediating the formation of protein complexes (17). In addition to the coiled-coil domain, the TFG protein also contains several consensus sites, which suggest possible interactions with other proteins. These include a PBI domain (18), putative phosphorylation sites for PKC and CK2, glycosylation sites, as well as SH2- and SH3-binding sites (19). Several of these sites

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‡ The abbreviations used are: SH2, Src homology 2; SHP, SH2 domain-containing phosphatase; NTRK1, neurotrophic tyrosine receptor kinase type 1; TFG, Trk-fused gene; PTP, phosphotyrosine phosphatase; CSF-1R, colony-stimulating factor 1 receptor; ALK, anaplastic lymphoma kinase; PB1, Phox and Bem1p domains; PKC, protein kinase C; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; TK, tyrosine kinase; SHC, Src homology-containing protein; FRB, fibroblast growth factor receptor substrate; PLC-γ, phospholipase C-γ; FL, full-length; DMEM, Dulbecco’s modified Eagle’s medium; WT, wild type; HEK, human embryonic kidney.
are identical in TFG proteins from different species, indicating that the protein might be involved in basic cellular processes (16). Interestingly, most of these sites are present in the TFG portion contained within the TRK-T3 and ALK oncogenes. The TFG sequences outside the coiled-coil domain play an important role in TRK-T3 oncogenic activation. Their deletion interferes with different mechanisms involving protein processing, formation of stable and/or functional complexes, and possible interactions with other proteins (18). In particular, the PB1 domain appears to have a crucial role since a single point mutation at a conserved Lys residue completely abrogates TRK-T3 activity, thus demonstrating the significance of this region in oncogenic activation. Another important contribution is further provided by a consensus site for binding to the SHP-1 SH2 domain, where the Y33F mutation drastically reduces TRK-T3-transforming activity.

In this study, we have tried to elucidate the role of the SHP-1 phosphatase in TRK-T3 oncprotein signaling and to explore the putative interaction between SHP-1 and TFG sequences. We show that SHP-1 is a negative modulator of the TRK-T3 oncprotein. Both the TFG and NTRK1 portions of the TRK-T3 oncprotein recruit SHP-1 by respectively interacting with either the SH2 or the catalytic domain. In addition, we identify TFG tyrosine 33 as the residue responsible for SHP-1 binding. Moreover, we provide evidence that TFG reduces SHP-1 catalytic activity. Our data suggest that TFG is a novel SHP-1 auxiliary docking protein, which participates in the regulation of phosphatase activity.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The T3/WT plasmid contains TRK-T3 cDNA inserted into the pRCCMV expression vector (12), which carries the neomycin resistance gene. The T3/Y291F (20) and T3/Y586F (21) mutants respectively carry the Tyr291 and Tyr586 to Phe mutations. The T3/Y291F mutant carries both of these mutations (21). All the mutants were inserted into the pRCCMV expression vector. The T3/L2 mutant, inserted into the pRESneo expression vector, is deleted for all the TFG sequences except the coiled-coil domain (18). The T3/ABN kinase-defective mutant, inserted into the pRCCMV expression vector, carries the Tyr586 to Ala mutation of the HD domain.

The T3/Y291F plasmid, in the pRKSRS vector, was kindly provided by Dr. A. Ullrich. SHP-1 cDNA was transferred into the pREShyg vector as follows. SHP-1 cDNA in the original pRKSRS vector was amplified by PCR using the following oligonucleotides, both containing NotI sites (underlined): 5'-ATAAGAATCCGGCGCGCATACAGAGATGCTGCTGCGTGGATAGCTTAAGTAA TTCAC-3' (containing the NotI site, underlined); 5'-CGCCCAAGCTTTCTGGAGTGATCATGGTTGATAG-3' (containing the HindIII site, underlined). Amplified cDNA was excised by BamHI/HindIII digestion and was inserted into the pCDNA 3.1/myc-His(-A) vector (Invitrogen), linearized with the same restriction enzymes. The resulting plasmid was subjected to nucleotide sequence analysis.

The N-TFG/Y33F site-specific mutant was constructed by QuikChange II XL site-directed mutagenesis, according to the manufacturer's instructions (Stratagene), using N-TFG DNA as a template. The following oligonucleotides were used: 5'-ATTATTTGTGATCATGACCGA CAGTTGGAT-3' (containing the BamHI site, underlined); 5'-CGCCCAAGCTTTCTGGAGTGATCATGGTTGATAG-3' (containing the HindIII site, underlined). Amplified cDNA was excised by BamHI/HindIII digestion and was inserted into the pCDNA 3.1/myc-His(-A) vector (Invitrogen), linearized with the same restriction enzymes. The resulting plasmid was subjected to nucleotide sequence analysis.

Cell Culture and Transfection—NIH3T3 mouse fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum. Human embryo kidney HEK293T cells were cultured in DMEM supplemented with 10% fetal calf serum. The NPT79 cell line, derived from NIH3T3 cells transformed by the TRK-T3 oncogene, were cultured in DMEM supplemented with 5% calf serum. SHP-1 cDNA was transferred into the pIREShyg vector previously linearized with the same restriction enzymes. SHP-1 cDNA was amplified by PCR using the following oligonucleotides, both containing NotI sites (underlined): 5'-ATAAGAATCCGGCGCGCATACAGAGATGCTGCTGCGTGGATAGCTTAAGTAA TTCAC-3' (containing the NotI site, underlined); 5'-CGCCCAAGCTTTCTGGAGTGATCATGGTTGATAG-3' (containing the HindIII site, underlined). Amplified cDNA was excised by BamHI/HindIII digestion and was inserted into the pCDNA 3.1/myc-His(-A) vector (Invitrogen), linearized with the same restriction enzymes. The resulting plasmid was subjected to nucleotide sequence analysis.

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(S-H2, residues 2–90); 5'-CCCGAATTCCGGCCAAGCTTCGTCGTTCTACG-3' (nucleotides 847–867) for the C-terminal SH2 domain (C-SH2, residues 107–204); 5'-CCCGAATTCCGGCCAGCTTCGTCGTTCTACG-3' (nucleotides 847–867) for the two SH2 domains (NC-SH2, residues 2–204); 5'-CCCGAATTCCGGCCAAGCTTCGTCGTTCTACG-3' (nucleotides 847–999); 5'-CCCGAATTCCGGCCAGCTTCGTCGTTCTACG-3' (nucleotides 1801–1821) for the PTP domain (PTP, residues 242–522). The amplified products were digested by EcoRI for full-length SHP-1 or EcoRI/XhoI for the other fragments and inserted in-frame into the pGEX-4T-2 vector respectively linearized with the same restriction enzymes. The fusion genes were then confirmed by DNA sequencing (see Fig. 6A).

GST-SHP-1 fusion proteins were expressed in bacteria. In the case of GST-SHP-1 FL, induction was performed by adding 0.2 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and incubating at 30 °C. The induction of other GST-SHP-1 proteins was performed in 0.5 mM IPTG at 37 °C. The fusion proteins were purified by incubation with glutathione-conjugated-Sepharose beads (Amersham Biosciences). Protein concentration was determined by SDS-PAGE and subsequent Coomasie Blue staining.

**GST-SHP-1 Fusion Protein Pull-down Assay**—To monitor the binding of TRK-T3 and N-TFG proteins to either full-length SHP-1 or specific SHP-1 domains, 10 μg of GST-SHP-1 fusion proteins or GST were coupled to 20 μl of glutathione-Sepharose. Lysates from NF/797 cells (800 μg) or HEK298T cells transfected with TRK-T3 constructs (800 μg) or N-TFG constructs (1 mg) were incubated with the immobilized fusion proteins for 3 h at 4 °C. The beads were washed three times with HNTG, and boiled in Laemmli sample buffer. Protein samples were then separated by 7% SDS-PAGE. Immunoblotting was performed as described above. The bound TRK-T3 proteins were visualized using anti-TRK antibodies; the bound N-TFG proteins were visualized using anti-TFG antibodies.

**Phosphatase Assay**—To assay SHP-1 phosphatase activity, SHP-1 was immunoprecipitated as described above from 800 μg of cell lysate proteins prepared from HEK298T cells co-transfected with SHP-1 and one of different amounts of TFG cDNA. Cells were used with radioligand precipitation assay buffer (RIPA) (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40) supplemented with aprotinin, pepstatin, leupeptin, and phenylmethylsulfonyl fluoride. Immunoprecipitated were washed three times in RIPA buffer and once in phosphatase buffer (50 mM Heps, pH 7.25, 50 mM NaCl, 5 mM dithiothreitol) and then incubated at 37 °C for 30 min in 200 μl of phosphatase buffer containing 4 mM p-nitrophenyl phosphate (Sigma). Reactions were terminated by addition of 1 ml of 0.2 M NaOH, and absorbance was measured at 410 nm.

**RESULTS**

**SHP-1 Is a Negative Regulator of the TRK-T3 Oncoprotein**—SHP-1 modulates NTRK1 activity after NGF-induced activation and therefore exerts an important role in the development and maintenance of the nervous system (10). The SHP-1/ NTRK1 interaction is mediated by tyrosine 490 of NTRK1. This residue is also involved in the recruitment of SHC and FRS2 adaptor proteins (Fig. 1A). We were interested in determining the effect of SHP-1 on TRK-T3, a cyttoplasmic oncoprotein derived from the rearrangement of NTRK1 with the TFG gene (Fig. 1A) (12). This was performed by investigating whether SHP-1 and TRK-T3 formed complexes in living cells. Wild type TRK-T3 cDNA (T3/WT) was transfected into HEK298T cells together with either SHP-1 cDNA, the SHP-1 C455S catalytically inactive mutant, or the empty pSV3neo vector. Cell extracts were subsequently immunoprecipitated with anti-TRK antibodies. Western blot hybridization with anti-SHP-1 antibodies detected SHP-1 proteins in TRK-T3/SHP-1 co-transfected cells (Fig. 1B, first panel). This indicated that both wild type and mutated SHP-1 could form complexes with the oncoprotein. Hybridization of a twin blot with anti-phosphotyrosine antibodies showed that the level of TRK-T3 oncoprotein phosphorylation was reduced in the presence of SHP-1 but not in the presence of the SHP-1 C455S mutant (Fig. 1B, second panel). This effect is not related to the amount of the TRK-T3 oncoprotein, which was comparable in all the samples (Fig. 3, third panel). These data indicate that SHP-1 interacts with and dephosphorylates TRK-T3. Therefore, oncogenic rearrangement and the consequent delocalization of the NTRK1 kinase activity do not affect SHP-1 mediated down-regulation.

Similar to the NGF-activated NTRK1 receptor, the TRK-T3 oncoprotein triggers multiple signal transduction pathways, including that of ERK1/2. We next investigated the effect of SHP-1-mediated TRK-T3 down-regulation on the activation of the downstream target ERK1/2. HEK298T cells were co-transfected with the TRK-T3 and SHP-1 constructs as described above. Cell extracts were immunoblotted with site-specific phosphotyrosine antibodies to measure the phosphorylation of...
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Fig. 2. Effect of SHP-1 phosphatase on ERK phosphorylation induced by TRK-T3. Serum-starved HEK293T cells transfected with SHP-1 alone (3 μg) or co-transfected with TRK-T3 (T3/WT) (1 μg) together with wild type SHP-1 (SHP-1) or the catalytically inactive SHP-1 mutant (SHP-1 C455S) or the empty pSV3neo vector (3 μg) were lysed, and proteins were subjected to 10% SDS-PAGE. Cell lysates were evaluated for endogenous ERK activity by immunoblotting with antibodies specific for phosphorylated ERK (anti-pERK) (top panel). The same membrane was re-probed with anti-ERK antibodies to show ERK expression (bottom panel). Similar results were obtained in three independent experiments.

As shown in Fig. 2 (top panel), phosphorylation of ERKs was detected in the presence of the TRK-T3 oncoprotein. Interestingly, the phosphorylation level of both p44 and p42 ERKs was reduced in cells expressing wild type SHP-1, but not in those expressing the catalytically inactive SHP-1 C455S mutant. Such differences are not related to ERK1/2 expression level, which were comparable in all the samples (Fig. 2, bottom panel). These data indicate that TRK-T3 down-regulation by SHP-1 phosphatase affects downstream signaling.

As a consequence of dimerization/oligomerization mediated by the activating portions, TRK oncoproteins display constitutive tyrosine kinase activity. In vitro cell culture systems, this results in oncogene-mediated transformation of NIH3T3 mouse fibroblasts. We investigated the effect of SHP-1 on TRK-T3 biological activity by performing NIH3T3 transfection/focus formation assays. The constructs used for these assays were the T3/WT inserted into the pRC/CMV vector, carrying the neomycin resistance gene, and SHP-1hyg wild type and SHP-1hyg C455S mutant inserted into the pIREShyg vector, carrying the hygromycin resistance gene (see “Experimental Procedures”). T3/WT was transfected in the presence of different amounts of either the SHP-1hyg or the SHP-1hyg C455S construct. Co-transfection efficiency was detected by selection in the presence of both G418 and hygromycin. The transforming activity was subsequently determined by selection in 5% serum medium. The relative transforming activity (RTA) was determined as the ratio between transforming activity and transfection efficiency. As shown in Fig. 3, transforming activity was comparable when T3/WT was co-transfected with an equal amount of either SHP-1 wild type or the catalytically inactive SHP-1 C455S mutant. Increasing amounts of wild type SHP-1 reduced TRK-T3 transforming activity in a dose-dependent manner. In contrast, different amounts of the SHP-1 C455S mutant did not affect TRK-T3 transforming activity. These data demonstrate that the negative regulation of TRK-T3 by SHP-1 phosphatase results in the inhibition of transforming activity.

TRK-T3 Recruits SHP-1 through Both the TFG and TK Portions—NTRK1 down-regulation by SHP-1 phosphatase is mediated by the Tyr490 residue of NTRK1, which is required for optimal complex formation between the two proteins (10). With respect to the TRK-T3 oncoprotein, in addition to the SHP-1 interaction site in the NTRK1-derived portion (Tyr291), a potential SHP-1-specific SH2-binding site is present within the TFG portion (Tyr35) (Fig. 1A). Indirect evidence of SHP-1 recruitment by the TRK-T3-activating portion is provided by the experiment reported in Fig. 4A. SHP-1 cDNA was co-transfected together with the following TRK-T3 mutants: 1) T3/Y291F, in which tyrosine 291, corresponding to Tyr490 of NTRK1 and representing the docking site for SHC, FRS2, and FR3, was mutated to phenylalanine. 2) T3/Y586F, in which tyrosine 586, corresponding to Tyr285 of NTRK1 and representing the docking site for PLC-ε, was changed to phenylalanine. 3) T3/Y291F/S586F, in which both tyrosine 291 and 586 to phenylalanine mutations were introduced. 4) T3/ABN, carrying the lysine 339 to alanine mutation of the ATP-binding site. Anti-TRK immunocomplexes were hybridized with anti-SHP-1 and anti-TRK antibodies to respectively detect SHP-1/TRK-T3 interactions and TRK-T3 construct expression (Fig. 4A, top and middle panels). The expression of SHP-1 was detected by Western blot of total extracts with anti-SHP-1 antibodies (Fig. 4A, bottom panel). All the mutants were capable of recruiting a considerable amount of the SHP-1 protein with respect to T3/WT. Interestingly, both the T3/ABN and T3/Y291F mutants behaved differently from the equivalent NTRK1 mutants, which were previously shown to coimmunoprecipitate with very little SHP-1 when compared with the wild type receptor (10). In vitro GST pull-down confirmed the results reported above. The full-length GST-SHP-1 fusion protein was incubated with protein extracts from HEK293T cells transfected with either the T3/WT or the T3/Y291F construct and the eluted complexes were analyzed by Western blot with anti-TRK antibodies. As shown in Fig. 4B, SHP-1 not only interacted with T3/WT but also with T3/Y291F, albeit with reduced capability. These findings strongly supported the possibility of an additional TRK-T3/SHP-1 interaction, which was not mediated by the tyrosine kinase domain.

Fig. 3. Transforming activity of NIH3T3 cells co-transfected with T3/WT and different amounts of SHP-1hyg or SHP-1hyg C455S. NIH3T3 cells were co-transfected with T3/WT (250 ng) together with different amounts of SHP-1hyg or SHP-1hyg C455S constructs: 250 ng (1:1), 500 ng (1:2), 1000 ng (1:4). Different amounts of the empty pIREShyg vector were added in order to transfect the same quantity (1250 ng) of DNA for each cell plate. Transfected cells were subjected to foci and G418-hyg selection. The bar graph represents the relative transforming activity (RTA), calculated as the ratio between number of foci and number of G418-hyg-resistant colonies. The data represent mean ± S.D. of triplicate samples. Similar results were obtained in four independent transfection experiments.

More direct evidence of the interaction between TFG and SHP-1 was provided by the coimmunoprecipitation experiments reported in Fig. 5A. SHP-1 cDNA was transfected into HEK293T cells together with either full-length TFG or N-TFG (corresponding to the portion contained in TRK-T3), both of which were tagged with the Myc epitope. Cell extracts were thus immunoprecipitated with anti-Myc antibodies. Western blot hybridization with anti-SHP-1 antibodies detected SHP-1 protein when coexpressed with TFG proteins (Fig. 5A, top panel). As a control, the expression of the transfected TFG and...
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The interaction between TFG and SHP-1 was further demonstrated by GST pull-down experiments (Fig. 5A). SHP-1 cDNAs is shown (Fig. 5A, middle and bottom panels). The interaction between TFG and SHP-1 was further demonstrated by GST pull-down experiments (Fig. 5B). The full-length TFG construct was transfected into HEK293T cells and cell extracts were incubated with the full-length GST-SHP-1 fusion protein. Western blot analysis with anti-TFG antibodies detected TFG in the eluted complexes (Fig. 5B, left panel). These data demonstrate that TFG is a new partner of the SHP-1 phosphatase, suggesting that the two proteins may be involved in common cellular pathways.

**Different SHP-1 Domains Interact with TFG- and NTRK1-derived Portions of TRK T3**—To better define the contribution of the TFG and NTRK1 portions of TRK-T3 oncoprotein in the recruitment of the SHP-1 phosphatase, we constructed GST fusion proteins containing the following SHP-1 regions fused in-frame with glutathione S-transferase: the N-terminal (N-SH2), the C-terminal (C-SH2), both the N-terminal and the C-terminal (NC-SH2) and the PTP (PTP) domains (Fig. 6A). The full-length GST-SHP-1 fusion protein (FL) was used as a control. The different fusion proteins were incubated with cell extracts expressing TRK-T3 and the eluted complexes were analyzed by Western blot with anti-TRK antibodies. The TRK-T3 oncoprotein interacted strongly with both full-length SHP-1 and the PTP domain, whereas it displayed a weaker, but still significant interaction, with the SHP-1 SH2 domains (Fig. 6B). These differences most likely reflect the intrinsic strength of interaction and are not related to the amount of GST fusion protein, which was comparable in all cases (data not shown). To understand the contribution of TFG in the above reported interaction, we performed GST pull-down experiments on N-TFG-expressing cell extracts using fusion proteins containing full-length SHP-1 or either the SH2 or the PTP domains. Western blot of the eluted complexes with anti-TFG antibodies showed that the N-TFG protein interacted with both full-length and SH2, but not with the PTP domain of SHP-1 (Fig. 6C). This demonstrated that the interaction of TRK-T3 with SHP-1 SH2 domains was mediated by TFG. This is in keeping with the presence of a putative SH2-binding motif (ITYDEL) within TFG. Altogether these data demonstrate that the TRK-T3 oncoprotein displays a dual interaction with the SHP-1 phosphatase; the NTRK1 portion binds the SHP-1 catalytic domain whereas the TFG portion interacts with SHP-1 SH2 domains. The latter interaction was further confirmed by GST pull-down experiments performed with the T3/L2 mutant, in which all the TFG sequences except the coiled-coil domain had been deleted (18). As shown in Fig. 6D, the T3/L2 protein...
lost the capacity to interact with the NC-SH2 fusion protein, whereas it was efficiently pulled-down by the PTP fusion protein.

The TFG Interaction with SHP-1 Is Mediated by TFG Tyrosine 33 Residue—To investigate whether the putative TFG consensus site (ITYDEL), containing the tyrosine 33 residue, represented a functional binding site for SHP-1 SH2 domains and thus mediates the TFG/SHP-1 interaction, we performed the experiment shown in Fig. 7. Tyrosine 33 of N-TFG was mutated to phenylalanine by site-directed mutagenesis and the resulting N-TFG/Y33F construct was transfected into HEK293T cells. Wild type N-TFG was used as a control. Cell extracts were used for pull-down experiments with the full-length GST-SHP-1 fusion protein. Western blot analysis of the eluted complexes using anti-TFG antibodies showed that the Y33F mutation abrogated the capacity of N-TFG to bind SHP-1. This demonstrated that TFG tyrosine 33 was responsible for the interaction with the phosphatase.

Effect of TFG on SHP-1 Phosphatase Activity—To evaluate the potential biologic relevance of SHP-1 association with TFG, SHP-1 was immunoprecipitated from HEK293T cells co-transfected with N-TFG and different amounts of TFG protein.
immunocomplexes were assessed for the associated phosphatase activity, evaluated by the dephosphorylation of p-nitrophenol phosphate substrate. As control, the catalytically inactive SHP-1 C455S mutant was used. The results, expressed as percentage of phosphatase activity obtained from SHP-1-transfected cells, are reported in Fig. 8. No phosphatase activity was detected in cells mock transfected or expressing the SHP-1 C455S mutant, indicating that the endogenous SHP-1 activity is not detectable in these experimental conditions. In cells expressing both SHP-1 and TFG, the SHP-1 activity decreased in the presence of TFG protein in a dose-dependent manner (reduction from 19 to 33%). Statistical analysis of the data with a regression model showed a highly significant association between TFG amount and the SHP-1 phosphatase activity \((p < 0.001\) for both linear and quadratic TFG terms; adjusted \(R^2 = 0.96\)). These data suggest that TFG protein may negatively regulate the SHP-1 phosphatase activity.

**DISCUSSION**

The thyroid TRK-T3 oncogene is generated by a chromosomal rearrangement juxtaposing the NTRK1 tyrosine kinase domain to sequences of TFG, a gene highly conserved among species which encodes a protein of unknown function (12). TFG is also the fusion partner of ALK kinase in anaplastic large cell lymphomas (13, 14), and of NORI in extraskeletal myxoid chondrosarcoma (15). An attractive hypothesis is the possibility that cellular pathways involving TFG protein might be deregulated in tumors carrying TFG rearrangements. TFG displays a coiled-coil domain that mediates dimerization/oligomerization of the TRK-T3 oncoprotein, which is strictly related to its constitutive tyrosine kinase activity (17). Among the other TRK-activating genes, TFG displays the peculiarity of a single coiled-coil domain flanked by sequences containing several consensus sites, which suggest interactions with other proteins (18, 19). We have recently demonstrated the importance of TFG sequences outside the coiled-coil domain in TRK-T3 activity. In particular, the drastic effect of point mutations within the PB1 domain and a putative binding site for the SHP-1 SH2 domain strongly supports the hypothesis that the interaction of TFG with other proteins may contribute to TRK-T3 oncogenic activation (18).

SHP-1 is a cytosolic tyrosine phosphatase consisting of two SH2 domains followed by a single catalytic domain and a C-terminal tail (11). The SHP-1 phosphatase is primarily a negative regulator of signaling downstream of a wide range of receptors including cytokine receptors (22), tyrosine kinase receptors (5, 6, 9, 10, 23, 24), and receptors of the immune system (25). SHP-1 is also considered a negative regulator of oncogenesis since it acts as a tumor suppressor gene in several cancers (3).
By employing GST fusion proteins containing the different SHP-1 domains, we identified the regions involved in the interaction between TRK-T3 and SHP-1. We showed that the SHP-1 catalytic domain (PTP) binds to the NTRK1-derived portion of TRK-T3. This is a novel finding since the phosphatase domains involved in binding were not investigated in a recent paper reporting the interaction between NTRK1 and SHP-1 (10). On the other hand, in a report characterizing the interaction of ROS with SHP-1, NTRK1 was used as a control and was shown to be unable to bind to the phosphatase SH2 domains (8). Interestingly, we have demonstrated that SHP-1 SH2 domains interact with the TFG portion of the TRK-T3 oncoprotein and have identified the TFG site responsible for this interaction. Mutation of TFG tyrosine 33, within the consensus SH2-binding site, abrogates the binding to SHP-1 SH2 domains. In a previous report, we showed that the equivalent mutation introduced into TRK-T3 cDNA strongly reduced oncogenic transforming activity (18). This finding appears to be in contrast with the negative regulation exerted by SHP-1 on TRK-T3. However, we have no evidence about the contribution of TFG-mediated interaction on TRK-T3 down-regulation by SHP-1. The possibility exists that interaction through the TFG portion of the TRK-T3 kinase domain; alternatively, SH2- and PTP-mediated SHP-1 interactions with the TRK-T3 oncoprotein may be unrelated. Moreover, we cannot exclude that the TFG SH2-binding site may interact with proteins other than SHP-1, and the lack of such interactions may account for the reduction of TRK-T3 activity in the presence of the Y33F mutation.

Our study demonstrates that the interaction of TFG with SHP-1 SH2 domains may lead to the modulation of SHP-1 phosphatase activity. The SH2 domains play an important role in the regulation of SHP-1 enzymatic activity. Crystal structure studies have shown that SHP-1 exists in a closed autoinhibitory conformation, in which the N-terminal SH2 domain hides the catalytic center. Binding of a phosphorylated ligand to the C-terminal SH2 domain, results in conformational changes, which allow the N-terminal SH2 domain to interact with a second phosphopeptide molecule. This weakens the autoinhibitory interaction between the N-terminal SH2 and PTP domains and permits the synergistic opening of the active site of the PTP domain (11, 28).

Recently, a model of negative regulation of SHP-1 has been proposed. Jones et al. (29) have shown that in human platelets SHP-1 is held in a basally active state by occupation of its SH2 domains by a protein complex that includes PKCa. Upon cellular activation PKCa phosphorylates a serine residue at the SHP-1 C-terminal tail, leading to the inactivation of SHP-1 catalytic activity. The negative effect of TFG on SHP-1 activity would suggest the participation of TFG in mechanism(s) of switching off SHP-1 thus attenuating the phosphatase inhibitory effect on protein phosphorylation.

In conclusion, our data demonstrate that SHP-1 phosphatase is a negative regulator of the thyroid TRK-T3 oncoprotein. Moreover, our evidence indicates that TFG is capable of interacting with and negatively regulating SHP-1. More studies are required in order to: 1) assess the precise contribution of the TFG protein in the regulation of SHP-1 activity; 2) define the effect of possible deregulation of SHP-1 activity in tumors carrying TFG rearrangements; and 3) determine the contribution of the TFG/SHP-1 interaction in the context of TRK-T3 oncogene down-regulation.

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Analysis of SHP-1-mediated Down-regulation of the TRK-T3 Oncoprotein Identifies Trk-fused Gene (TFG) as a Novel SHP-1-interacting Protein

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