Direct Binding of the Signaling Adapter Protein Grb2 to the Activation Loop Tyrosines on the Nerve Growth Factor Receptor Tyrosine Kinase, TrkA*

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We demonstrate that the signaling adapter, Grb2, binds directly to the neurotrrophic receptor tyrosine kinase, TrkA. Grb2 binding to TrkA is independent of Shc, FRS-2, phospholipase Cγ-1, rAPS, and SH2B and is observed in in vitro binding assays, yeast two-hybrid assays, and in co-immunoprecipitation assays. Grb2 binding to TrkA is mediated by the central SH2 domain, requires a kinase-active TrkA, and is phosphotyrosine-dependent. By analyzing a series of rat TrkA mutants, we demonstrate that Grb2 binds to the carboxyl-terminal residue, Tyr794, as well as to the activation loop tyrosines, Tyr668 and Tyr684. By using acidic amino acid substitutions of the activation loop tyrosines on TrkA, we can stimulate constitutive kinase activity and TrkA- Shc interactions but, importantly, abolish TrkA/Grb2 binding. Thus, in addition to providing the first evidence of direct Grb2 binding to the neurotrophin receptor, TrkA, these data provide the first direct evidence that the activation loop tyrosines of a receptor tyrosine kinase, in addition to their essential role in kinase activation, also serve a direct role in the recruitment of intracellular signaling molecules.

Signaling by the nerve growth factor (NGF) receptor tyrosine kinase, TrkA, has been intensively studied and involves the integration of both Ras-dependent and Ras-independent pathways. Although a number of signaling proteins (Shc, phospholipase Cγ-1 (PLC-γ-1), FRS-2/SNT, rAPS, SH2-B, Ras-GRF1, and the Cek homologous kinase) directly bind to TrkA (1–5), and others have been identified as downstream targets of NGF/TrkA (1), many questions still remain with respect to how the pathways are integrated and what factors influence NGF-stimulated differentiation in neuronal cells versus NGF-stimulated mitogenesis in non-neuronal cells. In this respect, Rap-1 stimulates a long term activation of MAP kinase (MAPK; Ref. 6) that is thought to be a deterministic event in regulating differentiation versus proliferation (7, 8).

Ligand activation results in the phosphorylation of five tyrosine residues in the intracellular domain of rat TrkA (Tyr499, Tyr679, Tyr683, Tyr684, and Tyr794). Tyr199 and Tyr794 mediate the phosphorylation and activation of Shc, FRS-2, and PLC-γ-1, whereas the phosphorylation of Tyr279, Tyr683, and Tyr684 are essential to kinase activity. In NGF-dependent signaling, the tyrosine phosphorylation and receptor binding of Shc and FRS-2, and their subsequent binding to Grb2, result in the activation of Ras and MAPK (9, 10). We have also recently shown that FRS-2 binds the adapter protein Crk in a phosphotyrosine-dependent manner (2). Since Crk also activates MAPK, via Rap1 (6), it is clear that there are multiple pathways to activate MAPK in response to NGF stimulation.

Moreover, TrkA receptors mutated at Tyr499, in which Shc and FRS-2-dependent pathways are lost, retain NGF-dependent activation of MAP kinase (11) suggesting that there are additional receptor-dependent mechanisms activating MAP kinase other than through the Shc and FRS-2 adapters. In this respect, PLC-γ-1, which binds TrkA at Tyr279, also contains a Grb2-binding site and thus could also contribute to the activation of MAP kinase in response to NGF stimulation. Interestingly, receptors in which both Tyr499 and Tyr794 are mutated show a dramatic reduction, but not a complete loss, of MAP kinase activity (11) suggesting a possible role of other Tyr(P) sites on TrkA in independent routes to MAP kinase activation.

As mentioned above, Grb2 is recruited both directly and indirectly into the signaling cascades of receptor tyrosine kinases (RTKs). For example, in addition to binding to tyrosine-phosphorylated signaling molecules such as Shc, Gab-1, Shp2, rAPS, SH2B, PLC-γ-1, and FRS-2, Grb2 can also bind directly to the activated epidermal growth factor receptor (EGFR) (12, 13), the Ret receptor (14), the Tpr-Met oncprotein (15), and the ErbB2 receptor (16). This redundancy in Grb2 recruitment provides alternative routes to the activation of Ras-dependent MAPK. Moreover, since the SH3 domain of Grb2 has a variety of binding partners, other than nucleotide exchange factors for Ras, it is likely that the direct recruitment of Grb2 can couple receptors to a variety of alternative signaling cascades and cellular functions.

To understand better Trk receptor signaling, we have uti-
lized the yeast two-hybrid system to identify and analyze proteins that interact with the intracellular domain (ICD) of TrkA (3). Here, we demonstrate that Grb2 binds directly to the ICD of TrkA in both yeast two-hybrid assays as well as in vitro binding assays. Moreover, we demonstrate that Grb2 co-immunoprecipitates with TrkA in NGF-stimulated cell lysates containing wild type TrkA as well as the Y499F and the Y499F/Y794F TrkA receptor mutants. Even in lysates pre-cleared of known NGF-induced Grb2-binding proteins (Shc, PLCγ-1, rAPS, and SH2B2), co-immunoprecipitation of TrkA and Grb2 is still observed with wild type TrkA and the Y499F/Y794F mutant. This interaction is mediated through the SH2 domain of Grb2 and is dependent upon a kinase-active TrkA. By analyzing a series of single, double, triple, and quadruple site-directed TrkA mutants and a combination of in vitro binding assays, co-immunoprecipitation assays, and yeast two-hybrid assays, we demonstrate that Grb2 binds TrkA at two independent sites, namely the carboxyl-terminal tyrosine, Tyr794, as well as the activation loop tyrosines, Tyr683 and Tyr684. Collectively, these data are the first evidence of direct Grb2 binding to a neurotrophin Trk receptor. Moreover, these data provide the first direct evidence that the activation loop tyrosines, in addition to their essential role in kinase activation, also serve a direct role in the recruitment of intracellular signaling molecules.

**Materials—** Glutathione-Sepharose and the pGex-4T1 and -4T2 bacterial expression vectors were from Amersham Pharmacia Biotech. The anti-hemagglutinin (HA) antigen antibodies, 3F10 or 12CA5, were from Roche Molecular Biochemicals, and the Renaissance Western Blot Chemiluminescence kit was from NEN Life Science Products. A rabbit antibody to Shc was the gift of Jane McGlade (The Hospital for Sick Children, Toronto, Canada); rabbit antisera to rAPS and SH2B2 were gifts from David Ginty (The Johns Hopkins University School of Medicine, Baltimore); the rabbit antibody to Grb2 was from Santa Cruz Biotechnology; the mouse monoclonal anti-PLCγ-1 and the horseradish peroxidase-coupled rabbit anti-phosphotyrosine antibodies (RC20) were from Transduction Laboratories; the rabbit antibody to MAPK was from Steve Peich (Kinetek Biotechnology Corp., Vancouver, Canada); and the rabbit anti-phospho-specific MAPK antibody was from Promega. Rabbits antibodies to the carboxyl-terminal 14 residues of TrkA (203) were prepared using standard techniques. The pGEX vectors containing Y499F/Y794F mutant TrkA (S8S9) were stimulated with 100 ng/ml of NGF (10 min), and lysates prepared and purified as described previously (3). In general, fusion proteins were stored bound to glutathione-Sepharose; however, in some instances the proteins were eluted with 10 mM reduced glutathione and dialyzed against phosphate-buffered saline containing 10% (v/v) glycglycerol. The protein concentrations were estimated by comparison with known amounts of purified GST following SDS-PAGE or by MicroBeads readings of purified material (A280 reading of 1.4 = 1 mg/ml).

**In Vitro Co-immunoprecipitations, SDS-PAGE, and Western Blotting—** The Shc PTB domain, the PLCγ-1 SH2 domain, and full-length Grb2 were expressed as fusion proteins with glutathione S-transferase (GST) in Escherichia coli strain BL21. GST fusion proteins were expressed and purified as described above. In general, fusion proteins were stored bound to glutathione-Sepharose; however, in some instances the proteins were eluted with 10 mM reduced glutathione and dialyzed against phosphate-buffered saline containing 10% (v/v) glycglycerol. The protein concentrations were estimated by comparison with known amounts of purified GST following SDS-PAGE or by MicroBeads readings of purified material (A280 reading of 1.4 = 1 mg/ml).

High-Five insect cells were infected with recombinant baculoviruses expressing the various receptors, stimulated with 100 ng/ml of NGF (10 min), and lysates prepared as described (18). Clarified lysates were assayed for total protein (Bio-Rad D Protein Assay Kit), and the level of Trk expression was determined by titration and Western blotting with the anti-HA antibody as described above.

Receptors were precipitated by mixing GST fusion proteins (1–2 μg) with baculovirus-infected insect cell lysates containing equal amounts of expressed TrkA receptors as described (3). Proteins were resolved on a 6% SDS-polyacrylamide gel, transferred to Immobilon-P, and blotted as described above. Purified GST served as the negative control.

For immunoprecipitation experiments, nrr5, B, and nrr5 cells expressing the Y499F/Y794F mutant TrkA (S8S9) were stimulated with NGF (100 ng/ml) for 10 min and lysed for (2). Equal amounts of protein (2 μg) were immunoprecipitated with rabbit anti-Ste (5 μg) or anti-HA (12CA5; 2 μg), with either 10 μl of Pansorbin (Calbiochem) or 50 μl of Tachsorb (Calbiochem). Where indicated, lysates were pre-cleared with either anti-Shc antibodies or a mixture of four different antibodies (anti-Ste (5 μg), anti-PLCγ-1 (1 μg), anti-SH2B2 (1,300), and anti-rAPS (1,300)) for 1.5 h at 4 °C, and the immune complexes were collected by centrifugation at 4 °C. The precipitated (3) lysates were then re-precipitated with either anti-Shc, the antibody mixture, or 12CA5 (5 μg) overnight at 4 °C. Immune complexes were collected and analyzed by SDS-PAGE and Western blotting (2). Membranes were incubated in blotto for 2 h at room temperature and then probed with rabbit anti-Grb2 (1,3000) and horseradish peroxidase goat anti-rabbit IgG (1:20,000) and visualized by chemiluminescence. When indicated, membranes were stripped at 50 °C for 15 min (62.5 mM Tris-PH (pH 6.8), 1% SDS, 100 mM β-mercaptoethanol and re-probed with anti-Ste (1 μg/ml) or the anti-HA monoclonal, 3F10 (1:4000).

To determine the level of MAPK activation, cells were stimulated for 10 min with 100 ng/ml NGF, lyse, and assayed for protein content as described above. Ten micrograms of total protein from each line was analyzed by 12% SDS-PAGE and Western blotting with an anti-phospho-specific MAPK antibody (1:10,000). Blots were stripped and re-probed with a polyclonal rabbit anti-MAPK antibody (1:10,000) to determine absolute levels of MAPK per lane.
Grb2 Binds TrkA at the Activation Loop Tyrosines (Tyr^{683} and Tyr^{684})

Grb2 Interacts Directly with TrkA in Yeast Two-hybrid Assays—The recruitment of the signaling adapter, Grb2, into Trk receptor signaling has been primarily described through interactions with Shc, FRS-2, RAPs, SH2B, and PLCγ-1. Since there are multiple examples in which Grb2 also directly associates with RTKs, we utilized the yeast two-hybrid system to determine whether Grb2 could directly interact with the ICD of TrkA. We have previously shown that dimerization of the DNA binding domain of the ICD of TrkA fused to the DNA binding domain of the yeast gal4 transcription factor (pAS2-TrkA), full-length Grb2 fused to the activation domain of the gal4 (pGAD-Grb2), or co-transformed with both plasmids. Yeast were also co-transformed with a kinase-inactive TrkA ICD (S11; K547A) fused to the gal4 DNA binding domain and pGAD-Grb2. Cells were plated under restrictive conditions as follows: pAS2-ICD (S11; K547A) fused to the gal4 DNA binding domain and pGAD-plasmids. Yeast were also co-transfected with a kinase-inactive TrkA ICD of the gal4 (pGAD-Grb2), or co-transformed with both transcription factor (pAS2-TrkA), full-length Grb2 fused to the activation domain of gal4 (amino acids 1–147) is sufficient to activate the kinase activity of TrkA (3); thus, yeast expressing Grb2 could directly interact with the ICD of TrkA. We have previously shown that dimerization of the DNA binding domain of gal4 (amino acids 1–147) is sufficient to activate the kinase activity of TrkA (3); thus, yeast expressing the TrkA-ICD were transfected with full-length Grb2 fused to the gal4 transcription factor activation domain (amino acids 761–881). Yeast co-expressing Grb-2 and either a kinase-active TrkA-ICD or a kinase-inactive TrkA-ICD (K547A, S11 mutant) were plated in the absence of His, Ade, Leu, and Trp (HALT medium) and monitored for growth in the presence of 3-AT (A), pAS2-TrkA on SC – His, – Ade, – Trp (B), and pGAD-Grb2 on SC – His, – Ade, – Leu (C).

RESULTS

Grb2 Interacts Directly with TrkA in Yeast Two-hybrid Assays—As previously shown, the amounts of TrkAS8 (Y499F) served with the kinase-inactive TrkAS11 (K547A) mutant (Fig. 1B, Grb2 precipitated all of the single point mutants to levels considerably greater than GST alone. No interaction was observed with either Shc domain. The SH2 domain and the SH3 domains of Grb2 were expressed as fusions with GST and used to precipitate TrkA from insect cell lysates. Full-length Grb2 and the PTB domain of Shc were included as controls. A first test of TrkA-Grb2 interaction outside of yeast, we used in vitro binding assays with GST fusion proteins encoding the SH2 and the carboxyl- and amino-terminal SH3 domains of Grb2 and baculovirus-infected insect cell lysates expressing TrkA. As shown in Fig. 2, the interaction with TrkA was mediated solely through the central SH2 domain of Grb2 with no interaction observed with either SH3 domain. This observation, together with the fact that the interaction is kinase-dependent (Fig. 1), suggests that the binding site(s) on TrkA are phosphorysine-dependent.

Grb2 Binds to Two Independent Phosphotyrosine Sites on TrkA—Upon ligand binding, rat TrkA is phosphorylated at five intracellular tyrosines, namely Tyr^{683}, Tyr^{679}, Tyr^{682}, Tyr^{684}, and Tyr^{794} (19). As previously stated, Tyr^{683} serves as the binding site for the PTB domains of Shc (11, 23) and FRS-2 (2), whereas Tyr^{794} serves as the binding site for PLCγ-1 and Chk (5, 11, 23). The central core “activation loop” tyrosines (679, 683, and 684) are essential for allosteric changes involved in kinase activation (24). To determine the Grb2-binding site(s) on TrkA, a series of substitution mutants (Table I) were assayed in an in vitro binding assay. In generating these mutants, we had to take into account the fact that Tyr^{683} and Tyr^{684} are essential for kinase activity (25), and replacement of them could inactivate TrkA and indirectly affect binding interactions. However, substitution of the activation loop tyrosines in TrkA with acidic amino acids (Glu and Asp) can mimic the acidic nature of the phosphotyrosines resulting in constitutive kinase activity and the subsequent activation of intracellular signaling molecules in the absence of exogenous NGF (20). This mutation effectively allows us to maintain the kinase activity of TrkA and to then determine the role of the activation loop phosphotyrosines as direct binding sites for intracellular signaling molecules. Baculovirus stocks expressing HA-tagged wild type TrkA, kinase-inactive TrkAS11 (K547A), the TrkAS13a constitutively active mutant (Y683D/Y684E), and the single tyrosine receptor mutants TrkAS8 (Y499F), TrkAS9 (Y794F), and TrkAS26 (Y679A) (Table I; Fig. 3A) were expressed in insect cells, and lysates were quantified for receptor expression. Equivalent amounts of receptor were precipitated with GST fusion proteins corresponding to the PTB domain of Shc, the SH2 domain of PLCγ-1, and full-length Grb2. As shown in Fig. 3B, Grb2 precipitated all of the single point mutants to levels considerably greater than GST alone. No interaction was observed with the kinase-inactive TrkAS11 (K547A) mutant (Fig. 3B). As previously shown, the amounts of TrkAS8 (Y499F) precipitated with GST-Shc and TrkAS9 (Y794F) precipitated with GST-PLCγ-1 are reduced relative to wild type TrkA (2).

The data in Fig. 3B indicate that Grb2 interacts with TrkA either at multiple Tyr(P) sites or at a site that does not contain Tyr(P) but that could be exposed in a kinase-active receptor conformation. Although SH2 domains are most commonly known to bind phosphorylated residues, it has also been shown that the SH2 domain of Grb2 can bind non-phosphorylated...
ligands (26). Thus, the in vitro binding assays were performed with two additional mutants containing deletions in conserved intracellular motifs, namely D450KFG452 (S17) and D493IMENP497 (S3). Neither of these TrkA mutants support NGF-dependent differentiation in nmr5 cells and both show a decrease in the stoichiometry of FRS-2/SNT phosphorylation and binding to TrkA (2, 19, 27). As shown in Fig. 3C, Grb2 efficiently precipitates both TrkAS3 and TrkAS17. As previously shown, the TrkA deletion significantly reduces interaction with the PTB domain of Shc (Fig. 3C; see Refs. 2 and 19) providing further evidence that Grb2 binds to TrkA independently of Shc/FRS-2.

Collectively, these data indicate that no single mutation affects Grb2 binding suggesting that there is more than one site of interaction. Thus, we assayed a series of combinatorial TrkA Tyr(P) mutants (Table I) for Grb2 binding. The first double TrkA mutant assayed substituted phenylalanine at Tyr499 and Tyr794 (TrkA-S8S9). We found that this mutant did not affect Grb2 binding (data not shown) suggesting that the activation loop tyrosines themselves could be involved. We next assayed the role of Tyr679, the first phosphotyrosine in the activation loop that is predicted to be solvent-exposed in the insulin receptor kinase (24) suggesting that it might interact with intracellular signaling molecules. We generated a triple mutant containing Y499F/Y679A/Y794F (S27) and found that it cannot bind to Shc and PLCγ-1, as expected, but retains binding to Grb2 (Fig. 3D). These data indicate that Tyr679 is also not a binding site for Grb2 and suggests that the core activation loop tyrosines 683 and 684 could themselves be a site(s) of interaction. A second triple mutant, termed S28, which contains Y679A in addition to Y683D/Y684E was then assayed. This combination of substitutions will retain constitutive kinase activity but will essentially remove all activation loop tyrosines as potential binding sites. Interestingly, as shown in Fig. 3D, this mutant also retains the ability to bind all three proteins. The ability to bind Shc and PLCγ-1 serves as a positive control and indicates that the receptor is still kinase-active and can phosphorylate both Tyr499 and Tyr794. However, the ability of Grb2 to bind TrkAS28, in the absence of the activation loop tyrosines, together with the ability of Grb2 to

| TrkA mutant | Mutation | Affected TrkA-binding proteins | Refs. |
|-------------|----------|--------------------------------|-------|
| S11         | K547 A   | All                            |       |
| S3          | 493IMENP497 | All                        | 2, 19 |
| S17         | 450KFG452 | SHT/FRS-2                      | 2, 27 |
| S8          | Y499F    | Shc, SHT/FRS-2                 | 2, 11, 23 |
| S9          | Y794F    | Shc, SNT/FRS-2                 | 5, 11, 23 |
| S889        | Y499F/Y794F | Shc, SHT/FRS-2          | 2, 11, 23 |
| S13a        | Y683D/Y684E | Plcγ-1, CHK                |       |
| S26         | Y679 A   | Grb2, rAPS, SH2B               | 4, 20, this manuscript |
| S27         | Y499F/Y679A/Y794F | Shc, Plcγ-1, CHK            |       |
| S28         | Y679A/Y683D/Y684E | Grb2, rAPS, SH2B          |       |
| S29         | Y679A/Y683D/Y684E/Y794F | Grb2, rAPS, SH2B, CHK, Plcγ-1 |       |
| S30         | Y499F/Y679A/Y683D/Y684E/Y794F | All                      |       |

**FIG. 3.** In vitro association of Grb2 with a series panel of TrkA mutants. Full-length Grb2 was expressed as a fusion protein with GST and used to precipitate TrkA mutants from insect cell lysates. A, schematic of the TrkA-ICD indicating the mutations. B, in vitro binding assay with wild type TrkA and single point mutants as follows: TrkAS8 (Y499F), TrkAS9 (Y794F), TrkAS11 (K547A), TrkAS13a (Y683D/Y684E), and TrkAS68 (Y679A). B, in vitro binding assay with the deletion mutants, TrkAS42 (493IMENP497) and TrkAS17 (450KFG452). C, in vitro binding assay with the combinatorial mutants as follows: TrkAS27 (Y499F/Y679A/Y794F), TrkAS28 (Y679A/Y683D/Y684E), TrkAS29 (Y679A/Y683D/Y684E/Y794F), and TrkAS30 (Y499F/Y679A/Y683D/Y684E/Y794F). Both the PTB domain of Shc and the SH2 domain of Plcγ-1 were included as controls.
Grb2 Binds TrkA at the Activation Loop Tyrosines (Tyr<sup>683</sup> and Tyr<sup>684</sup>)

The B2, B5, and TrkA-S8 nmr5-derived cell lines, in addition to PC12 and nmr5 cells, were stimulated with NGF. Lysates were immunoprecipitated with either anti-Shc or anti-Trk antibodies and analyzed by immunoblotting with antibodies against Grb2. As shown in Fig. 5A, the amount of Grb2 precipitating with Shc is significantly higher in NGF-stimulated PC12, B5, and B2 cells than in NGF-stimulated nmr5 cells. In contrast, Shc/Grb2 was not co-precipitated from TrkA-S8S9 cells to levels greater than nmr5 cells. Stripping and re-probing with anti-Shc antibodies indicate similar amounts of all three Shc isoforms in each lane (Fig. 5A). These data are consistent with previous reports that the Y499F mutant is incapable of NGF-dependent Shc phosphorylation and Grb2 binding (11, 23).

In contrast to the anti-Shc immunoprecipitation studies, analysis of the anti-TrkA immunoprecipitates indicates co-immunoprecipitation of Grb2 with TrkA in PC12, B5, B2, and S8 cells with insignificant amounts observed in nmr5 cells. Grb2 does not co-immunoprecipitate with TrkA in the absence of NGF (data not shown). Stripping and re-probing the blot with an anti-HA antibody reveal that similar levels of TrkA were detected from PC12 cells. Since B2, B5, and S8 cells all express more receptors per cell than PC12 cells (19), this result is somewhat unexpected. Significantly, however, the data indicate Shc and FRS-2-independent association of Grb2 with TrkA in NGF-stimulated cells.

The in vitro binding studies shown above suggest that Grb2 binds TrkA at both the activation loop tyrosines and at the carboxyl-terminal residue Tyr<sup>794</sup>. To test this directly, we assayed naive and NGF-stimulated B5 and TrkA-S8S9 (Y499F/Y794F) cells for TrkA-Grb2 interaction in lysates that were pre-cleared with the NGF-stimulated Grb2-binding proteins,
Shc, rAPS, SH2B, and PLC-1. As shown in Fig. 6A (top panel), TrkA-S8S9-expressing cells stimulate NGF-dependent TrkA tyrosine phosphorylation to levels lower than B5 cells consistent with the loss of 2 major sites of receptor tyrosine phosphorylation; however, TrkA-S8S9 cells also express approximately 2-fold fewer receptors than B5 cells. Lysates were prepared and precipitated with a mixture of anti-Shc, rAPS, SH2B, and PLC-1. Lysates from unstimulated and NGF-stimulated cells were precipitated with a mixture of anti-Shc/PLC-1/rAPS/SH2B antibodies. The clarified lysate was re-precipitated with the same mixture of 4 antibodies. The twice clarified lysate was re-precipitated with anti-HA antibodies to isolate TrkA from B5 cells and S8S9 cells (Y499F/Y794F). Western blots were probed with anti-Grb2 antibodies.

![Fig. 6](http://www.jbc.org/)

**Fig. 6. Immunodepletion analysis of TrkA/Grb2 co-immunoprecipitation.** A, level of TrkA expression in cells expressing wild type TrkA (B5) and the Y499F/Y794F mutant (S8S9). Lysates from NGF-stimulated cells (100 ng/ml, 5 min) were immunoprecipitated (IP) with anti-Trk antibodies and analyzed by Western blotting with anti-Tyr(P) and anti-HA antibodies. B, Grb2 co-immunoprecipitates with TrkA independently of Shc, rAPS, SH2B, and PLC-1. Lysates from unstimulated and NGF-stimulated cells were precipitated with a mixture of anti-Shc/PLC-1/rAPS/SH2B antibodies. The clarified lysate was re-precipitated with the same mixture of 4 antibodies. The twice clarified lysate was re-precipitated with anti-HA antibodies to isolate TrkA from B5 cells and S8S9 cells (Y499F/Y794F). Western blots were probed with anti-Grb2 antibodies.

The TrkA Y499F/Y794F Mutant Supports Low Levels of NGF-dependent MAPK Activation and Neurite Outgrowth—Collectively, these data indicate that Grb2 can bind directly to NGF-activated TrkA independent of Shc, FRS-2, PLC-1, rAPS, and SH2B. The obvious question then arises as to the functional role of direct Grb2 (SH2 domain-mediated) binding to TrkA. In this respect, Grb2 is a multifunctional signaling adapter that can couple receptors to a variety of intracellular pathways. Of these, the best understood is the activation of Ras and MAPK. Previous studies have shown a dramatic reduction but not a complete loss of NGF-induced MAPK activation by Shc and PLC-1 uncoupled human TrkA (11). Thus, we assayed cells expressing the rat TrkAY499F/Y794F receptor (S8S9) for NGF-induced MAPK activation using a specific antiphospho-MAPK antibody. As shown in Fig. 8A, cells expressing the S8S9 mutant show NGF-dependent activation of MAPK, although the levels are reduced relative to NGF-stimulated wild type TrkA (B5 cells). These observations thus raised a subsequent question, namely can the Y499F/Y794F receptor support sufficient levels of MAPK activation to support neurite outgrowth? Since prolonged levels of MAPK activation correlate with neurite outgrowth (7) and overexpression of compo-
Grb2 binds TrkA at the activation loop tyrosines (Tyr\textsuperscript{683} and Tyr\textsuperscript{684})

Fig. 7. TrkA-Grb2 interaction in yeast requires pY\textsuperscript{683} and pY\textsuperscript{684}. Yeast expressing the ICD of wild type TrkA, the TrkA-S27 mutant (Y499F/Y679A/Y794F), and the TrkA-S29 mutant (Y679A/Y683D/Y794F) (pAS constructs) were transfected with pGAD-Shc(PTB) or pGAD-Grb2(SH2) and assayed for interaction on HAT plates containing 20 mM 3-AT.

DISCUSSION

To date a number of signaling proteins have been shown to associate directly with TrkA. Among these are the signaling adapters Shc (11, 23), FRS-2 (2), rAPS, and SH2-B (4) as well as PLC\textgamma-1 (11, 23), the Csk homologous kinase (5) and the guanine nucleotide exchange protein Ras-GRF1 (3). In this report, we demonstrate that the adapter protein Grb2 also interacts with the intracellular domain of TrkA at more than one site. That the interaction is direct and not due to a complex between TrkA and other intracellular signaling molecules (Shc, FRS-2, rAPS, SH2-B, and PLC\textgamma-1) is based on a number of observations. 1) Grb2 is co-immunoprecipitated with TrkA mutants (TrkAS8 (Y499F) and TrkASS8 (Y499F/Y794F)) which are incapable of binding Shc, FRS-2, or PLC\textgamma-1. 2) The interaction is observed in lysates that are effectively pre-cleared of Shc, PLC\textgamma-1, rAPS, and SH2-B. 3) Grb2 interacts with kinase-active TrkA but not kinase-inactive TrkA in the yeast two-hybrid assay. 4) Grb2 binding to TrkA in yeast is independent of Tyr\textsuperscript{499}/Tyr\textsuperscript{679}/Tyr\textsuperscript{794} but requires the activation loop tyrosines Tyr\textsuperscript{683}/Tyr\textsuperscript{684}. As stated earlier, it is not unprecedented for Grb2 to be recruited both directly and indirectly into the signaling cascades of RTKs. For example, Grb2 directly binds the activated EGFR (12, 13), the PDGF receptor (31), the Ret receptor (14), the Tpr-Met oncoprotein (15) and the ErbB2 receptor (16).

Because the interaction between TrkA and Grb2 requires a kinase-active receptor and is mediated through the central SH2 domain, it was predicted that the interaction with TrkA would be mediated through one or more phosphoryrosine residues. In general, the canonical sequence for SH2 domain-mediated Grb2 binding is Tyr(P)-X\textsuperscript{Y}-Asn-X\textsuperscript{Y} where the residues Gln, Tyr, Val, Leu, Phe, or Met are favored at position X\textsuperscript{1} and Gln, Tyr, Val, Pro, or Phe are favored at position X\textsuperscript{3} (26, 28, 32, 33); however, Grb2 has also been shown to bind Tyr(P)\textsuperscript{1172} of the EGFR at the sequence pYLR (13). Of the five phosphoryrosine residues in TrkA identified to be phosphorylated in response to NGF (Tyr\textsuperscript{499}, Tyr\textsuperscript{679}, Tyr\textsuperscript{683}, Tyr\textsuperscript{684}, and Tyr\textsuperscript{794}), none corresponds to the canonical pYXY sequence, and only Tyr\textsuperscript{683}, within the activation loop, contains a positive charge two amino acids downstream from the phosphoryrosinated tyrosine (pYpY).

To determine which tyrosine residue(s) on TrkA is/are involved in Grb2 binding, each tyrosine was mutated, singly and in combination, and the mutant receptors were assayed in \textit{in vitro} binding assays. Since a kinase-active receptor was essential for the interaction, mutation of the activation loop tyrosines (Tyr\textsuperscript{683} and Tyr\textsuperscript{684}) presented a challenge as their substitution could potentially lead to loss of kinase activity (25, 34). However, this problem was overcome by replacing the activation loop tyrosines with acidic amino acids (Glu and Asp) to retain NGF-independent kinase activity (20).

We found that no single tyrosine was identified as the docking site for Grb2 raising the possibility that Grb2 binds at multiple phosphoryrosines on TrkA. Such a scenario is not unprecedented; for example, Shc binds at five sites on the PDGF receptor and competes at several of these sites with Grb2, phosphatidylinositol 3-kinase, Nck, and GAP (33, 35). Moreover, Grb2 binds three phosphoryrosine residues on the EGFR (13). Interestingly, we found that substitution of the first activation loop tyrosine (Y679A), the S26 mutant, retained binding to all signaling molecules tested (Shc, Grb2, and PLC\textgamma-1). This result was surprising given a previous report in which phenylalanine substitution at this site in human TrkA (Y670F) significantly reduced both the tyrosine phosphorylation and receptor binding of PLC\textgamma-1 in co-immunoprecipitation assays. This discrepancy may be explained by either slight differences in substituting alanine \textit{versus} phenylalanine or by the greater sensitivity of \textit{in vitro} binding studies.

Given the facts that no single tyrosine substitution mutant or the two separate deletion mutants were identified as the docking site for Grb2, we generated a series of combinatorial TrkA mutants involving all five intracellular tyrosines. We found that the combination mutants, Y499F/Y794F (SS8S9) and Y679A/Y683D/Y684E (S28), still bound Grb2, indicating that Grb2 must bind TrkA at more than one site. Through a combination of triple and quadruple mutants, we demonstrate that the activation loop tyrosines, Tyr\textsuperscript{683} and Tyr\textsuperscript{684}, and the carboxyl-terminal residue Tyr\textsuperscript{794} are independently involved in binding to Grb2. Specifically, only when the carboxyl-terminal Y794F mutation is combined with the activation loop TrkA mutant Y683D/Y684E is Grb2 binding effectively terminated. Under these conditions, the receptor is still tyrosine-phosphorylated (Fig. 4), presumably at Tyr\textsuperscript{499}, and still binds the PTB domain of Shc (Fig. 3D). Importantly, however, this mutant is unable to bind either the SH2 domain of PLC\textgamma-1 or Grb2.

The amino acid sequences surrounding these two binding sites do not correspond to the well described pYXY binding site for the SH2 domain of Grb2 (28); however, one of the two Grb2-binding sites identified on TrkA, Tyr\textsuperscript{683}, does correspond...
Grb2 Binds TrkA at the Activation Loop Tyrosines (Tyr683 and Tyr684)

Fig. 8. TrkAS8S9 mutant receptors (Y499F/Y794F) support NGF-dependent MAPK activation and low levels of NGF-dependent neurite outgrowth. A, lysates from unstimulated and NGF-stimulated nmr5, S8S9 cells, and wild type TrkA cells (B5 cells) were assayed for MAPK phosphorylation (anti-phosphospecific MAPK). Blots were stripped and re-probed with an antibody that recognizes both unstimulated and activated forms of MAPK. B, Nmr5 cells were transiently transfected with EGFP and/or with the TrkA-S8S9 mutant. EGFP-positive cells were scored for NGF-stimulated neurite outgrowth at 5 days.

The obvious question then arises as to what is the role and/or need of redundancy in Grb2 binding to an activated RTK. In this respect, both the EGFR and the PDFR contain multiple docking sites for Shc-Grb2 complexes as well as direct binding sites for the SH2 domain of Grb2 (12, 13, 33). The most well known function of Grb2 is to bind, via its SH3 domains, to the guanine nucleotide exchange factors (Sos, Vav, and C3G) and to activate Ras-dependent signaling (41, 42). However, it is also clear that the Grb2 SH3 domains can bind to a variety of other intracellular substrates, including components of endocytic machinery (dynamin; see Ref. 43), adapters involved in cytoskeletal re-arrangements (CMS (44), Ajuba (45), and N-WASP (46)), as well as other intracellular signaling molecules (Deltex (47); Disabled-2 (48); c-Cbl (49), and the stress-activated protein kinase IHPK1 (50)), indicating that the complexity of Grb2 signaling, in different biological scenarios, far exceeds a single role in the activation of Ras. Thus, the direct binding of Grb2 to TrkA, via its SH2 domain, could facilitate subsequent Grb2/SH3 domain-mediated recruitment and activation of other signaling molecules to the activated TrkA receptor complex. The identification and elucidation of these pathways and their potential role(s) in neurotrophin signaling remain to be investigated.

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Direct Binding of the Signaling Adapter Protein Grb2 to the Activation Loop Tyrosines on the Nerve Growth Factor Receptor Tyrosine Kinase, TrkA
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