Brain-derived Neurotrophic Factor Binding to the p75 Neurotrophin Receptor Reduces TrkA Signaling While Increasing Serine Phosphorylation in the TrkA Intracellular Domain*

(Received for publication, June 27, 1997)

Ian J. MacPhee‡ and Philip A. Barker§

From the Center for Neuronal Survival, Montreal Neurological Institute, McGill University, 3801 University Avenue, Montreal, Quebec H3A 2B4, Canada

We have examined whether the low affinity neurotrophin receptor p75NTR modulates TrkA function by intracellular signaling. Using ligands that selectively bind p75NTR or TrkA, we found that a p75NTR-derived signal reduces TrkA activation. Short term treatment of PC12 cells with ceramide analogues also resulted in reduced NGF-stimulated TrkA activation, suggesting that p75-mediated increases in sphingomyelinase activity may contribute to this modulatory effect. Phosphoamino acid analysis was performed to determine if brain-derived neurotrophic factor- or ceramide-mediated phosphorylation of the TrkA intracellular domain correlated with a reduction in its ligand-induced activation. A specific increase in TrkA phosphoserine content was observed in response to both C2-ceramide and brain-derived neurotrophic factor. These results suggest that ligand binding of p75NTR can activate a signaling cascade that results in reduced TrkA activity through phosphorylation of its intracellular domain.

The neurotrophins are a well conserved family of proteins that play critical roles in the maintenance and development of the nervous system (1–7). Their cellular effects are mediated by two distinct classes of cell surface receptors. Highly related high affinity receptors TNFR1 and TNFR2 (reviewed in Ref. 8). The physiological role of the p75NTR has proven elusive, but recent data suggest that p75NTR may be functionally grouped with other members of the TNF receptor superfamily, such as TNFR1 and Fas.

Recent analyses of p75NTR-TrkA interactions have focused on ligand binding events that might account for modulation of TrkA function. This report, we tested the hypothesis that a p75NTR-activated signaling mechanism alters TrkA function. Using receptor-selective ligands, we found that BDNF binding to p75NTR results in a strong attenuation of TrkA signaling. TrkA signaling was also reduced following a short exposure to ceramide, suggesting that ceramide production which results from p75NTR activation may be responsible for this effect. To test if the reduction in TrkA signaling correlated with serine or threonine phosphorylation of the TrkA intracellular domain, TrkA was subjected to phosphoamino acid analysis; both BDNF and ceramide treatment were found to increase phosphoserine content. These findings indicate that ligand binding to p75NTR activates a ceramide-dependent signaling cascade that regulates TrkA activity through serine phosphorylation of the TrkA intracellular kinase domain.

EXPERIMENTAL PROCEDURES

Materials—NGF was purchased from Collaborative Research, TNF was purchased from R & D Systems, and C2-ceramide and dihydrothiophosphine were obtained from BioMol. BDNF was provided by Regeneron Pharmaceuticals (Tarrytown, NY), the pan-Trk antibody 203 was a gift from David Kaplan (Montreal Neurological Institute), and NGF3T was prepared as described (12). All other reagents were purchased from either Sigma or ICN.

Cell Culture—PC12 cells (18) and PC12-6/24 cells (19) were maintained in Dulbecco's modified Eagle's medium containing 6% horse serum and 6% bovine calf serum in 7% CO2 at 37 °C. MG139–2 cells (20), a subline of 3T3 fibroblasts stably expressing rat TrkA (but not p75NTR), were maintained in Dulbecco's modified Eagle's medium containing 10% bovine calf serum and 200 δ/ml G418 in 7% CO2 at 37 °C.

Immuno precipitation and Immunoblotting—PC12 cells or MG139–2 cells were plated on 100-mm dishes and 24 h later were washed twice in Dulbecco's modified Eagle's medium containing 0.1% bovine serum albumin (DMEM) and then incubated in 5 ml of DMEM supplemented as described in the figure legends. Control incubations received appropriate vehicle control solutions. After induction, the medium was removed, and plates were placed on ice, rinsed with ice-cold Tris-buffered saline (20 mM Tris (pH 8.0), 137 mM NaCl, 1% Nonidet P-40, 10 δ glycercyl, 1 δ/ml leupeptin, 100 δ propylthiourea, and 1 mM orthovanadate). Lysates were normalized for protein content, incubated with 2 δ of rabbit pan-Trk antibody 203 overnight at 4 °C, supplemented with 30 δ of protein A-Sepharose, and then incubated at
4°C for 90 min. Beads were pelleted, washed three times in lysis buffer, resuspended in Laemmli sample buffer, and boiled for 5 min. Immunoprecipitates were separated on 10% Laemmli acrylamide gels and transferred to nitrocellulose. Phosphoamino acid blotting was performed in 10 mM Tris (pH 7.4), 150 mM NaCl, 2% bovine serum albumin, 0.2% Tween 20, and primary and secondary antibody incubations were performed in 10 mM Tris (pH 7.4), 150 mM NaCl, and 0.2% Tween 20. Blocking, primary (using antibody 203), and secondary (using protein A-conjugated horseradish peroxidase) incubations for TrkA immunoblots were all performed in 10 mM Tris (pH 7.4), 150 mM NaCl, 0.2% Tween 20, and 5% (w/v) dry skim milk powder. Reactive bands on all immunoblots were detected using enhanced chemiluminescence (ECL) according to the manufacturer’s instructions (DuPont).

Phosphoamino Acid Analysis—PC12 cells were grown to confluence in 150-mm plates, washed once in serum-free medium, and then preincubated 2 h in phosphate-free DMEM containing 750 μCi of [32P]orthophosphate/ml. Cells were then supplemented with 11 × stock solutions made in phosphate-free DMEM to produce final NGF, BDNF, or C2-ceramide concentrations of 100 ng/ml, 200 ng/ml, and 100 μM, respectively. After a 30-min incubation, the medium was removed, and cells were washed once with Tris-buffered saline (pH 7.4). Wash buffer was replaced with lysis buffer (10 mM Tris (pH 7.4), 10% glycerol, 150 mM NaCl, 1 mM EGTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM orthovanadate). Lysates were centrifuged at 16,000 × g, and the supernatant was precleared with preimmune serum-coated protein A-Sepharose beads for 1 h at 4°C. The beads were removed by centrifugation, and TrkA was immunoprecipitated as described above. Immunoprecipitates were split into two aliquots that were both separated on SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. One set of labeled lysates was analyzed for TrkA content by immunoblot as described above, and the other was exposed to XAR film to detect labeled TrkA protein. Polyvinylidene difluoride membrane fragments containing the labeled TrkA samples were cut from the transfer membrane and hydrolyzed under vacuum in 6 N HCl vapor for 2.5 h at 110°C. Phosphoamino acids were eluted from the membrane with H2O for 16 h then were concentrated in a Speed-Vac. Phosphoamino acids were separated using two-dimensional thin layer electrophoresis on a Hunter apparatus. Phosphoamino acid standards were visualized by spraying the plates with a solution of 0.2% ninhydrin in acetone (w/v), and 32P-labeled phosphoamino acids were identified by autoradiography. Quantitation of phosphoamino analysis was performed on scanned autoradiographs using NIH IMAGE.

RESULTS

Signaling pathways originating from p75NTR and TrkA can be activated in PC12 cells (15, 19). We have previously shown that BDNF treatment of PC12 cells, which binds p75NTR but not TrkA, results in a dramatic reduction in TrkA receptor activation that is at least partially due to its ability to disrupt ligand transfer between p75NTR and TrkA (12). To test whether other mechanisms might also contribute to the effect of BDNF on TrkA activation, we have used ligands that selectively bind either TrkA or p75NTR. A mutant form of NGF (NGF3T) that does not bind p75NTR (21) was used to selectively activate TrkA and BDNF was used to selectively activate p75NTR. To determine if a p75NTR-derived signal may affect TrkA activation, PC12 cells were preincubated with various concentrations of BDNF for 3 h, exposed to NGF3T for 5 min, and then analyzed for TrkA tyrosine phosphorylation levels by immunoblotting (Fig. 1A). A clear reduction in NGF3T-stimulated TrkA tyrosine phosphorylation levels was observed in the presence of BDNF, densitometry reveals reductions of 53, 70, and 81% at BDNF concentrations of 50, 100, and 500 ng/ml, respectively. Direct TrkA immunoblotting showed that these decreases in phosphotyrosine signal did not reflect a reduction in TrkA tyrosine phosphorylation because these were maintained throughout all treatment conditions (Fig. 1B). This effect of BDNF was rapid; a BDNF preincubation lasting 5 min (Fig. 1C) produced effects on TrkA tyrosine phosphorylation similar to that observed using a 3-h preincubation (Fig. 1A).

To test if the BDNF-mediated reduction in TrkA tyrosine phosphorylation is reflected in downstream signaling events, NGF3T-stimulated c-fos mRNA accumulation was examined in the presence and the absence of BDNF. Fig. 2A shows that the robust increase in c-fos mRNA expression, which normally occurs in response to NGF3T, was reduced (by 71%) following BDNF treatment. We next considered the possibility that the effect of BDNF on TrkA activation and signaling could be due some direct interference with NGF3T binding. We have previously shown that BDNF does not directly interact with TrkA and does not interfere with the interaction of wild type NGF with TrkA (9, 12), but to ensure that BDNF has no effect on the interaction of TrkA with NGF3T, a mutant form of NGF, we examined the effect of BDNF on the activation of TrkA in fibroblasts that do not express p75NTR. Fig. 2B shows that the NGF3T-mediated increase in TrkA tyrosine phosphorylation in these cells was unaffected by BDNF preincubation.

Binding of BDNF to p75NTR on PC12 cells activates sphingomyelinase activity and results in the accumulation of intracellular ceramide (15). To test if BDNF-mediated ceramide accumulation might mediate the reduction in TrkA activity, PC12 cells were pretreated with increasing concentrations of C2-ceramide, a cell-permeable ceramide analogue, and then supplemented with either NGF or NGF3T for 5 min and analyzed for TrkA phosphotyrosine levels. Fig. 3A shows that C2-ceramide levels greater than 15 μM cause a profound reduction in the level of TrkA tyrosine phosphorylation induced by NGF,
similar to the effects observed with BDNF. Densitometry shows an average decrease in NGF-stimulated tyrosine phosphorylation of 61\% at 20 \( \mu \text{M} \) C2-ceramide, 71\% at 50 \( \mu \text{M} \) C2-ceramide, and 80\% at 100 \( \mu \text{M} \) C2-ceramide. Very similar results were obtained when NGF3T was used in place of NGF (data not shown). Levels of TrkA protein remain unchanged under these conditions (Fig. 3B), indicating that C2-ceramide reduced the ability of TrkA to respond to NGF. To determine whether this represents a specific effect of ceramide or a nonspecific lipid effect, these experiments were repeated using dihydrosphingosine, the inactive saturated analog of C2-ceramide. Dihydrosphingosine had no effect on NGF-mediated TrkA activation (Fig. 3C).

Tyrosine kinase receptors are ligand-regulated transmembrane enzymes, but kinase activity of the intracellular domain is subject to intracellular regulation, with phosphorylation of key intracellular serine or threonine residues playing an important regulatory role (22–25). To determine if BDNF or ceramide treatment results in alterations in the level of serine or threonine phosphorylation within the TrkA intracellular domain, we performed phosphoamino acid analysis of the TrkA receptor. PC12-6/24 cells, which overexpress the TrkA receptor, were grown in \([32\text{P}]\)orthophosphate for 2 h, treated with NGF, BDNF, or C2-ceramide for 30 min, and then analyzed for phosphoamino acid content. Fig. 4 shows that TrkA obtained from untreated cells contained low levels of phosphoserine, and as expected, TrkA from cells treated with NGF treatment contained abundant phosphoserine and also showed an increase in phosphoserine levels of about 50-fold. TrkA receptor from cells treated with either BDNF or C2-ceramide in the absence of NGF also showed a clear increase in phosphoserine content, with maximal increases in phosphoserine content approaching 12-fold with either reagent. These results indicate that the phosphorylation state of the TrkA intracellular domain is regulated by BDNF-activated signal and by ceramide and indicate that ligand-dependent activation of the TrkA receptor may be regulated by increased serine phosphorylation of its intracellular domain.

**DISCUSSION**

p75NTR and TrkA have a complex functional interaction. TrkA activation is increased in cells coexpressing the two receptors compared with those expressing TrkA alone; at least part of this effect is due to a p75NTR-mediated increase in the amount of NGF that ultimately becomes bound to the TrkA receptor (9, 12, 13). Intriguingly, whereas p75NTR increases the ability of TrkA to become activated by preferred ligands such as NGF, correlative evidence suggests that p75NTR reduces the ability of TrkA to become activated by nonpreferred ligands such as neurotrophin-3 and neurotrophin-4 (26–29). In our studies, we have found that treatment of PC12 cells with BDNF results in a pronounced attenuation in subsequent signaling through TrkA. This effect of BDNF is not due to disruption of NGF binding to p75NTR, since the NGF3T mutant used in these experiments does not bind this receptor. Furthermore, BDNF does not interfere with NGF3T-mediated activation of TrkA on cells that do not express p75NTR, indicating that the effects observed on PC12 cells are not due to disruption of...
NGF3T binding to TrkA. Instead, these effects are most easily explained by a mechanism that involves BDNF binding to p75NTR and subsequent activation of a signaling cascade that regulates TrkA activity.

Regulation of receptor activity by intracellular signaling paths can occur through a process termed receptor transmodulation. Selective phosphorylation of specific serine and threonine residues within the epidermal growth factor and insulin receptors results in a reduction in subsequent ligand-mediated receptor activity (22, 24, 30, 31). The mechanisms controlling these regulatory events are not well understood, but recent analyses of the effect of TNF receptor activation on insulin receptor signaling has been revealing. TNF treatment results in a reduction in subsequent ligand-stimulated insulin receptor signaling, and recent reports have shown that this attenuation is due to TNF-induced serine and threonine phosphorylation of the insulin receptor and its downstream tyrosine kinase signaling partner, IRS1. TNFR1 and TNFR2-mediated activation of sphinomyelinase activity and the subsequent accumulation of ceramide appear to be key components of this signaling cascade (32–38). Because BDNF, acting through p75NTR, also stimulates sphingomyelinase activity within PC12 cells (15), we reasoned that the BDNF-mediated attenuation in TrkA activation may likewise function through ceramide-dependent phosphorylation events. Consistent with this, we found that C2-ceramide, a cell-permeable ceramide analogue, mimics the effect of BDNF on TrkA activation and that BDNF and C2-ceramide lead to an increase in TrkA phosphoserine content. NGF treatment also results in a profound increase in TrkA phosphoserine, but p75-mediated activation of sphenomyelinase is apparently suppressed when TrkA is activated (15). Therefore, NGF-dependent serine phosphorylation of TrkA is unlikely to be a ceramide-dependent event but may instead be an indirect consequence of TrkA activation. Comparing the specific TrkA phosphoserine residues phosphorylated in response to NGF with those phosphorylated by BDNF and C2-ceramide treatment will allow us to define the specific activities mediating these effects.

We have not yet determined the mechanism by which BDNF and ceramide lead to an increase in TrkA phosphoserine content. BDNF and ceramide may activate cascades that result in activation of a regulated serine-directed kinase or could result in the stable association of some constitutively active kinase with TrkA. Alternatively, the increase in phosphoserine content observed in response to BDNF or ceramide treatment may reflect an induced dephosphorylation of TrkA with subsequent replacement with labeled phosphate through the process termed "back-phosphorylation." Identification of the serine residues within the TrkA intracellular domain that are substrates of this modulatory activity should allow us to dissect the mechanism by which ceramide and BDNF alter levels of TrkA phosphoserine.

Our studies indicate that a p75-activated signaling pathway that involves ceramide acts to negatively regulate ligand-stimulated TrkA activity. Combined with work by others that suggests that a TrkA-derived signal may reduce the capacity of p75NTR to signal (15), this suggests a complex trans-receptor regulatory loop between the two receptors. When exposed to NGF, a preferred ligand, the primary function of p75NTR appears to be to increase TrkA signaling, and the transmodulatory activity of p75NTR is likely inhibited under these conditions. Conversely, in the presence of nonpreferred ligands, our results suggest that p75NTR may inhibit nonpreferred ligand-stimulated TrkA activity through ceramide-dependent receptor transmodulation.

Acknowledgments—We are grateful to Dr. David Kaplan (Montreal Neurological Institute) for providing the anti-Trk sera used in these studies and to Dr. Wayne Sossin, Dr. Christian Lachance, and Christine Zeindler for helpful comments on the manuscript.

REFERENCES
1. Klein, R., Smekey, R. J., Warst, W., Long, I. K., Auerbach, B. A., Joyner, A. L., and Barabac, M. (1993) Cell 75, 113–122
2. Smekey, R. J., Klein, R., Schnapp, A., Long, I. K., Bryant, S., Lewin, A., Lira, S. A., and Barabac, M. (1994) Nature 368, 246–249
3. Klein, R., Silos, S. I., Smekey, R. J., Lira, S. A., Brambilla, R., Bryant, S., Zhang, L., Snider, W. D., and Barabac, M. (1994) Nature 368, 249–251
4. Crowley, C., Spencer, S. D., Nishimura, M. C., Chen, K. S., Pitts, M. S., Armanini, M. P., Ling, L. H., McMahon, S. B., Shelton, D. L., Levinson, A. D., and Phillips, H. S. (1994) Cell 76, 1001–1011
5. Ernfors, P., Lee, K. P., and Jaenisch, R. (1994) Nature 368, 147–150
6. Ernfors, P., Lee, K. P., Kucera, J., and Jaenisch, R. (1994) Cell 77, 503–512
7. Jones, K. R., Fariñas, I., Backus, C., and Reichardt, L. F. (1994) Cell 76, 989–999
8. Bazan, J. F. (1995) Curr. Biol. 5, 603–606
9. Barker, P. A., Barbee, G., Misko, T. P., and Shooter, E. M. (1994) J. Biol. Chem. 269, 30945–30950
10. Hantapoulo, P. A., Suri, C., Glass, D. J., Goldfarb, M. P., and Yancopoulos, G. D. (1994) Neuron 13, 187–201
11. Verdi, J. M., Birren, S. J., Ibáñez, C. F., Persson, H., Kaplan, D. R., Benedetti, M., Chao, M. V., and Anderson, D. J. (1994) Neuron 12, 733–745
12. Barker, P. A., and Shooter, E. M. (1994) Neuron 13, 203–215
13. Mahadeo, D., Kaplan, L., Chao, M. V., and Hemptstead, B. L. (1994) J. Biol. Chem. 269, 6884–6891
14. Dobrowsky, R. T., Wernert, M. H., Castellino, A. M., Chao, M. V., and Hannun, Y. A. (1994) Science 265, 1596–1598
15. Dobrowsky, R. T., Jenkins, G. M., and Hannun, Y. A. (1995) J. Biol. Chem. 270, 22135–22142
16. Carter, B. D., Kaltschmidt, C., Kaltschmidt, B., Offenhausen, N., Bohm, C., Rüegg, M. A., and Barde, Y. A. (1996) Science 272, 542–545
17. Braccau, L., Aloe, L., Stenfors, C., Tiras, P., Theodorsson, E., and Lundberg, T. (1996) Neuron 17, 485–488
18. Greene, L. A., and Tischler, A. S. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 4324–4329
19. Hempstead, B. L., Rabin, S. J., Kaplan, L., Reid, S., Parada, L. F., and Kaplan, D. R. (1992) Neuron 9, 833–836
20. Barker, P. A., Lomen-Hoerth, C., Genrich, E. M., Meakin, S. O., Glass, D. J., and Shooter, E. M. (1993) J. Biol. Chem. 268, 15150–15157
21. Ibáñez, C. F., Ebdell, T., Barbany, G., Murray-Rust, J., Blundell, T. L., and Persson, H. (1992) Cell 69, 329–341
22. Théroux, S. J., Latour, D. A., Stanley, K., Raden, D. L., and Davis, R. J. (1992) J. Biol. Chem. 267, 16620–16626
23. Tanti, J.-F., Gremenaux, T., Van Obberghen, E., and Le Marchand-Brustel, Y. (1994) J. Biol. Chem. 269, 6051–6057
24. Olson, J. E., and Pledger, W. J. (1990) Cell 64, 248–260
25. Lewis, R. E., Volle, D. J., and Sanderson, S. D. (1994) J. Biol. Chem. 269, 26259–26266
Regulation of TrkA Signaling by p75NTR

26. Davies, A. M., Lee, K. F., and Jaenisch, R. (1993) Neuron 11, 565–574
27. Ip, N. Y., Stitt, T. N., Tapley, P., Klein, R., Glass, D. J., Fandl, J., Greene, L. A., Barbacid, M., and Yancopoulos, G. D. (1993) Neuron 10, 137–149
28. Berkemeier, L. R., Winslow, J. W., Kaplan, D. R., Nikolios, K., Goeddel, D. V., and Rosenthal, A. (1991) Neuron 7, 857–866
29. Benedetti, M., Levi, A., and Chao, M. V. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7859–7863
30. Northwood, I. C., and Davis, R. J. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 6107–6111
31. Countaway, J. L., Northwood, I. C., and Davis, R. J. (1989) J. Biol. Chem. 264, 10828–10835
32. Kanety, H., Feinstein, R., Papa, M. Z., Hemi, R., and Karasik, A. (1995) J. Biol. Chem. 270, 23780–23784
33. Kanety, H., Hemi, R., Papa, M. Z., and Karasik, A. (1996) J. Biol. Chem. 271, 9895–9897
34. Feinstein, R., Kanety, H., Papa, M. Z., Lunenfeld, B., and Karasik, A. (1993) J. Biol. Chem. 268, 26055–26058
35. Hotamisligil, G., Murray, D., Choy, L., and Spiegelman, B. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 4854–4858
36. Hotamisligil, G., Peraldi, P., Budavari, A., Ellis, R., White, M., and Spiegelman, B. (1996) Science 271, 665–668
37. Peraldi, P., Hotamisligil, G. S., Buurman, W. A., White, M. F., and Spiegelman, B. M. (1996) J. Biol. Chem. 271, 13018–13022
38. Mothe, I., and Van Obberghen, E. (1996) J. Biol. Chem. 271, 11222–11227
Brain-derived Neurotrophic Factor Binding to the p75 Neurotrophin Receptor Reduces TrkA Signaling While Increasing Serine Phosphorylation in the TrkA Intracellular Domain

Ian J. MacPhee and Philip A. Barker

J. Biol. Chem. 1997, 272:23547-23551.
doi: 10.1074/jbc.272.38.23547

Access the most updated version of this article at http://www.jbc.org/content/272/38/23547

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 38 references, 21 of which can be accessed free at http://www.jbc.org/content/272/38/23547.full.html#ref-list-1