Differential Utilization of Trk Autophosphorylation Sites*

(Rosalind A. Segal†, Anita Bhattacharyya†, Lori A. Ruan†, John A. Albertas§, Robert M. Stephens†, David R. Kaplan, and Charles D. Stiles‡)

Dana-Farber Cancer Institute Dept. of Health and Human Services under Contract No. N01-CO-74101 with ABL (to R.M.S. and D.R.K.).

†From the Department of Neurology, Harvard Medical School and the Beth Israel Hospital, Boston, the Department of Microbiology and Molecular Genetics, Harvard Medical School and the Dana-Farber Cancer Institute, Boston, Massachusetts 02115, and the ABL-Basic Research Program, Frederick Cancer Research and Development Center, Frederick, Maryland 21702

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The neurotrophins are a family of soluble peptide factors that can trigger a wide diversity of biological responses in susceptible cells, including proliferation, commitment, differentiation, and survival (1, 2). The primary receptor for NGF is TrkA, and TrkB for BDNF primarily mediated by the Trk family of receptor tyrosine kinases (3–5). Binding of the appropriate ligand induces or stabilizes a dimeric conformation of Trk receptor subunits (7). Within the ligand-receptor complex, Trk receptor subunits phosphorylate each other in-trans on specific tyrosine residues. Tyrosine autophosphorylation is required for both the catalytic and signaling activities of the Trk receptors (8–14).

Within the TrkA catalytic (SH1) domain, three tyrosines (Tyr-670, Tyr-674, and Tyr-675) have been identified as po-
tential phosphorylation sites based on phosphopeptide analysis (12, 17, 18). The phosphorylation state of Tyr-674 within this three-tyrosine cluster may be especially significant. This tyro-

sine is the positionally equivalent of Tyr-1162 within the insulin receptor β subunit. Crystallographic analysis of the insulin receptor β subunit suggests that phosphorylation of Tyr-1162 and its positionally equivalent in other receptor tyrosine kinases could be the initial event in receptor activation (19).

Outside the catalytic domain, three putative phosphorylation sites have been identified. One of these, Tyr-490 in the juxtamembrane domain of TrkA, is an NPXY motif. Tyrosine to phenylalanine substitutions at this site indicate that tyrosine (and, by implication, tyrosine phosphorylation) at this site is critical for Shc binding and hence for activation of the Ras-MAP kinase signaling cascade (10, 12). Another site in the carboxy-
terminal tail of TrkA, Tyr-785, has been shown by mutational analysis to play a functional role in binding phospholipase Cγ to activated NGF receptors (8, 12). A third site at Tyr-751 is a YXXM motif and thus a candidate recognition site for the SH2 adapter protein p85 that is a component of the phosphatidy-
inositol 3-kinase (10). However, phosphorylation at Tyr-751 has not been detected in TrkA overexpression systems that do detect phosphorylation at Tyr-490 and Tyr-785 (12, 18).

It is important to note that the tyrosine autophosphorylation events outlined above have not been observed or analyzed in normal neurons. Since Trks are expressed at low abundance levels in neurons, key autophosphorylation sites have been identified by radioactive phosphopeptide mapping of receptors isolated from non-neuronal cells genetically engineered to over-
express Trk protein. Biological functions of these autophospho-
ylation sites have been confirmed by mutational analysis in established cell lines. This approach has several limitations. First, radioactive labeling is a stress that can, in and of itself, alter the pattern of phosphorylation. Second, overexpression of a tyrosine kinase in systems such as baculovirus can lead to nonphysiological phosphorylation events. Third, mutational analysis demonstrates that a designated tyrosine is important for proper functioning of the Trk receptor; however, it does not prove that the site is phosphorylated in vivo. Finally, it is not known whether autophosphorylation sites identified in this manner are used in actual neurons.

We have used an alternative approach (20, 21) to study the tyrosine phosphorylation of Trks. Using synthetic tyrosine phosphopeptides as immunogens, we generated a panel of antibo-
dies that report the phosphorylation state of individual tyrosines common to the cytoplasmic domain of the Trk family of neurotrophin receptors. We targeted pY-674 and pY-675 in

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the catalytic domain. As a representative signal generating tyrosine, we targeted pY-490 in the Shc binding site. Using this approach we determined that the phosphorylation state of individual tyrosine sites is not coordinately controlled following ligand binding. The differential time course of the individual phosphorylation state changes has functional ramifications for neurotrophin-mediated signal transduction in neurons.

EXPERIMENTAL PROCEDURES

Generation of Antibodies—Tyrosine-phosphorylated peptides, corresponding to predicted phosphorylation sites on the Trk receptors (12, 17, 18), were synthesized via TBOC chemistry on an ABI 430A peptide synthesizer using an ABI PAM resin. In this chemistry, coupled residues are protected on their α amino group by an N-tert-butyloxycarbonyl group. The N-tert-butyloxycarbonyl group is stable to alkali and nucleophiles but is rapidly removed by acid. Therefore, deprotection after each coupling step is carried out by exposing the growing peptide strand to trifluoroacetic acid. Following synthesis, cleavage from the resin with concomitant removal of side-chain protecting groups was carried out in a mixture of trifluoroethane sulfonyl fluoride, trifluoroacetic acid, m-cresol, and dimethyl sulfoxide. The peptides were then precipitated with diethyl ether and purified by reverse phase high performance liquid chromatography on a C18 column. Amino acid analysis on an ABI 130A Separation System in tandem with an ABI 420A Derivitizer, and MALDI-TOF (matrix-assisted laser desorption ionization time of flight) mass spectrometry on a Finngan Mat Lasermat. The peptides were desalted using a Sep-Pak C18 column and lyophilized overnight. An NH2-terminal cysteine was incorporated into each coupling site for immunization purposes.

The phosphorylated peptides were coupled to keyhole limpet hemocyanin (Pierce) and used to immunize rabbits. Antiserum were screened on protein extracts from NGF-stimulated Trk-PC12 cells (22). Reactive antisera were then purified by sequential affinity chromatography and the immunoglobulin fraction was purified on a protein A-Sepharose column (Pharmacia Biotech Inc.) and eluted with 0.1 M glycine, pH 2.2. The immunoglobulin fraction was then applied to a peptide affinity column, containing the corresponding unphosphorylated peptide coupled to Affi-Gel (Bio-Rad). The effluent was collected and used for further purification. The effluent was applied to a keyhole limpet hemocyanin Affi-Gel affinity column, and the second effluent was collected. In the case of the pY-674/pY-675 antibody, the second effluent was then applied to a phosphotyramine Affi-Gel affinity column, to remove general phosphotyrosine antibodies, and the effluent was again collected. The final effluent was then applied to an affinity column containing the phosphopeptide immunogen. The column was washed sequentially with 100 mM Tris, pH 8.0, and then 10 mM Tris, pH 8.0, 0.5 M NaCl, and then eluted with gentle Wash elution buffer (ß-lactoglobulin). Bovine serum albumin was added to the eluted antibody, and the purified antibody was dialyzed against Tris-buffered saline and concentrated by centrifugation through a Centriprep-10 (Amicon Inc., Beverly, MA). A Fluorimager apparatus (Molecular Dynamics, Sunnyvale, CA) was used to quantitate the IgG in the purified antibody preparations by immunoblot analysis and eluted for use by avidin. Therefore, phosphorylation state changes of the full-length Trk receptors, rabbits were immunized with two different antigen preparations.

Equal amounts of protein extracts from untransfected Trk-A, B, and C (22). The pan-Trk antibody was used at 1:200. A monoclonal antibody to phosphotyrosine was used at 1:7,000 (27). Blots were extensively washed in TBST and then incubated with alkaline phosphatase- or horseradish peroxidase-linked secondary antibodies according to standard procedures. Blots were visualized with enhanced chemiluminescence (Amersham Corp.), with the alkaline phosphatase substrate nitro blue tetrazolium/5-bromo-1-chloro-3-indolyolphosphate, or with a fluorescent alkaline phosphatase substrate. For quantitation, blots were developed with Attophos, a fluorescent alkaline phosphatase substrate (BL Scientific, San Luis Bisca, CA), and visualized and quantitated with a Fluorimag apparatus (Molecular Dynamics, Sunnyvale, CA). In immunoblotting experiments, the Fluorimag apparatus performs volume integration of the signal from the Attophos substrate. The quantitation of the signal is linear over three logarithms of applied protein.

Immunoprecipitation—Protein extracts were incubated with primary antibody (pY-674/pY-675 was used at 1:50 and pY-490 at 1:100) for 2 h at 4°C and then precipitated with protein A-Sepharose (Pharmacia) according to standard procedures (12, 22). The immunoprecipitates were washed three times with lysis buffer, twice with 20 mM Tris, pH 8.0, 1 M LiCl, twice with 20 mM Tris, pH 8.0, and then solubilized in sample buffer and run on SDS-PAGE. The immunoprecipitates were visualized by immunoblotting with anti-Trk, anti-pan Trk, or anti-phosphotyrosine, or the activation state-specific antibodies as indicated.

Peptide Competition—Phosphorylated and unphosphorylated peptides were synthesized as described above. Peptides were solubilized in water, and the concentration was calculated based on the OD at 260 nm for phosphorylated peptides and at 280 nm for unphosphorylated peptides. For immunoblots, peptides at 10 or 100 μM were incubated with antibody at the appropriate concentration in TBST for 30 min at room temperature. The antibody/peptide mixture was then used in place of primary antibody.

In Vitro Kinase Assay—Protein extracts of PC12 cells were immunoprecipitated with an antibody to the carboxy-terminal tail of Trk (22) as described above, washed twice with lysis buffer, once with 20 mM Tris + 1.0 M LiCl, and once with kinase buffer (10 mM Tris, pH 7.4, 10 mM MgCl2, 0.5 mM ATP). The immunoprecipitated samples were then incubated for 15 min at room temperature with 25 μl of kinase buffer + 2 μl of [γ-32P]ATP (3000 Ci/mmol from DuPont NEN). The final concentration of ATP was 260 μM. The samples were then precipitated; the supernatant was removed, and the immunoprecipitated proteins were analyzed by SDS-PAGE. The bands were visualized and quantitated using the Phosphimager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Antibody Targeting—Rabbits were immunized with synthetic phosphopeptides (12-14-mers) corresponding to three tyrosine autophosphorylation motifs common to the Trk family of receptor tyrosine kinases. These are shown schematically in Fig. 1. To generate antibodies to pY-674 and pY-675 in the catalytic domain, rabbits were immunized with two different peptides. One peptide corresponded to the bisphosphorylated state (pY-674/pY-675) and another corresponded to the monophosphorylated state (pY-674/pY-675). The third targeted tyrosine was pY-490, the recognition site for Shc. Rabbit antisera was collected and purified by affinity chromatography and reverse affinity chromatography procedures as described under "Experimental Procedures."
Phosphorylation State Specificity and Positional Specificity of an Antibody to the Shc Site—As shown in Fig. 2A, the pY-490 antibody recognizes TrkA protein from NGF-stimulated TrkA overexpressing PC12 cells, but not from unstimulated cells, indicating that pY-490 is an activation state-specific antibody. In some experiments, the pY-490 displays a weak immunoblot signal from unstimulated cell lysates. This probably reflects the fact that Trk PC12 cells have a low level of constitutively activated TrkA (22). Specificity of the pY-490 antibody was documented in two independent ways. First, we used a panel of synthetic peptides to compete with the antibodies. When the pY-490 antibody is preincubated with an excess of the phosphopeptide immunogen, no signal is observed. However, the corresponding unphosphorylated peptide does not compete, indicating that the antibody is specific for the phosphorylated sequence. As a control for the sequence specificity, the pY-490 antibody was preincubated with tyrosine phosphopeptides corresponding to other positions on TrkA. None of the other TrkA phosphopeptides competed with the pY-490 antibody. As a final control for sequence specificity we used a peptide containing a functionally homologous Shc binding motif, the NPY motif from the unrelated receptor tyrosine kinase, c-ErbB2 (sequence: CAENPEpYGLDVPV). This ErbB2 phosphopeptide did not compete. These experiments with PC12 demonstrate that the pY-490 is an activation and sequence-specific antibody to Trk receptors.

Positional specificity of the pY-490 antibody was independently validated by mutational analysis (Fig. 2B). Baculovirus expression vectors were used to generate insect cell lysates containing recombinant Trk proteins. In this insect cell expression system, recombinant Trk proteins are constitutively activated (22). As a positive control for the pY-490 antibody, we used the wild type TrkA. As a negative control, we used recombinant TrkA that had been mutated to ablate its tyrosine kinase activity. To establish positional specificity of the pY-490 antibody, we used mutated TrkA with tyrosine to phenylalanine substitutions at Tyr-490, Tyr-674, Tyr-675, and/or Tyr-785 as indicated (12). As shown, the pY-490 antibody recognizes wild type TrkA but not kinase-inactive TrkA. The pY-490 antibody also recognizes mutated TrkA with tyrosine to phenylalanine substitutions at any position other than Tyr-490. However, this antibody will not recognize any mutated TrkA with a substitution at Tyr-490. Mutations at Tyr-490 have no effect on the ability of TrkA to bind ligand and to become activated as a tyrosine kinase (12). Accordingly the pY-490 antibody detects the phosphorylation state of tyrosine at a single position in activated TrkA.

As shown in Fig. 2B, the pY-490 antibody recognizes activated TrkB and TrkC as well as TrkA. Peptide competition experiments using cell lysates from unstimulated or BDNF-stimulated TrkB expressing 3T3 cells (data not shown) indicate that the pY-490 antibody recognizes TrkB with positional specificity and activation state-specificity comparable with that demonstrated for TrkA. This is an expected result since the peptide used for immunization and competition studies was actually derived from the TrkB sequence data.

Phosphorylation State Specificity and Positional Specificity of Antibodies to Tyrosines in the Catalytic Domain—Similar methods were used to document the phosphorylation state and positional specificity of antibodies to the double phosphotyrosine motif pY-674/pY-675 that is located within the catalytic domain of the Trks. As shown in Fig. 3A, this antibody specifically recognizes activated TrkA from NGF-stimulated Trk-PC12 cells. The bisphosphorylated peptide used as an immunogen competes for antibody but the corresponding unphosphorylated peptide does not. The positional specificity of this antibody is further demonstrated by competition experiments with the monophosphorylated peptides pY-674/pY-675 and Y-674/pY-675. As shown (Fig. 3A) this antibody strongly prefers the bisphosphorylated motif pY-674/pY-675 over either
of the monophosphorylated peptides. Mutated Trk receptors produced by the baculovirus expression system were used to document further the specificity of the pY-674/pY-675 antibody. As shown in Fig. 3B, the antibody detects activated wild type TrkA. Tyr to phenylalanine substitutions at positions 674 and 675 abolished antibody recognition. Mutations at other positions were without effect.

A second antibody made to the monophosphorylated motif, pY-674/pY-675, recognizes both the mono and bisphosphorylated forms. As shown in Fig. 3C, the antibody to pY-674/pY-675 specifically recognizes Trk receptors of stimulated, but not unstimulated, Trk overexpressing PC12 cells. Both the monophosphorylated and bisphosphorylated peptides compete with the antibody, but the unphosphorylated peptide does not. These experiments indicate that the two antibodies we have generated recognize Y→F substitutions as indicated were analyzed by SDS-PAGE and immunoblotted with the pY-674/pY-675 antibody. The antibody was used alone or competed with the phosphopeptide immunogen, the corresponding unphosphorylated peptide, the monophosphorylated pY-674/pY-675, or Y-674/pY-675 peptide as indicated. All peptides were used at 10 nM concentration. As shown, the antibody strongly prefers the bisphosphorylated form of the catalytic domain site of Trk-A, B, and C. We targeted for antibody production are exactly identical in the antibody, but the unphosphorylated peptide does not.

The two catalytic domain autophosphorylation motifs that we targeted for antibody production are exactly identical in TrkA, B, and C (see Fig. 1) (16). Accordingly, we would expect these antibodies to react with TrkB and TrkC with activation state and position specificity comparable with that shown for TrkA in these studies. Immunoprecipitation and peptide competition experiments using cell lysates from TrkB expressing 3T3 cells indicate that this is the case (data not shown).

Sequential Phosphorylation at Distinct Tyrosine Residues Following Activation of Trk Receptors—To explore the sequence of tyrosine phosphorylation during Trk activation, we used TrkA overexpressing PC12 cells. TrkA phosphorylation in response to NGF occurs over a prolonged time in these cells, so that differences in the kinetics of phosphorylation of individual sites can be resolved (22). Trk-PC12 cells were stimulated by NGF for 30 s to 1 h and then analyzed by immunoblot analysis using each of the activation-specific antibodies. As shown in Fig. 4), phosphorylation state of the individual tyrosines is differentially regulated following exposure to NGF. Tyrosine 674 within the SH1 domain acquires phosphate prior to the Shc binding site at Tyr-490. Furthermore, phosphorylation at Tyr-490 is more persistent than phosphorylation at Tyr-674. The pY-674/pY-675 antibody showed a pattern of phosphorylation similar to that seen with pY-674 and distinct from that seen with pY-490 (Fig. 4 and Table I).

The experiment depicted in Fig. 4 was repeated a total of seven times. The intensity of the phospho-TrkA signals as a function of time was quantitated using the Fluorimag (see “Experimental Procedures”). Time to peak phosphorylation at Tyr-674 was used as an internal frame of reference for phosphorylation at targeted positions. The two catalytic domain autophosphorylation motifs that we targeted for antibody production are exactly identical in TrkA, B, and C (see Fig. 1) (16). Accordingly, we would expect

\[ \text{Phosphatase Inhibitors Stabilize Phosphorylation at Activation Loop Tyrosines—} \]

To determine the mechanism(s) that underlie the differential phosphorylation of tyrosine residues, we analyzed the effect of the tyrosine phosphatase inhibitor orthovanadate on Trk phosphorylation. As shown in Fig. 5, the

**Antibodies to the catalytic domain sites (Tyr-674 and Tyr-674/Tyr-675) are specific for phosphorylation state and position.** A, the pY-674/pY-675 antibody is specific for the bisphosphorylated Trk receptor. Protein extracts of Trk-PC12 cells, stimulated with 50 ng/ml NGF for 5 min (+), or control, unstimulated cells (−), were electrophoresed and immunoblotted with pY-674/pY-675 antibody. The antibody was used alone or competed with the phosphopeptide immunogen, the corresponding unphosphorylated peptide, the monophosphorylated pY-674/pY-675, or Y-674/pY-675 peptide as indicated. All peptides were used at 10 nM concentration. As shown, the antibody strongly prefers the bisphosphorylated form of the catalytic domain site of Trk-A, B, and C. Supernatants from the catalytic domain site, Tyr-674 and Tyr-675. The antibody can recognize Y→F substitutions at the corresponding unphosphorylated peptide, the monophosphorylated pY-674/pY-675, or Y-674/pY-675 peptide as indicated. The two catalytic domain autophosphorylation motifs that we targeted for antibody production are exactly identical in TrkA, B, and C (see Fig. 1) (16). Accordingly, we would expect...
Non-coordinate Phosphorylation of Trk Receptors

TABLE I
Noncoordinate phosphorylation of TrkA tyrosines following exposure to NGF

| Antibody | Time to maximum phosphorylation (relative units) | p value of difference (vs. pY-674) |
|----------|-----------------------------------------------|----------------------------------|
| pY-674   | 1.00                                          |                                  |
| pY-674/pY-675 | 1.71 (±0.38)          | 0.11                            |
| pY-490   | 4.91 (±1.51)                          | 0.04                            |

The NGF stimulation experiment depicted in Fig. 4 was repeated a total of seven times. The intensity of the phospho TrkA signals as a function of time was quantified using the Fluorimagier (“Experimental Procedures”). Time to peak phosphorylation at Tyr-674 was used as an internal frame of reference for phosphorylation at other positions.

time course of phosphorylation of the kinase domain Tyr-674/675 sites in response to NGF is dramatically altered by vanadate. Rather than transient phosphorylation noted in control cells, vanadate-treated cells display sustained phosphorylation at these positions following exposure to NGF. In contrast, the duration of phosphorylation at the Shc binding site Tyr-490 is not affected by vanadate. Collectively, these data suggest that the transient nature of phosphorylation at Tyr-674/Tyr-675 reflects tyrosine phosphatase activity and that Tyr-490 is less vulnerable to these phosphatases. Perhaps the binding of Shc to the phosphotyrosine at Tyr-490 protects this phosphotyrosine from phosphatases and allows for sustained phosphorylation at this signal generating site.

We also observed that vanadate seemed to have a small effect on the accumulation of tyrosine phosphate. At both activation loop sites and at Tyr-490, phosphorylation is slightly retarded by vanadate at early times following NGF stimulation. The basis of this effect is unclear.

Phosphorylation at Tyr-674/Tyr-675 in the Trk “Activation Loop” Correlates with Kinase Activity—Recent x-ray crystallographic analysis of a receptor tyrosine kinase suggests that the phosphorylation state of Tyr-674 within the SH1 domain could regulate catalytic activity of the Trk tyrosine kinase (19) (see “Discussion”). As shown already (Fig. 4) phosphorylation at Tyr-674 is the earliest detectable autophosphorylation event in NGF-treated Trk PC12 cells. In the experiment depicted in Fig. 6, we correlated activity of the Trk tyrosine kinase with the phosphorylation state of individual tyrosines in the Trk cytoplasmic domain. For these experiments we used PC12 cells rather than Trk-PC12 cells because background activity in the in vitro kinase assay was lower with the wild type cell line. Since NGF induces TrkA phosphorylation more rapidly in wild type PC12 cell than in Trk-PC12 cells, we could not resolve differences between the phosphorylation at catalytic domain sites (pY-674/pY-675) and the Shc binding site (pY-490) at early times after NGF stimulation. However, differences can be seen at the later time points. As noted previously with Trk-PC12 cells, phosphorylation at Tyr-490 is more persistent than phosphorylation at Tyr-674/Tyr-675 in PC12 cells.

As shown (Fig. 6), catalytic activity of the Trk tyrosine kinase in vitro correlates very well with the phosphorylation state of tyrosines 674 and 675 in the Trk activation loop. In contrast, the phosphorylation state of Tyr-490 is not in close accord with Trk catalytic activity. As noted under “Discussion,” the close coincidence of phosphorylation at Tyr-674 and Tyr-675 with Trk catalytic activity supports a role for these tyrosines in regulating catalytic activity of the receptor.

Tyrosines 490, 674, and 675 Are Autophosphorylated in Primary Nerve Cell Cultures—Which, if any, of the Trk autophosphorylation sites identified thus far are used in normal neurons? The sensitivity of the phosphotyrosine-directed antibodies allows us to detect activated receptors in 50 μg of protein lysate, which corresponds to approximately 2 × 10^5 cells. Accordingly, this question can now be addressed in primary neuronal cultures, which are difficult to grow in large quantities. We prepared primary cell cultures of mouse cerebellar granule neurons. These cells express TrkB, the receptor for BDNF (26, 28). As shown (Fig. 7), each of the three antibodies in our panel recognized TrkB in immunoblots of BDNF-treated cerebellar neuron cultures but not of control cultures. Thus, the three positional equivalents of Tyr-490 and Tyr-674 in TrkB (Tyr-515 and -705, respectively) are utilized as autophosphorylation sites in cultured primary neurons. The Tyr-675 positional equivalent (Tyr-706) is probably used as well in the primary neurons. However, the fact that the pY-674/pY-675 antibody recognizes the Tyr-674 monophosphorylated form, albeit with lower affinity than the bisphosphorylated form (Fig. 3), sheds some ambiguity on this interpretation.

DISCUSSION

A distinguishing characteristic of the Trk receptors is that they have a small cytoplasmic domain, with few potential sites for tyrosine phosphorylation (16). For comparison, the receptor for platelet-derived growth factor has 26 potential tyrosine phosphorylation sites (29), whereas TrkA has only 10 (16). Thus, the Trks are ideal receptors for analyzing individual tyrosine phosphorylation events. We have used a new approach to analyze Trk autophosphorylation sites. The method is sensitive and nonisotopic. It allows us to resolve the sequence of ligand-induced autophosphorylation events at high resolution. Our data establish that tyrosines within the Trk catalytic domain and a site in the juxtamembrane domain are phosphorylated in primary neurons in response to ligand binding. Finally, we show that the individual tyrosines are not coordinately phosphorylated.

In response to receptor activation, phosphorylation state changes within the kinase domain precede those within the Shc binding site. The sequence of phosphorylation appears to be an intrinsic property of Trk receptor. Previous studies have demonstrated that the level and duration of Trk phosphorylation vary as a function of receptor number (22) and the presence of the p75 neurotrophin receptor (30–32). However, we have observed similar patterns of phosphorylation in wild type PC12 cells that express p75 and low levels of TrkA (Fig. 6), in PC12 cells genetically engineered to express a 20-fold excess of Trk (Fig. 4), and in 3T3 cells that express TrkA but not the p75 receptor (data not shown).

How is this non-coordinate phosphorylation pattern achieved? In principle, differential utilization of tyrosine residues by the Trk tyrosine kinase or differential utilization of phosphotyrosine residues by cellular phosphatases are possible. Our data suggest that both differential kinase and differential phosphatase activity account for the observed pattern. Studies on a closely related receptor tyrosine kinase, the insulin receptor β subunit, suggest that differential utilization of tyrosines 674/675 as substrate contributes to the non-coordinate phosphorylation. In studies using synthetic peptides as substrates for a soluble form of the insulin receptor, phosphorylation of Tyr-1162 was found to proceed to completion before phosphorylation of Tyr-1163 began (33). Tyr-1162 and Tyr-1163 in the insulin receptor β subunit correspond to 674 and 675 in TrkA (34). By analogy, differential utilization of tyrosine residues by the Trk receptor tyrosine kinase is likely to account for our data showing that phosphorylation of Tyr-674 proceeds to completion in vivo before other sites are fully phosphorylated.

Substrate preferences of the Trk receptor kinase do not fully account for the non-coordinate pattern of phosphorylation state changes. We used the tyrosine phosphatase inhibitor orthovanadate to investigate the role of phosphatases in the
observed pattern. As shown in Figs. 4 and 5, while the activation loop sites are rapidly dephosphorylated after stimulation, phosphorylation at the Shc binding site persists for a longer time. This rapid dephosphorylation of the kinase domain sites can be prevented by vanadate (Fig. 5), suggesting that specific tyrosine phosphatases preferentially dephosphorylate the kinase domain sites.

The sequence in which the kinase domain sites and the Shc binding site are phosphorylated may explain the mechanism by which Trk kinase activity is induced following ligand binding. As shown in Fig. 6, the phosphorylation state of the kinase domain sites Tyr-674/Tyr-675 tracks closely with tyrosine kinase activity. This correlation can be interpreted in the context of crystallographic analysis of a related receptor tyrosine kinase. Crystallographic analysis of the insulin receptor β subunit places Tyr-1162 (the positional equivalent of Tyr-674 in TrkA) within a flexible activation loop of amino acids lying near the cleft between the amino-terminal, ATP binding, and the carboxyl-terminal, substrate binding lobes of the kinase (19).

Fig. 5. Phosphatase inhibitors synchronize phosphorylation of individual tyrosines. Extracts of Trk-PC12 cells were stimulated with 50 ng/ml NGF for the times indicated in the presence (+ Vanadate) or absence (− Vanadate) of 1 mM vanadate, analyzed by SDS-PAGE, and immunoblotted with pY-490 and pY-674/pY-675. For each data point 50 μg of protein were loaded.

Fig. 6. Tyrosine phosphorylation at the catalytic domain sites correlates with kinase activation. Protein extracts of PC12 cells stimulated with 50 ng/ml NGF for the indicated times were immunoprecipitated with pY-490 and then immunoblotted with pY-490. Parallel samples were immunoprecipitated with pY-674/pY-675 and then immunoblotted with pY-674/pY-675. An inset shows the immunoblots from one such experiment. The bands were quantitated with the Fluorimager, and the maximal response for each antibody was taken as 1.0. The values in the graph represent the average of two experiments. Similarly prepared extracts of PC12 cells stimulated with 50 ng/ml NGF for the indicated times were immunoprecipitated with anti-Trk antibody and used for an in vitro kinase assay as described under “Experimental Procedures.” The radioactively labeled bands corresponding to phosphorylated Trk were quantitated with the PhosphorImager. Maximal response was designated 1.0.

Fig. 7. Activation-specific antibodies recognize BDNF-activated TrkB receptors in primary neurons. Primary cultures of cerebellar cells from P5 mice were cultured in serum-free medium for 2 days and then stimulated for 5 min with 50 ng/ml BDNF (+) or vehicle control (−). Protein extracts (50 μg/lane, corresponding to approximately 2 × 10⁶ cells) were analyzed by SDS-PAGE and immunoblotted with the activation-specific antibodies, or with anti-phosphotyrosine (pY) as indicated. The positions of molecular mass markers are indicated.
viation loop exists in a thermodynamic equilibrium between two conformations. When Tyr-1162 is phosphorylated, the preferred conformation of the activation loop exposes the ATP and substrate binding sites. When Tyr-1164 is dephosphorylated, the preferred conformation of the activation loop occludes the ATP and substrate binding sites, and so decreases enzymatic activity. This model for ligand-induced tyrosine kinase activation predicts that phosphorylation at the critical tyrosines in the activation loop (Tyr-1162/Tyr-1163 for insulin receptor and the corresponding Tyr-674/Tyr-675 for TrkA) would be the initial event in ligand-induced receptor activation and that phosphorylation state of these critical tyrosines would correspond with the level of tyrosine kinase activity. Our data, presented in Figs. 4 and 6 and Table I, show that this is indeed the case. The relationship between phosphorylation state of Tyr-674/Tyr-675 and Trk enzyme activity also sheds light on previous studies with the Trk oncogene. Amino acid substitutions at Tyr-674 or Tyr-675 in the oncogene decreased transforming capacity and in vitro kinase activity (35). Phosphorylation at the Tyr-490 site does not correlate with kinase activity (Fig. 6). Instead, the prolonged phosphorylation at the Shc binding sites provides an explanation for neurotrophin-dependent sustained activation of Ras, MEK (MAP kinase/Erk kinase), and MAP kinase (36–38), which are downstream of Shc on a critical signal transduction pathway (13, 39, 40). It has been proposed that transient activation of the Ras/MAP kinase pathway is mitogenic, while sustained activation has a differentiating effect on PC12 cells (37, 40, 41). We demonstrate that the duration of activation of this pathway reflects prolonged phosphorylation of a specific site on the Trk receptor. Thus the kinetics of MAP kinase activation reflects intrinsic properties of the receptors themselves.

Why should Trk receptors have prolonged tyrosine phosphorylation of signaling sites, such as the Shc binding site? Perhaps this reflects the special requirements of signal transduction within a neuron. Target-derived neurotrophins bind to and activate Trk receptors present on nerve terminals. However, many cellular responses including survival and neurite elongation require changes in gene expression. Prolonged phosphorylation at sites critical for regulating gene expression may enable the signal to be conveyed along the distance of an axon.

Acknowledgments—We thank Dr. Andrew Welcher, Amgen Inc., for generously providing us with recombinant BDNF. We thank Michael Greenberg and Thomas Roberts for helpful comments. In compliance with Harvard Medical School guidelines on possible conflict of interest, we disclose that one of the authors (C. D. S.) has consulting relationships with Upstate Biotechnology and Sandoz Pharmaceuticals Inc.

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