Identification of Trk Binding Sites for SHC and Phosphatidylinositol 3'-Kinase and Formation of a Multimeric Signaling Complex*

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Phosphotyrosine-containing synthetic peptides were used to identify the binding sites for cellular polypeptides involved in nerve growth factor receptor/Trk-mediated signal transduction. In vitro association of SHC and the p85 subunit of phosphatidylinositol 3'-kinase with the Trk tyrosine kinase was prevented only by phosphorylated Y-490- and Y-751-containing peptides, respectively. In spite of the close proximity of the p85 binding site to that of phospholipase Cγ (Y-785), both target proteins are able to interact with the same receptor molecule simultaneously.

Tyrosine phosphorylation-mediated cellular signals are generated by extracellular interaction of receptor tyrosine kinase (RTK)1 binding domains with specific ligands, followed by dimerization and autophosphorylation of tyrosine residues within the intracellular signaling domain. This triggers the recruitment of src homology 2 (SH2) domain-containing proteins to specific phosphotyrosines (pY) and leads to the assembly of these primary signal transfer factors at the inner face of the plasma membrane in proximity to molecules involved in subsequent steps of signal transduction (Cantley et al., 1991; Koch et al., 1991; Schlessinger and Ullrich, 1992). This initial interaction between the RTK cytoplasmic domain and cellular substrates involves amino acid sequences flanking the receptor pY docking sites and the variant residues within the SH2 consensus motif of the substrate, which together define the binding affinity and thereby the specificity of the signal (Koch et al., 1991).

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Antibodies—Antibodies against the human EGF-R extracellular domain (108.1), phosphotyrosine (SE2), and the COOH termini of Trk, PLCγ, GAP, and p85 have been previously described (Herbst et al., 1992; Obermeier et al., 1993). Additionally, an oSHC polyclonal rabbit antibody, raised against the COOH-terminal 282 amino acids of SHC fused to glutathione S-transferase (Smith and Johnson, 1988), was employed.

Peptides—The (phospho-)peptides were synthesized as described in Kitas et al. (1989) and analyzed by ion spray mass spectrometry. For nerve growth factor receptor (NGF-R) Trk, phospholipase Cγ (PLCγ) and the noncatalytic p85 subunit of phosphatidylinositol (PI) 3'-kinase have been described to be direct tyrosine kinase substrates (Soltoff et al., 1992; Obermeier et al., 1993). Moreover, SHC, an oncopgenic SH2 domain-containing molecule (Pellici et al., 1992), was recently implicated in linking Trk to the p21ras signaling pathway (Rozakis-Adcock et al., 1992), which had been shown previously to be essential for NGF-induced neurite formation by PC12 cells (Bar-Sagi and Feramisco, 1985; Hagag et al., 1986).

Here we show that p85 and SHC specifically bind to phosphotyrosine residues at positions 751 and 490, respectively, of the Trk cytoplasmic domain sequence. The sequence flanking Tyr-751 contains the YXXM consensus motif for p85 association, while Tyr-490, located in the Trk juxtamembrane domain, represents the first receptor binding site to be described for SHC. Moreover, we demonstrate that p85 and PLCγ can bind simultaneously to the Trk cytoplasmic domain in vitro, in spite of very close proximity of their specific binding sites.

MATERIALS AND METHODS

Expression Plasmids—The construction of the chimeric receptor ET-R was described previously (Obermeier et al., 1993). ET-Y490F and ET-Y751F were generated by oligonucleotide-directed mutagenesis of ET-R with the 23-mer 5'-GAAACCACATTCTCTGATG-3' and the 20-mer 5'-CAGAGCTTCCGATCG-3', respectively. This was performed directly in the pCMV-1 expression vector employing the Double TakeTM System (Stratagene) according to the manufacturer's protocol. The epidermal growth factor receptor (EGF-R) was expressed using pCMV-1-HERc, containing the full-length cDNA of the human EGF-R. For the expression of the substrates PLCγ, p85, and SHC, their cDNA sequences were subcloned into pCMV-1.

Cell Culture, Transient Expression, and in Vitro Association—Human embryonic kidney fibroblasts (293; ATCC CRL 1573) were grown in transfected essentially as described previously (Obermeier et al., 1993). In vivo association experiments, lysates of either receptor or substrate overexpressing 293 cells were mixed, and relevant proteins were immunoprecipitated and analyzed as previously described (Obermeier et al., 1993). In order to reprobe proteins with a second antibody, filters were incubated for 1 h at 50 °C in strip buffer (62.5 mM Tris, pH 6.8, 100 mM β-mercaptoethanol, 2% SDS). Expression levels of different receptors were monitored by Coomassie staining. Aliquots of the lysates from receptor-over-expressing 293 cells were immunoprecipitated as described above, the precipitates fractionated by 7.5% SDS-PAGE, fixed 1 h in 40% methanol/10% acetic acid, stained 20 min in 0.025% Coomassie G250, destained in 10% acetic acid, and the gels dried.

Expression levels of different receptors were monitored by Coomassie staining. Aliquots of the lysates from receptor-over-expressing 293 cells were immunoprecipitated as described above, the precipitates fractionated by 7.5% SDS-PAGE, fixed 1 h in 40% methanol/10% acetic acid, stained 20 min in 0.025% Coomassie G250, destained in 10% acetic acid, and the gels dried.

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neutralization with hydrochloric acid was necessary. Addition of -1% v/v of concentrated triethylamine and immediate receptor association analysis, peptides were dissolved in lysis buffer at a concentration of 10 μM. For solubilization of Y-751, pY-585 and pY-695, addition of ~1% v/v of concentrated triethylamine and immediate neutralization with hydrochloric acid was necessary.

RESULTS

To analyze the molecular basis of the pleiotrophic cellular signal, which through the activation of the NGF-R/Trk tyrosine kinase leads to the formation of neurites by primary neurons in the peripheral and central nervous systems, we employed an in vitro assay that allows sensitive measurements of receptor-target protein interactions. In our system, reaction components such as a receptor tyrosine kinase and SH2 domain-containing substrates were separately overexpressed in 293 human embryonic fibroblasts and, after mixing of individual cell extracts, association between the components was determined by immunoprecipitation with anti-receptor antibody and immunoblotting with respective substrate antisera.

To determine the high affinity binding sites of two SH2 domain-containing proteins, the regulatory subunit of PI 3'-kinase, p85, and SHC, a series of peptides were synthesized, which corresponded to all but one of the 11 tyrosine residues of the Trk receptor cytoplasmic domain flanked by 3–6 amino acids on each side (Table I). The peptide containing Tyr-634 was omitted because of the hydrophobic nature of surrounding amino acids. Phosphorylated tyrosine-containing Trk peptides and nonphosphorylated controls were then examined for their ability to prevent the in vitro association between the autophosphorylated Trk cytoplasmic domain and p85 or SHC substrates.

Subconfluent human 293 cells were transfected with cytomegalovirus-based expression plasmids encoding either the juxtamembrane domain of Trk, a region previously suggested to the receptor was determined (Fig. 2). Both phosphopeptides and nonphosphorylated peptide, and association between receptor and substrate was determined by immunoprecipitation with the anti-NGF-R extracellular domain monoclonal antibody 108.1 (Honegger et al., 1989), polyacrylamide gel electrophoresis, and immunoblot analysis with anti-p85 or SHC antibodies. As shown in Fig. 1, A and B, both p85 and SHC specifically associated with the activated autophosphorylated receptor (lane 1), but not with nonphosphorylated ET-R (lane 2). Coprecipitation of p85 and SHC with ligand-activated ET-R was prevented by Trk phosphotyrosine peptides pY-751 (Fig. 1A, lane 13) and pY-490 (Fig. 1B, lane 3), respectively. In contrast, all other phosphopeptides were ineffective.

These data strongly suggest that p85 and SHC specifically bind to Trk via pY-751 and -490, respectively. While Tyr-751 is the most COOH-terminal tyrosine residue of the kinase core domain, Tyr-490 is the only tyrosine located in the cytoplasmic juxtamembrane domain of Trk, a region previously suggested to be involved in defining RTK-specific properties (Yarden and Ullrich, 1988).

To further characterize the interaction of p85 and SHC to the Trk cytoplasmic domain, the concentration dependence of pY-751 and pY-490 peptide competition with p85 and SHC binding to the receptor was determined (Fig. 2). Both phosphopeptides displayed similar inhibition properties, with p85 binding to ET-R being reduced to ~10–20% by a 10 μM concentration of pY-751 and pY-490 reducing SHC binding to about 50% at the same concentration. At 50 μM pY-490, less than 10% of SHC associated with the chimeric ET-R receptor. No further decrease in SHC binding was observed when all phosphopeptides (50 μM) were added to the same reaction mixture. In each case, the respective nonphosphorylated peptide had no inhibitory effect, even at a concentration of 1 μM (Fig. 2, lanes 9 and 10).

Our findings were confirmed by testing ET-R mutants with phenylalanine in place of Tyr-490 and -751 (ET-Y490F and ET-Y751F).

Table I

| Sequences of Trk phosphopeptides |
|---------------------------------|
| pY-490                          |
| pY-585                          |
| pY-670                          |
| pY-674                          |
| pY-675                          |
| ppY-674/675                     |
| pY-695                          |
| pY-717                          |
| ppY-717/723                     |
| pY-751                          |
| pY-785                          |
| IENPQpYFSDA                     |
| LMVFpYpYMHRGD                   |
| MSRDPysySTD                     |
| STDpYpYVGRG                     |
| STDpYpYVGGGR                    |
| PESILpYpKFFT                    |
| WIEITpYpKGKP                     |
| KGRPpYpYQLSNN                   |
| WIEITpYpKGKFpYpQYLSNT          |
| PFEVpYAIRG                     |
| QAFFPpYLDVLG                    |

FIG. 1. Association of p85 (A) and SHC (B) with ET-R and specific inhibition by phosphopeptides. Lysates of either EGF-stimulated (ET-R+) or unstimulated (ET-R−) ET-R chimera-expressing 293 cells (3 × 10^6 cells) were mixed with lysates of 293 cells overexpressing p85 (5 × 10^6 cells) (A) or SHC (8 × 10^6 cells) (B). Where indicated, phosphopeptides (Table I) were added to a final concentration of 50 μM before immunoprecipitation with anti-receptor antibody (108.1). Precipitates were subjected to SDS-PAGE and immunoblotted with p85- (A) or SHC (B) antiserum.

FIG. 2. Inhibition of p85 (A) and SHC (B) association with ET-R by pY-751/pY-751 (A) and pY-490/pY-490 (B). Lysates of transfected 293 cells overexpressing either autophosphorylated (ET-R+) or nonphosphorylated receptors (ET-R−) were mixed with lysates of cells overexpressing p85 (A) or SHC (B). Phosphopeptides or nonphosphorylated peptides were added to the indicated concentrations before immunoprecipitation with anti-receptor antibody (108.1). Precipitates were subjected to SDS-PAGE and immunoblotted with p85- (A) or SHC (B) antiserum.
ET-Y751F), together with ET-R and EGF-R, for their binding affinities to p85 and SHC (Fig. 3). Lysates of transfected 293 ce
ceptors ET-Y751F), together with ET-R and EGF-R, for their binding ET-Y490F failed almost completely to bind SHC, and the asso-
binds with very high affinity to tyrosine 785 (Obermeier both ET-Y490F and ET-Y751F retained full binding capacity for experimental conditions. Moreover, in comparison with ET-R, both ET-Y490F and ET-Y751F retained full binding capacity for p85 and SHC, respectively.

Since, as previously reported, another Trk substrate, PLCγ, binds with very high affinity to tyrosine 785 (Obermeier et al., 1993), only 34 amino acids downstream from the p85 binding site, we explored whether the two substrates compete for this region of the Trk cytoplasmic domain or whether they may be able to bind simultaneously to the same receptor molecule. Lysates from cells overexpressing p85, PLCγ, or ET-R were mixed, followed by immunoprecipitation with α-PLCγ-antibody. Aliquots of the precipitates were then analyzed by SDS-PAGE and immunoblotting with either α-PLCγ, αTrk, or αop85 antibody. As shown in Fig. 4, EGF-stimulated and therefore -auto-

![Fig. 3. Association of p85 (A) and SHC (B) with EGF-R, ET-R, and ET-YF mutants. Lysates containing equal amounts of autophosphorylated (+) or nonphosphorylated (−) receptors were mixed with p85 (A) or SHC lysates (B), and receptors were immunoprecipitated with anti-receptor antibody (108.1). Precipitates were subjected to SDSA-PAGE and immunoblotted with αp55 (A) or αSHC (B) antisera (upper panels) and reprobed with anti-phosphotyrosine antibody (αPY; lower panels). pCMV-1 denotes a control, where lysates of cells, transfected with only the expression vector and further treated like cells producing autophosphorylated receptors, were mixed with substrate containing lysates.](image)

![Fig. 4. Simultaneous binding of PLCγ and p85 to ET-R. Lysates containing either EGF-stimulated (+) or unstimulated (−) ET-R (5 × 10⁶ cells), p85 (1 × 10⁶ cells), and PLCγ (2 × 10⁶ cells) were mixed and precipitated with α-PLCγ antiserum. 5% (lanes 1 and 2), 25% (lanes 3 and 4), and 70% (lanes 5 and 6) of the precipitates were subjected in parallel to SDS-PAGE, blotted onto nitrocellulose, and probed with α-PLCγ, αTrk, or αop85 antiserum, respectively.](image)

nearly stoichiometric association between endogenous sub-
strate and ectopically expressed receptor (not shown), in con-
junction with our observation that the mutant receptor ET-
Y751F lacking the PLCγ binding site did not bind higher amounts of p85 than ET-R (Fig. 3), strongly indicated that the formation of a p85- and PLCγ-containing signaling complex was not mediated by separate ET-R molecules but by a single receptor within an ET-R dimer.

**DISCUSSION**

Receptors with intrinsic tyrosine kinase activity mediate the diverse functions attributed to their cognate ligands by pre-
senting autophosphorylated tyrosine residues as binding sites for SH2 domain-containing cellular substrates, which repre-
sent primary transducer molecules for a specific signal (Schlessinger and Ullrich, 1992). While this principle is well established and appears to be of general significance, the mechanisms and molecular parameters that define the signal's cell type- and receptor-specific characteristics are still poorly understood.

One of the most interesting and still unresolved issues in signal transduction is the apparent utilization of overlapping sets of substrates by different RTKs to mediate distinct cellular responses like cell proliferation and differentiation. This is ex-amplified by pheochromocytoma-derived PC12 cells, for which EGF is a weak mitogen and which are stimulated by NGF to differentiate. Both the EGF-R and the NGF-R (Trk) bind and phosphorylate PLCγ, GAP, p85, and SHC (Margolis et al., 1989, 1990a; 1990b; Vetter et al., 1991; Hu et al., 1992; Soltoff et al., 1992; Rotin et al., 1992; Obermeier et al., 1993; Pellieci et al., 1992; Rozakis-Adcock et al., 1992) and may also interact with additional, possibly specific, as yet unidentified SH2 domain-containing proteins. The specificity of the EGF- and NGF-mediated responses may be defined by such far unknown substrates, or, alternatively, specificity may be a manifestation of quantitative differences such as substrate binding affinity for distinct binding sites within the cytoplasmic domains of respective receptors.

To investigate the latter possibility, we determined the Trk tyrosine kinase binding sites for the p85 subunit of PI 3'-kinase and SHC, two signal transducer proteins that do not display any apparent enzymatic activity but seem to function as allo-
steric adapter polypeptides for one or possibly more catalytically active cellular factors. This is better described for p85, which mediates the engagement of the p110 catalytic subunit of PI 3'-kinase (Otsu et al., 1991) and has been suggested to play a role in Trk signaling (Raffioni and Bradshaw, 1992; Soltoff et al., 1992). While the phosphotyrosine docking sites for all SH2-containing tyrosine kinase substrates mapped so far were found to be located in hydrophilic COOH-terminal tail and kinase insertion sequence domains of various RTKs, our results localize the p85 binding site to Tyr-751 in the COOH-terminal
region of the kinase core domain and that of SHC to Tyr-490 in the juxtamembrane domain of Trk (Fig. 5). Together with the recently identified platelet-derived growth factor receptor binding sites for Src family kinases (Mori et al., 1993), Trk Tyr-490 is one of the first sites identified in the hydrophilic juxtamembrane region, whose sequence was found to be conserved in RTK subfamily members and had therefore been predicted to be involved in definition of sub-class-characteristic signaling properties (Yarden and Ullrich, 1988).

Upon comparison of the in vitro binding properties of EGF-R and Trk for p85 and SHC (Fig. 3), we found that p85 binds with higher affinity to EGF-R than to Trk, while SHC displayed respective tyrosine residues in intact cells. In contrast, our findings suggest a role for SHC in signaling functions that are similar for Trk and EGF-R. In addition to the receptor-characteristic binding affinity, the location of a substrate binding site in the cytoplasmic domain of a receptor may have significance for the interaction with secondary signal transducer molecules and may therefore contribute to signal definition. The multiplicity of substrate interaction sites within spatially separated RTK subdomains, shown for Trk in Fig. 5, indicates the possibility that the RTK and its substrates form signal transfer complexes containing more than one polypeptide bound to the receptor. Such an organized multienzyme complex, which we proposed earlier (Ullrich and Schlessinger, 1990), could include membrane-associated signaling factors such as myristoylated Src-like kinases and farnesylated Ras, as well as cytoplasmic proteins that are recruited by the activated RTK. Our in vitro association experiments with p85 and PLCγ (Fig. 4) demonstrate simultaneous binding of these factors, with binding sites that are separated by only 33 amino acids, to the same receptor and thereby suggest the possibility of a multimeric complex consisting of Trk, PLCγ, and the p85 subunit of PI 3'-kinase mediating the binding of the p110 catalytic subunit. Although we cannot exclude the possibility of an in vitro artifact that may not be relevant for intact cells, our findings provide important insights into the spatial parameters defining SH2 domain/phosphotyrosine interactions.

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FIG. 5. Substrate binding sites within the Trk cytoplasmic domain. Receptor subdomains are indicated. TM, transmembrane domain; JM, juxtamembrane domain; TK, tyrosine kinase domain; CT, C-tail.