Two Signaling Molecules Share a Phosphotyrosine-Containing Binding Site in the Platelet-Derived Growth Factor Receptor

R. NISHIMURA,¹ W. LI,¹ A. KASHISHIAN,² A. MONDINO,¹ M. ZHOU,¹ J. COOPER,²
AND J. SCHLESSINGER^{1*}

Department of Pharmacology, New York University Medical Center, 550 First Avenue, New York, New York 10016,¹ and Fred Hutchinson Cancer Research Center, Seattle, Washington 98104²

Received 26 May 1993/Returned for modification 12 July 1993/Accepted 27 July 1993

Autophosphorylation sites of growth factor receptors with tyrosine kinase activity function as specific binding sites for Src homology 2 (SH2) domains of signaling molecules. This interaction appears to be a crucial step in a mechanism by which receptor tyrosine kinases relay signals to downstream signaling pathways. Nck is a widely expressed protein consisting exclusively of SH2 and SH3 domains, the overexpression of which causes cell transformation. It has been shown that various growth factors stimulate the phosphorylation of Nck and its association with autophosphorylated growth factor receptors. A panel of platelet-derived growth factor (PDGF) receptor mutations at tyrosine residues has been used to identify the Nck binding site. Here we show that mutation at Tyr-751 of the PDGF β -receptor eliminates Nck binding both in vitro and in living cells. Moreover, the Y751F PDGF receptor mutant failed to mediate PDGF-stimulated phosphorylation of Nck in intact cells. A phosphorylated Tyr-751 is also required for binding of phosphatidylinositol-3 kinase to the PDGF receptor. Hence, the SH2 domains of p85 and Nck share a binding site in the PDGF receptor. Competition experiments with different phosphopeptides derived from the PDGF receptor suggest that binding of Nck and p85 is influenced by different residues around Tyr-751. Thus, a single tyrosine autophosphorylation site is able to link the PDGF receptor to two distinct SH2 domain-containing signaling molecules.

Autophosphorylation of growth factor receptors with protein tyrosine kinase activity is an immediate event that occurs upon ligand binding. Accumulating evidence indicates that receptor autophosphorylation has at least two functions. For some receptors, such as insulin and insulin-like growth factor 1 (IGF1) receptors, tyrosine autophosphorylation enhances the intrinsic kinase activity (for a review, see reference 41). However, certain autophosphorylation sites in growth factor receptors and in cytoplasmic tyrosine-phosphorylated proteins function as binding sites for signaling molecules containing Src homology 2 (SH2) domains, such as phospholipase C- γ (PLC- γ) *ras*-GTPase-activating protein (GAP), and phosphatidylinositol-3 (PI-3) kinase (for reviews, see references 18, 24, 30, and 35). The phosphorylated tyrosine residues and their flanking amino acids determine both the specificity and affinity of the receptor-SH2 protein interactions. Site-directed mutagenesis studies of receptor autophosphorylation sites have resulted in the identification of binding sites for a number of SH2 domain-containing signaling molecules. It has been shown that PLC- γ binds to phosphorylated tyrosine Y-992, Y-1068, and Y-1173 residues of the epidermal growth factor (EGF) receptor (33), tyrosine Y-766 of the fibroblast growth factor (FGF) receptor (28), and Y-1021 and Y-1009 of the platelet-derived growth factor (PDGF) β -receptor (12, 32, 42). Other proteins bind to other phosphorylation sites. GAP binds to phosphorylated Y-771, and the p85 subunit of PI-3 kinase binds to both phosphorylated Y-740 and Y-751 of the PDGF receptor (PDGFR) (8, 13).

The interaction between tyrosine-phosphorylated receptors and the SH2 domains of signaling molecules appears to be important in regulating the function of these signaling proteins. It has been shown that replacement of Y-766 of the

FGF receptor with phenylalanine (Y766F) abolishes PLC- γ binding to the FGF receptor, its tyrosine phosphorylation, and its activation in response to FGF treatment (27, 31). Recent studies indicate that binding of p85 to the tyrosine-phosphorylated insulin receptor substrate 1 activates PI-3 kinase (2).

SH2 domains are structural modules composed of approximately 100 amino acids (for reviews, see references 24, 30, and 35). SH2 domains have been identified in a wide range of molecules, which can be divided into two classes. One class contains proteins with enzymatic or other known functional elements and includes cytoplasmic tyrosine kinases such as the *src* product, PLC- γ , GAP, protein tyrosine phosphatase, the transcription factor interferon-stimulated gene factor 3 (ISGF-3), and the product of the putative GDP-GTP exchanger *vav* (for reviews, see references 24, 30, and 35). Proteins in the second class lack any known catalytic domain and are composed virtually of SH2 and SH3 domains. Members of this group include the Crk protein, GRB2/Sem-5, and Nck. It is thought that these proteins function as adaptors, using their SH2 and SH3 domains to link receptor tyrosine kinases to a downstream effector molecule(s). The functional importance of SH3 domains was suggested by their regulatory role in *src* and *abl* transforming activity (for reviews, see references 24 and 30), in the transforming ability of *v-crk* (25), and in the function of the product of the *sem-5* gene involved in vulval induction of *Caenorhabditis elegans* (5). Recent studies indicate that the SH3 domains of GRB2/Sem-5 function as independent and specific binding modules which are responsible for binding to a guanine nucleotide releasing factor (hSOS1) of *ras* (5, 20, 23, 34, 39).

Nck is a ubiquitously expressed protein consisting of one SH2 and three SH3 domains (19). Nck is phosphorylated on serine residues in quiescent cells and on serine, threonine, and tyrosine residues in response to a variety of extracellular stimuli, including EGF, PDGF, and nerve growth factor

* Corresponding author.

(NGF), and to stimulation of the immunoglobulin E receptor (FcεRI) on mast cells, as well as activation of T- and B-cell antigen receptors (4, 21, 26, 29). Upon growth factor stimulation, Nck associates with activated EGF and PDGF receptors via its SH2 domain (21). Overexpression of Nck protein leads to cell transformation and tumor formation in nude mice (4, 21). These studies indicate that Nck is an oncogenic protein and a common target for tyrosine and serine/threonine kinases. In order to gain further insight into the role of Nck in growth factor receptor signal transduction, we have identified the binding site of Nck on the human PDGF β-receptor and explored the role of Nck binding on PDGFR signaling. We present data demonstrating that Nck shares a binding site at Tyr-751 of the PDGFR with one of the SH2 domains of the p85 subunit of PI-3 kinase, indicating that a single mutation at an autophosphorylation site of a growth factor receptor can recruit at least two signaling molecules.

MATERIALS AND METHODS

Dog kidney epithelial cells (TRMP) expressing wild-type or mutant human PDGFRs were maintained as described previously (16, 17). Human recombinant PDGF-BB was purchased from INTERGEN (Purchase, N.Y.). Anti-PDGFR antibodies were used as described elsewhere (15). Polyclonal rabbit anti-human Nck antibodies were generated and used as previously described (21). Generation and production of glutathione *S*-transferase (GST) fusion proteins of Nck and p85 were as described elsewhere (11, 21). Iodine-125 (in dilute sodium hydroxide solution; specific activity; 17.4 mCi/μg) was from Amersham.

Iodination of GST-Nck fusion protein and denaturation of cell lysates. Purified GST-Nck was iodinated with iodine-125 by using Iodo-Gen iodination reagent (1,3,4,6-tetrachloro-3α-6α-diphenylglycoluril) (Pierce, Rockford, Ill.). A specific activity of 10⁴ cpm/ng of Nck protein was obtained. Cells were lysed in a small volume of lysis buffer (see below) containing additional 1% sodium dodecyl sulfate (SDS), heated to 95°C for 5 min, cooled on ice, and then diluted with the Triton X-100 lysis buffer (see below) so that the final concentration of SDS was 0.1%. The samples either were incubated with GST-p85 or GST-Nck fusion protein immobilized on agarose beads or were incubated with antibodies against Nck or p85.

Detection of Nck-PDGFR complex in lysates of PDGF-stimulated cells. TRMP cells were grown to 80% confluency in 6-cm-diameter tissue culture dishes and incubated in 0.5% serum for 16 h. Cells were then treated with or without human recombinant PDGF (400 ng/ml) for 5 min at 37°C, washed three times with ice-cold phosphate-buffered saline buffer, and solubilized in lysis buffer as described previously (21). The supernatants of the centrifuged cell lysates were incubated with anti-Nck antiserum for 3 h at 4°C, and the immunocomplexes were precipitated by incubation with protein A-Sepharose beads for 45 min at 4°C. The beads were washed three times with lysis buffer without bovine serum albumin and boiled in 1× electrophoresis sample buffer containing 0.5 M β-mercaptoethanol. The supernatants were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose membranes, and blotted with anti-PDGFR antibodies. The PDGFR was visualized by labeling with ¹²⁵I-protein A followed by autoradiography. The amount of PDGFR was ascertained by determining the radioactive content of the excised bands.

In vitro binding experiments with GST fusion proteins.

GST-Nck, GST-p85-2SH2, GST-p85-N'SH2, and GST-p85-C'SH2 fusion proteins were freshly prepared as described elsewhere (11, 21) for the in vitro binding experiments. Lysates of cells stimulated with or without PDGF were incubated with GST-Nck protein or GST-p85 proteins immobilized on glutathione agarose beads for 1 h at 4°C. In certain experiments, cell lysates were preincubated with soluble GST-Nck protein or GST-p85 proteins before the addition of GST fusion proteins. Accordingly, the beads were preincubated with phosphotyrosine peptides as competitors prior to the addition of cell lysates. The beads were then washed three times with cold lysis buffer and boiled in 1× sample buffer. The supernatants were analyzed by immunoblotting with anti-PDGFR antibodies as described above.

Phosphorylation of Nck protein in TRMP cells in response to PDGF. The tyrosine phosphorylation of Nck was examined by immunoblotting using antiphosphotyrosine (anti-PY) antibodies. Cell lysates were immunoprecipitated with anti-Nck antibodies (21). The samples were resolved by SDS-PAGE, and the proteins were transferred to nitrocellulose membranes and immunoblotted with anti-PY antibodies. The samples were visualized by labeling with ¹²⁵I-protein A followed by autoradiography. Phosphorylation of the Nck proteins was assessed by labeling cells with ³²P_i and then performing phosphoamino acid analysis of the ³²P-labeled Nck protein of stimulated or unstimulated cells (21, 22).

RESULTS

Direct binding of Nck via its SH2 domain to the PDGFR. We have previously shown that Nck coimmunoprecipitates with tyrosine-phosphorylated PDGFRs and that the SH2 domain of Nck is responsible for this association (21). However, these experiments did not determine whether Nck binds PDGFRs directly or associates via some other PDGFR-bound protein. In order to address this issue, PDGFRs were immunoprecipitated from lysates of unstimulated cells and cells stimulated with PDGF, resolved by SDS-PAGE, and transferred to nitrocellulose filters. Samples were blotted with either anti-PDGFR antibodies or ¹²⁵I-GST-Nck fusion proteins. Figure 1A shows that GST-Nck (lanes 1 and 2), but not GST alone (data not shown), binds to the PDGFR from stimulated (lane 1) but not from unstimulated (lane 2) cells. Equal amounts of PDGFRs from either PDGF-stimulated or PDGF-unstimulated cell lysates were present, as detected by blotting with anti-PDGFR antibodies followed by ¹²⁵I-protein A labeling and visualization by autoradiography (Fig. 1, lanes 3 and 4). Similar results were obtained with a GST fusion protein containing only the SH2 domain of Nck (data not shown). These results were further confirmed by analysis of denatured PDGFR. In this experiment, cell lysates were denatured by boiling for 5 min in the presence of 1% SDS, cooled on ice, and diluted with Triton X-100 lysis buffer (see Materials and Methods) to a final concentration of 0.1% SDS. These conditions dissociated the PDGFR from associated molecules, as revealed by immunoprecipitation with antibodies against Nck or p85 and assaying for tyrosine-autophosphorylated PDGFRs (data not shown). These lysates were incubated with GST-Nck fusion protein immobilized on agarose beads to test whether Nck was able to bind the denatured PDGFRs. The results presented in Fig. 1B clearly demonstrate that Nck is capable of binding to the denatured wild-type PDGFRs in lysates from stimulated (lane 2) but not unstimulated (lane 1)

FIG. 1. Nck protein binds the activated PDGFR directly in vitro. (A) PDGFR immunoprecipitated from lysates of either unstimulated (lanes 2 and 3) or PDGF-stimulated (lanes 1 and 4) TRMP cells was resolved by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with either ^{125} I-labeled Nck (^{125} I-NCK) (lanes 1 and 2) or anti-PDGFR antibodies (α PDGFR) followed by incubation with ^{125} I-protein A (lane 3 and 4). Protein bands were visualized by autoradiography. IP, immunoprecipitate. (B) The lysates of TRMP cells treated with PDGF-BB (400 ng/ml) or untreated were boiled for 5 min in the presence of 1% SDS (see Materials and Methods), cooled on ice, and diluted (1:10) with Triton X-100 lysis buffer (see Materials and Methods) prior to addition of GST-Nck beads. The beads were washed, boiled in sample buffer, and analyzed by immunoblotting using anti-PDGFR antibodies. WT, wild type. Mutant PDGFRs: 740, Y740F; 740/751, Y740/Y751F; 751, Y751F.

cells. However, Nck did not bind directly to either active or denatured EGF receptor (data not shown).

Phosphorylation on tyrosine 751 of the PDGFR is required for Nck binding. We next analyzed the binding site(s) for Nck on the human β type PDGFR using previously characterized dog kidney epithelial cells (TRMP) expressing either wild-type or various mutant PDGFRs (12–14). The point mutations of tyrosine (Y) to phenylalanine (F) of the PDGFR expressed in the cell lines include five previously identified autophosphorylation sites (Y740F, Y751F, Y771F, Y1009F, and Y1021F) and other tyrosine residues that are not known to be phosphorylated (Y716F, Y763F, Y778F, Y775F, Y857F, Y966F, and Y970F). Figure 2 summarizes the various PDGFR mutants used in this study and the established assignment of the binding sites for PI-3 kinase, GAP, and PLC- γ . We first screened these cell lines for association between the PDGFR and Nck protein by means of coimmunoprecipitation. Serum-starved cells were unstimulated or stimulated with PDGF-BB (400 ng/ml) and solubilized in lysis buffer. Cell lysates were immunoprecipitated with anti-Nck antibodies. The immunocomplexes were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and blotted with anti-PDGFR antibodies. Figure 3A shows the PDGFRs coimmunoprecipitated by anti-Nck antibodies from cells expressing either wild-type (lanes 1 and 2) or mutant (lanes 3 to 16) PDGFRs. This experiment clearly shows that wild-type, Y740F, Y771F, Y1009F, Y1021F, and

FIG. 2. Summary of mutations of human PDGFR and schematic representation of known autophosphorylation sites and their binding proteins. The tyrosines with asterisks are the previously identified phosphorylation sites. Phosphorylation of other tyrosine residues has not been determined.

Y1009/1021F PDGFRs were coimmunoprecipitated by anti-Nck antibodies. However, the PDGFRs with Y751F or Y740/751F mutations were not coimmunoprecipitated with the Nck protein (Fig. 3, lanes 5 to 8). Other point mutations listed in Fig. 2 had little effect on the coimmunoprecipitation of PDGFRs with Nck (Fig. 3B). These results indicate that phosphorylation of Tyr-751 in the kinase insert of the PDGFR is required for Nck binding.

To further explore the specificity of the interaction with the PDGFR, we carried out *in vitro* binding experiments using GST-Nck fusion proteins. Lysates of cells treated with or without PDGF were incubated with GST alone or with GST-Nck fusion proteins immobilized to glutathione-agarose beads. The beads were then washed to remove the unbound molecules, and the samples were analyzed by SDS-PAGE and immunoblotting using anti-PDGFR antibodies. As shown in Fig. 3C, the binding of PDGFR to GST-Nck is eliminated by mutations at Y740/751F (lanes 7 and 8) or Y751F (lanes 5 and 6), whereas other mutations (Y771F and Y1009/1021F) had no effect on the binding to GST-Nck. GST protein alone did not bind any of the PDGFRs (Fig. 3C, lanes 13 and 14). We also tested the binding of Nck to denatured PDGFR mutants (Fig. 1B). GST-Nck failed to bind to Y751F or Y740/751F mutant receptors (Fig. 1B, lanes 5 to 8) but did bind to wild-type and Y740F receptors (lanes 1 to 4). Thus, consistent with the coimmunoprecipitation results from lysates of living cells, the *in vitro* binding data clearly show that Nck protein binding requires phosphorylated Tyr-751 in the PDGFR.

To further test whether Nck binds directly to the phosphorylated Tyr-751 region of the receptor, we carried out competition experiments using synthetic phosphopeptides corresponding to the amino acid sequences around Y-751, Y-740, Y-771, and Y-1021 (Fig. 4B). Figure 4A shows that Nck binding to the PDGFR was inhibited by synthetic phosphopeptide Y-751(1) (lanes 3 to 5) but not by phosphopeptide Y-740 (lane 6), Y-771 (lane 7), or Y-1021 (lane 8). A 10-fold-lower concentration of Y-751 phosphopeptide also

FIG. 3. Phosphorylation of Y-751 is required for Nck binding. Lysates of cells which were treated with 400 ng of PDGF-BB per ml at 37°C for 5 min or untreated were either immunoprecipitated with anti-Nck antiserum (A and B) or incubated (total lysate, 100 μ g) with GST beads or GST-Nck beads (C). The immunocomplexes on the beads were washed and analyzed by immunoblotting using anti-PDGFR antibodies, followed by incubation with ^{125} I-protein A and autoradiography. This experiment was performed six times, and the apparent reduction in coimmunoprecipitation of Nck with the Y1009F and Y1021F mutants was not reproducible. WT, wild type. Numbers at the top of each panel represent the sites of the Y-to-F mutation.

inhibited the binding of p85 to PDGFR (Fig. 4A, lanes 11 to 13) to a similar degree (lane 12 versus lane 5). Interestingly, a shorter phosphopeptide [Y-751(2)] lacking N-terminal amino acid residues competed less efficiently for Nck binding (Fig. 4C, lanes 2 to 5) but still competed efficiently for p85 binding to the PDGFR (data not shown). Phosphopeptide Y-740 had no inhibitory effect (Fig. 4A). These results indicate that the YMXM motif, which determines the binding specificity of p85, is not sufficient for Nck binding. It is therefore possible that phosphorylated Tyr-751 together with C-terminal residues of the PDGFR represent the binding site for p85, whereas Tyr-751 with N-terminal residues is crucial for Nck binding.

Nck competes with the N-terminal SH2 domain of p85 for binding to Y-751 of the PDGFR. It has been previously shown that the p85 subunit of PI-3 kinase binds via its two SH2 domains to the human PDGFR at tyrosines 740 and 751 (8, 13) and that p85 binds to Y-740 with higher affinity than it binds to Y-751 (13). Therefore, we set up experiments to test whether the SH2 domains of p85 and Nck compete for binding to activated PDGFRs. Lysates of PDGF-stimulated cells were preincubated with GST alone or with GST-p85 fusion proteins containing both SH2 (p85-2SH2) domains, the C-terminal SH2 (p85-C'SH2) domain alone, or the N-terminal SH2 (p85-N'SH2) domain alone for 1 h at 4°C prior to the addition of GST-Nck beads. To avoid the binding of the glutathione of the GST-Nck beads to the GST portion of GST-p85, GST-Nck beads were presaturated with purified GST protein alone. After incubation, the GST-Nck beads were washed, and the GST-Nck-bound PDGFRs were sub-

FIG. 4. Y-751 phosphopeptides inhibit Nck binding to the PDGFR. (A and C) GST-Nck or GST-p85 fusion protein attached to beads was preincubated with the phosphopeptides listed (B) at the indicated concentrations (Pept conc.) and then added to the lysates of either PDGF-stimulated or unstimulated TRMP cells expressing wild-type PDGFR. The peptide concentrations in panel C were 10 μ M (lanes 2 and 4) and 50 μ M (lanes 3 and 5), respectively. The GST-Nck or p85 fusion protein-bound PDGFRs were analyzed by immunoblotting followed by incubation with ^{125} I-labeled protein A and autoradiography. Y*, phosphorylated tyrosine.

jected to SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-PDGFR antibodies. The results presented in Fig. 5A show that GST-Nck binds to the PDGFR only from stimulated cells (lane 1 versus lane 2) and that the binding was not affected by preincubation with GST protein alone (lane 3). However, preincubation with p85-2SH2 dramatically reduced the binding of Nck to the PDGFR (Fig. 5A, lane 4). The inhibitory effect of p85-2SH2 was apparently due to the N-terminal SH2 domain of p85, since p85-N'SH2 (Fig. 5A, lane 6), but not p85-C'SH2 (lane 5), was able to prevent Nck binding. These results are consistent with the finding that the N-terminal SH2 domain of p85 binds mainly to Tyr-751 whereas the C-terminal SH2 binds mainly to Tyr-740 of the PDGFR. The competition with soluble GST-p85-SH2 domains was concentration dependent and specific for p85. As shown in Fig. 5B, approximately 100 nM GST-p85-2SH2 (lanes 3 to 6), 300 nM GST-p85-N'SH2 (lanes 11 to 14), and 600 nM GST-p85-C'SH2 (lanes 7 to 10) reduced Nck binding to the PDGFR by approximately 50%. GST-PLC- γ (lanes 15 to 18) or GST-GAP SH2 domains (data not shown) had no inhibitory effect on Nck binding. We have also performed experiments to examine inhibition of p85 binding with the soluble GST-Nck. Cell lysates were preincubated with GST-Nck protein prior to incubation with immobilized p85-2SH2, p85-C'SH2, or p85-N'SH2 fusion proteins. The beads were washed and analyzed by immunoblotting with anti-PDGFR antibodies. As shown in Fig. 5C, 1 μ M GST-Nck was able to inhibit binding to the PDGFR of p85-2SH2 (lane 3), p85-C'SH2 (lane 6), and p85-N'SH2 (lane 9), by 40, 40, and 70%, respectively. The competitive effect of GST-p85-C'SH2 for Nck binding (Fig. 5B) and of Nck for p85-C'SH2 binding (Fig. 5C, lane 6) may be due to steric hindrance between two

FIG. 5. p85 SH2 domains compete with Nck for PDGFR binding. (A) Lysates of cells expressing wild-type PDGFR treated in the presence or absence of 400 ng of PDGF per ml were preincubated with GST protein (250 nM), GST-p85-2SH2 (125 nM), GST-p85-C'SH2 (250 nM), or GST-p85-N'SH2 (250 nM) prior to the addition of GST-Nck fusion protein (10 nM) beads. (B) The lysates were preincubated with different concentrations (Conc.) of the GST-p85 fusion protein (lanes 1 to 14) or GST-PLC-γ (lanes 15 to 18) prior to incubation with Nck beads. The labeled beads were washed and analyzed for the associated PDGFR by immunoblotting and autoradiography. (C) Lysates of PDGF-stimulated or unstimulated cells were preincubated with GST-Nck fusion protein (105 nM) prior to addition of p85-2SH2, p85-C'SH2, or p85-N'SH2 (10 nM) beads. The associated PDGFRs were analyzed by SDS-PAGE followed by immunoblotting and autoradiography.

proteins that bind to phosphorylated residues, which are separated by only 11 amino acid residues.

Mutation at Tyr-751 of the PDGFR abolishes PDGF-stimulated phosphorylation of Nck. Nck was shown to be phosphorylated on serine in quiescent cells and on serine, threonine, and tyrosine residues in response to a variety of extracellular signals (4, 21, 26, 29). To study the relationship between Nck binding to the PDGFR and phosphorylation of Nck, we examined tyrosine phosphorylation of Nck in cells expressing wild-type or mutant PDGFRs. Nck proteins were immunoprecipitated from the lysates of untreated cells and cells treated with PDGF, subjected to SDS-PAGE, and transferred to a nitrocellulose membrane. The tyrosine phosphorylation of Nck was examined by immunoblotting with anti-PY antibodies. Figure 6A shows that tyrosine phosphorylation of Nck was observed in cells expressing wild-type PDGFR (lane 1) and Y740F (lane 3), Y771F (lane 9), and Y1009F (lane 11) PDGFR mutants but not in cells expressing either the PDGFR with Y740/751F (lane 7) or that with Y751F (lane 5) in response to PDGF. We also examined the effect of the Y751F mutation on PDGF-stimulated phosphorylation of Nck in metabolically labeled cells. Cells labeled with $^{32}\text{P}_i$ were incubated in the presence or absence of PDGF, solubilized, and subjected to immunoprecipitation using anti-Nck antibodies followed by SDS-PAGE and autoradiography. The results presented in Fig. 6B show that PDGF stimulation leads to a twofold increase in the phosphorylation of Nck in cells expressing wild-type PDGFR (lanes 1 and 2) as well as Y740F, Y771F, and Y1009/1021F (data not shown) PDGFR mutants. However, phosphorylation of Nck in cells expressing the Y751F PDGFR mutant was not stimulated in response to PDGF (lanes 3 and 4). Phosphoamino acid analysis showed that Nck is phosphorylated on serine only in unstimulated cells and that increased phosphorylation on serine and tyrosine residues was observed in cells expressing wild-type PDGFR but not in cells expressing the Y751F PDGFR mutant. Taken together, these results demonstrate that the Y751F mutation abolishes binding of Nck to the PDGFR and also its capacity to undergo PDGF-dependent increases in phosphorylation.

DISCUSSION

Several laboratories have recently shown that Nck is a target for a variety of cell surface receptors (4, 21, 26, 29). The fact that overexpression of Nck causes cellular transformation and tumor formation in nude mice suggests that it plays a role in the control of cell proliferation. We have recently shown that GRB2, which contains SH2 and SH3 domains, binds to the activated EGF receptor via its SH2 domain and to the guanine nucleotide releasing factor hSOS1 via its SH3 domains, thus linking receptor tyrosine kinases to *ras* signaling (5, 20, 23, 34, 39). Because of its structural similarity to GRB2, it is likely that Nck functions also as an adaptor protein that links receptor tyrosine kinases to downstream effector proteins with the SH2 domain of Nck binding to the activated PDGFR while its SH3 domains bind to downstream signaling protein(s) (Fig. 7). In this report, we show that Nck binds directly to the activated PDGFR receptor and that phosphorylation of Tyr-751 is required for the association between the PDGFR and Nck. Moreover, the association of Nck with the PDGFR is required for PDGF-induced tyrosine and serine phosphorylation of Nck. Previous studies identified Tyr-751 as one of the two binding sites for the SH2 domains of p85 (8, 13). In accord with this finding, we show that Nck and the amino-terminal SH2 domain of p85 compete for binding to the PDGFR. Our study shows that two signaling proteins can share a tyrosine phosphorylation site on a growth factor receptor.

The importance of the kinase insert region in PDGF α- and β-receptor-mediated signaling has been addressed in several studies (1, 7, 10, 16, 36, 37, 46). Similar studies were also done with the colony-stimulating factor 1 (CSF-1) receptor in macrophage cell lines (38). On the basis of mutations made at the PI-3 kinase-Nck binding site, it was proposed that PDGF-induced PI-3 kinase is crucial for PDGFR-mediated mitogenic signaling (8, 43). Our study indicates that any defects which are associated with a mutation at Tyr-751 are not necessarily due to elimination of the binding and activation of PI-3 kinase, because the binding of the oncogenic protein Nck is also decreased.

Replacement of tyrosine residues in the FGF and PDGF receptors that are required for binding PLC-γ also prevents

FIG. 6. Mutation at Y-751 eliminates PDGF-stimulated phosphorylation of Nck. TRMP cells expressing either wild-type or PDGF β -receptor mutants were either unlabeled (A) or labeled with $^{32}\text{P}_i$ (B) and stimulated in the presence or absence of 400 ng of PDGF per ml for 5 min at 37°C. Lysates of the cells were immunoprecipitated by using anti-Nck antibodies. (A) The immunocomplexes from unlabeled cells were analyzed by SDS-PAGE and immunoblotting with anti-PY antibodies. Molecular weight (in thousands) is indicated at the left. (B) The ^{32}P -labeled samples were analyzed by SDS-PAGE and autoradiography. (C) The Nck bands shown in panel B were excised and subjected to phosphoamino acid analysis as described previously (21). S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine; WT, wild type; numbers at the top of panels A and B represent the sites of the Y-to-F mutation.

the tyrosine phosphorylation and activation of PLC- γ , indicating that PLC- γ must associate with the autophosphorylated FGF or PDGF receptor for activation (27, 28, 31, 32, 42). Consistent with this notion, we have shown that elimination of Nck binding to the PDGFR prevents PDGF-stimulated tyrosine phosphorylation of Nck. This suggests that binding of Nck to the PDGFR is required for Nck protein to be phosphorylated.

The specificity of the receptor-signaling molecule interactions has been demonstrated for phosphopeptide sequences and for SH2 domains (for a review, see reference 35). The SH2 domains of p85 bind to a YXXM motif found in the PDGF and CSF-1 receptors, middle T antigen, and insulin receptor substrate 1 (for reviews, see references 3 and 35).

FIG. 7. A schematic model for the interaction of Nck and PI-3 kinase with the PDGF receptor. The p85 subunit of PI-3 kinase links the p110 catalytic subunit (not shown here) to the PDGFR by binding to phosphorylated Tyr-740 and Tyr-751. The binding sites are composed of the phosphotyrosine moiety and the immediate C-terminal residues (YXXM). It is proposed that the SH2 domain of Nck binds to phosphorylated Tyr-751 together with the immediate N-terminal residues. Tyr-771 functions as a binding site for GAP, and Tyr-1009 and Tyr-1021 function as binding sites for PLC- γ .

Interestingly, although Nck and the N-terminal SH2 domain of p85 share the binding site Tyr-751 in the PDGF β -receptor, their binding specificity appears to be determined by different flanking amino acid residues. It is noteworthy that the phosphopeptides used so far in quantitative binding experiments with various SH2 domains and for the analysis of the structure of the complexed SH2 domains were biased towards amino acid residues that are C terminal to the phosphotyrosine moiety (6, 9, 40, 44, 45). The results presented in this report demonstrate that amino acids that are N terminal to the phosphotyrosine moiety can also contribute to the specificity of SH2 domain binding. Moreover, a single phosphotyrosine moiety may provide an anchor to two different SH2 domains that recognize different features of the sequence surrounding the phosphotyrosine.

ACKNOWLEDGMENTS

We thank P. Hu (Da-JiJi), E. Skolnik, and T. Spivak-Kroizman for critical reading of the manuscript and K. Nelson for technical assistance.

This work was supported by grants from SUGEN (J.S.), HFSP (J.S.), and NIH (grant CA54786 to J.C.).

The first two authors contributed equally to this work.

REFERENCES

- Arridsson, A.-K., C.-H. Heldin, and L. Claesson-Welsh. 1992. Transduction of circular membrane ruffling by the platelet-derived growth factor β -receptor is dependent on its kinase insert. *Cell Growth Differ.* 3:881-887.
- Backer, J. M., M. G. Myers, Jr., S. E. Shoelson, O. J. Chin, X.-J. Sun, M. Miralpeix, P. Hu, B. Margolis, E. Y. Skolnik, J. Schlessinger, and M. F. White. 1992. Phosphatidylinositol 3' kinase is activated by association with IRS1 during insulin

- stimulation. *EMBO J.* 11:3469–3479.
3. Cantley, L. C., K. R. Auger, C. Carpenter, B. Duckworth, A. Graziani, R. Kapeller, and S. Soltoff. 1991. Oncogenes and signal transduction. *Cell* 64:281–302.
4. Chou, M. M., J. E. Fajardo, and H. Hanafusa. 1992. The SH2- and SH3-containing Nck protein transforms mammalian fibroblasts in the absence of elevated phosphotyrosine levels. *Mol. Cell. Biol.* 12:5834–5842.
5. Clark, S. G., M. J. Stern, and H. R. Horvitz. 1992. *C. elegans* cell-signaling gene *sem-5* encodes a protein with SH2 and SH3 domains. *Nature (London)* 356:340–344.
6. Eck, M. J., S. E. Shoelson, and S. C. Harrison. 1993. Recognition of a high affinity phosphotyrosyl peptide by the Src homology-2 domain of p56^{lck}. *Nature (London)* 362:87–91.
7. Escobedo, J. A., and L. T. Williams. 1988. A PDGF receptor domain essential for mitogenesis but not for many other responses to PDGF. *Nature (London)* 335:85–87.
8. Fantl, W. J., J. A. Escobedo, G. A. Martin, C. W. Turck, M. del Rosario, F. McCormick, and L. T. Williams. 1992. Distinct phosphotyrosines on a growth factor receptor bind to specific molecules that mediate different signaling pathways. *Cell* 69:413–423.
9. Felder, S., M. Zhou, P. Hu, J. Ureña, A. Ullrich, M. Chaudhuri, M. White, S. E. Shoelson, and J. Schlessinger. 1993. SH2 domains exhibit high-affinity binding to tyrosine-phosphorylated peptides yet also exhibit rapid dissociation and exchange. *Mol. Cell. Biol.* 13:1449–1455.
10. Heidaran, M. A., J. H. Pierce, D. Lombardi, M. Ruggiero, J. S. Gutkind, T. Matsui, and S. A. Aaronson. 1991. Deletion or substitution within the α platelet-derived growth factor receptor kinase insert domain: effects on functional coupling with intracellular signaling pathways. *Mol. Cell. Biol.* 11:134–142.
11. Hu, P., B. Margolis, E. Y. Skolnik, R. Lammers, A. Ullrich, and J. Schlessinger. 1992. Interaction of phosphatidylinositol 3-kinase-associated p85 with epidermal growth factor and platelet-derived growth factor receptors. *Mol. Cell. Biol.* 12:981–990.
12. Kashishian, A., and J. A. Cooper. 1993. Phosphorylation sites in the C-terminus of the platelet-derived growth factor receptor bind phospholipase C γ 1. *Mol. Biol. Cell* 4:49–51.
13. Kashishian, A., A. Kazlauskas, and J. A. Cooper. 1992. Phosphorylation sites in the PDGF receptor with different specificities for binding GAP and PI3 kinase *in vivo*. *EMBO J.* 11:1373–1382.
14. Kazlauskas, A., and J. A. Cooper. 1989. Autophosphorylation of the PDGF receptor in the region regulated interactions with cell proteins. *Cell* 58:1121–1133.
15. Kazlauskas, A., and J. A. Cooper. 1991. Phosphorylation of the PDGF receptor β subunit creates a tight binding site for phosphatidylinositol 3 kinase. *EMBO J.* 9:3279–3286.
16. Kazlauskas, A., D. L. Durden, and J. A. Cooper. 1991. Functions of the major tyrosine phosphorylation site of the PDGF receptor β subunit. *Cell Regul.* 2:413–425.
17. Kazlauskas, A., A. Kashishian, J. A. Cooper, and M. Valius. 1992. GTPase-activating protein and phosphatidylinositol 3-kinase bind to distinct regions of the platelet-derived growth factor receptor β subunit. *Mol. Cell. Biol.* 12:2534–2544.
18. Koch, C. A., D. Anderson, M. F. Moran, C. Ellis, and T. Pawson. 1991. SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. *Science* 252:669–674.
19. Lehmann, J. M., G. Riethmuller, and J. P. Johnson. 1990. Nck, a melanoma cDNA encoding a cytoplasmic protein consisting of the src homology units SH2 and SH3. *Nucleic Acids Res.* 18:1048.
20. Li, N., A. Batzer, R. Daly, V. Yalnik, E. Y. Skolnik, P. Chardin, D. Bar-Sagi, B. Margolis, and J. Schlessinger. 1993. The guanine nucleotide releasing factor, hSOS1, binds to GRB2 linking receptor tyrosine kinase to *ras* signaling. *Nature (London)* 363:85–88.
21. Li, W., P. Hu, E. Y. Skolnik, A. Ullrich, and J. Schlessinger. 1992. The SH2 and SH3 domain-containing Nck protein is oncogenic and a common target for phosphorylation by different surface receptors. *Mol. Cell. Biol.* 12:5824–5833.
22. Li, W., Y. G. Yeung, and E. R. Stanley. 1991. Tyrosine phosphorylation of a common 57-kDa protein in growth factor-stimulated and -transformed cells. *J. Biol. Chem.* 266:6808–6814.
23. Lowenstein, E. J., R. J. Daly, A. G. Batzer, W. Li, B. Margolis, R. Lammers, A. Ullrich, and J. Schlessinger. 1992. The SH2 and SH3-containing protein GRB2 links receptor tyrosine kinases to *ras* signaling. *Cell* 70:431–442.
24. Mayer, B. J., and D. Baltimore. 1993. Signaling through SH2 and SH3 domains. *Trends Cell Biol.* 3:8–13.
25. Mayer, B. J., and H. Hanafusa. 1990. Mutagenic analysis of the *v-crk* oncogene; requirement for SH2 and SH3 domains and correlation between increased cellular phosphotyrosine and transformation. *J. Virol.* 64:3581–3589.
26. Meisenhelder, J., and T. Hunter. 1992. The SH2/SH3 domain-containing protein Nck is recognized by certain anti-phospholipase C- γ 1 monoclonal antibodies, and its phosphorylation on tyrosine is stimulated by platelet-derived growth factor and epidermal growth factor treatment. *Mol. Cell. Biol.* 12:5843–5856.
27. Mohammadi, M., C. A. Dionne, W. Li, N. Li, T. Spivak, A. M. Honegger, M. Laye, and J. Schlessinger. 1992. A point mutation at tyr 766 of FGF receptor eliminates FGF-induced tyrosine phosphorylation of PLC γ and phosphatidylinositol hydrolysis without affecting mitogenesis. *Nature (London)* 358:681–684.
28. Mohammadi, M., A. M. Honegger, D. Rotin, R. Fischer, F. Bellot, W. Li, C. A. Dionne, M. Jaye, M. Rubinstein, and J. Schlessinger. 1991. A tyrosine-phosphorylated carboxy-terminal peptide of the fibroblast growth factor receptor (Fgf) is a binding site for the SH2 domain of phospholipase C- γ 1. *Mol. Cell. Biol.* 11:5068–5078.
29. Park, D., and S. G. Rhee. 1992. Phosphorylation of Nck in response to a variety of receptors, phorbol myristate acetate, and cyclic AMP. *Mol. Cell. Biol.* 12:5816–5823.
30. Pawson, T., and G. D. Gish. 1992. SH2 and SH3 domains: from structure to function. *Cell* 71:359–362.
31. Peters, K. G., J. Marie, E. Wilson, H. E. Ives, J. Escobedo, M. Del Rosario, D. Mirda, and L. T. Williams. 1992. Point mutation of an FGF receptor abolishes phosphatidylinositol turnover and Ca²⁺ flux but not mitogenesis. *Nature (London)* 358:678–681.
32. Ronnstrand, L., S. Mori, A.-K. Arridsson, A. Eriksson, C. Wernstedt, U. Hellman, L. Claesson-Welsh, and C.-H. Heldin. 1992. Identification of two C-terminal autophosphorylation sites in the PDGF β -receptor: involvement in the interaction with phospholipase C- γ . *EMBO J.* 11:3911–3919.
33. Rotin, D., B. Margolis, M. Mohammadi, R. Daly, G. Daum, N. Li, E. Fisher, W. Burgess, A. Ullrich, and J. Schlessinger. 1992. SH2 domains prevent tyrosine dephosphorylation of the EGF receptor: identification of tyr 992 as the high-affinity binding site for SH2 domains of phospholipase C- γ . *EMBO J.* 11:559–567.
34. Rozakis-Adcock, M., J. McGlade, G. Mbamalu, G. Pelicci, R. Daly, W. Li, A. Batzer, S. Thomas, J. Brugge, P. G. Pelicci, J. Schlessinger, and T. Pawson. 1992. Association of the Shc and Grb2/Sem5 SH2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinases. *Nature (London)* 360:689–692.
35. Schlessinger, J., and A. Ullrich. 1992. Growth factor signaling by receptor tyrosine kinases. *Neuron* 9:383–391.
36. Seedorf, K., S. Felder, B. Millaner, J. Schlessinger, and A. Ullrich. 1991. Analysis of platelet-derived growth factor receptor domain function using a novel chimeric receptor approach. *J. Biol. Chem.* 266:12424–12431.
37. Severinsson, L., B. Ek, K. Mellström, L. Claesson-Welsh, and C.-H. Heldin. 1990. Deletion of the kinase insert sequence of the platelet-derived growth factor β -receptor affects receptor kinase activity and signal transduction. *Mol. Cell. Biol.* 10:801–809.
38. Shurtleff, S. A., J. R. Downing, C. O. Rock, S. A. Hawkins, M. F. Roussel, and C. J. Sheer. 1991. Structural features of colony-stimulating factor 1 receptor that affect its association with phosphatidylinositol 3-kinase. *EMBO J.* 9:2415–2421.
39. Skolnik, E. Y., C.-H. Lee, A. Batzer, L. M. Vicentini, M. Zhou, R. Daly, M. J. Myers, J. M. Backer, A. Ullrich, M. F. White, and J. Schlessinger. 1993. The SH2/SH3 domain containing protein

- GRB2 interacts with tyrosine phosphorylated IRS-1 and Shc: implications for insulin control of ras signaling. *EMBO J.* 12:1929-1936.
40. Songyang, Z., S. E. Shoelson, M. Chaudhuri, G. Gish, T. Powson, W. G. Haser, F. King, T. Roberts, S. Ratnoffsky, R. J. Lechleider, B. G. Neel, R. B. Birge, J. E. Fajardo, M. M. Chou, H. Hanafusa, B. Schaffhausen, and L. C. Cantley. 1993. SH2 domains recognize specific phosphopeptide sequences. *Cell* 72:767-778.
  41. Ullrich, A., and J. Schlessinger. 1990. Signal transduction by receptors with tyrosine kinase activity. *Cell* 61:203-212.
  42. Vallius, M., C. Bazenet, and A. Kazlauskas. 1993. Tyrosines 1021 and 1009 are phosphorylation sites in the carboxy terminus of the platelet-derived growth factor receptor β subunit and are required for binding of phospholipase C γ and a 64-kilodalton protein, respectively. *Mol. Cell. Biol.* 13:133-143.
  43. Vallius, M., and A. Kazlauskas. 1993. Phospholipase C- γ 1 and phosphatidylinositol 3 kinase are the downstream mediators of the PDGF receptor's mitogenic signal. *Cell* 73:321-334.
  44. Waksman, G., D. Kominos, S. C. Robertson, N. Pant, D. Baltimore, R. B. Birge, D. Cowburn, H. Hanafusa, B. J. Mayer, M. Overduin, M. D. Resh, C. B. Rios, L. Silverman, and J. Kuriyan. 1993. Binding of a high affinity phosphotyrosyl peptide to the *src* SH2 domain: crystal structures of the complexed and peptide-free forms. *Nature (London)* 358:646-653.
  45. Waksman, G., S. E. Shoelson, N. Pant, D. Cowburn, and J. Kuriyan. 1993. Binding of a high affinity phosphotyrosyl peptide to the *src* SH2 domain: crystal structures of the complexed and peptide-free forms. *Cell* 72:779-790.
  46. Yu, J.-C., M. A. Heidaran, J. H. Pierce, J. S. Gutkind, D. Lombardi, M. Ruggiero, and S. A. Aaronson. 1991. Tyrosine mutations within the α platelet-derived growth factor receptor kinase insert domain abrogate receptor-associated phosphatidylinositol-3 kinase activity without affecting mitogenic or chemotactic signal transduction. *Mol. Cell. Biol.* 11:3780-3785.