MOLECULAR AND CELLULAR BIOLOGY, Dec. 1992, p. 5834–5842
0270-7306/92/125834-09$02.00/0
Copyright © 1992, American Society for Microbiology
Vol. 12, No. 12

The SH2- and SH3-Containing Nck Protein Transforms Mammalian Fibroblasts in the Absence of Elevated Phosphotyrosine Levels

MARGARET M. CHOU, J. EDUARDO FAJARDO, AND HIDESABURO HANAFUSA*

The Rockefeller University, 1230 York Avenue, New York, New York 10021

Received 23 June 1992/Returned for modification 26 August 1992/Accepted 15 September 1992

We have established the human *nck* sequence as a new oncogene. Nck encodes one SH2 and three SH3 domains, the Src homology motifs found in nonreceptor tyrosine kinases, Ras GTPase-activating protein, phosphatidylinositol 3-kinase, and phospholipase C-γ. Overexpression of human *nck* in 3Y1 rat fibroblasts results in transformation as judged by alteration of cell morphology, colony formation in soft agar, and tumor formation in nude BALB/c mice. However, overexpression of *nck* does not induce detectable elevation of the phosphotyrosine content of specific proteins, as is observed for v-*crk*, another SH2/SH3-containing oncogene. Despite this fact, we demonstrate that Nck retains the ability to bind tyrosine phosphorylated proteins in vitro, using a fusion protein of Nck with glutathione-S-transferase (GST). Moreover, when incubated with lysates prepared from v-*src*-transformed 3Y1 cells or the *nck*-overexpressing cell lines, GST-Nck binds to both $p60^{v\text{-}src}$ and serine/threonine kinases, respectively. Although phosphotyrosine levels are not elevated in the *nck*-expressing fibroblasts, vanadate treatment of these cells results in a phosphotyrosine pattern that is altered from the parental 3Y1 pattern, suggestive of a perturbation of indigenous tyrosine kinase pathways. These results suggest the possibility that human *nck* induces transformation in 3Y1 fibroblasts by virtue of its altered affinity or specificity for the normal substrates of its rat homolog and that Nck may play a role in linking tyrosine and serine/threonine kinase pathways within the cell.

During the last several years a flurry of experiments have contributed to the elucidation of the signalling pathways involved in receptor-mediated growth control. Precipitated by the discovery of the v-*crk* oncogene (27), a unifying theme in tyrosine kinase-initiated pathways became evident from the work of many groups with the discovery that SH2 domains bind to tyrosine-phosphorylated substrates. Identified as the transforming gene of the avian CT10 retrovirus, v-*crk* is expressed as a Gag fusion protein, $p47^{gag\text{-}v\text{-}crk}$. Expression of this protein was found to elevate cellular phosphotyrosine levels, yet it encodes no kinase activity of its own (27). Rather, v-Crk contains sequences that are homologous to the modulatory domains of nonreceptor tyrosine kinases, the prototype of which is Src; hence, these motifs were designated SH2 and SH3 (for Src homology regions 2 and 3) (28, 36). These sequences were simultaneously identified in phospholipase C-γ (PLC-γ) (41, 42) and subsequently found in other signalling molecules such as Ras GTPase-activating protein (GAP) (43) and phosphatidylinositol 3-kinase (PI3-kinase) (5, 33, 39).

Further characterization of v-*crk*-transformed chicken embryo fibroblasts (CEF) revealed that specific proteins are hyperphosphorylated on tyrosine when compared with those in normal CEF and that the ability of *crk* mutants to elevate phosphotyrosine levels correlates with their transforming ability (29). Moreover, these phosphoproteins are associated in a stable complex with Crk, and the SH2 domain of Crk is necessary and sufficient to mediate this interaction (26, 31). Concomitantly, the work of many groups demonstrated that the SH2 domains of GAP, PLC-γ, and PI3-kinase also mediate their association with phosphotyrosine-containing proteins, including activated receptors such as the platelet-derived growth factor (PDGF) receptor and the epidermal growth factor (EGF) receptor (2, 15–17, 19, 23, 24, 31, 32). Thus, SH2 domains proved to be a convergent means by which signalling molecules become associated with and, in some cases, activated by autophosphorylated receptors.

Since the discovery of the v-*crk* oncogene, other proteins containing SH2 and SH3 domains, but lacking any recognizable catalytic activity, have been identified. These include the c-*crk* proto-oncogene product (35), the p85 regulatory subunit of PI3-kinase, the hematopoietic factor Vav (4), and Nck (21). These proteins may serve as adaptor molecules that link tyrosine kinase receptors to their downstream effectors.

Nck was fortuitously cloned from a human melanoma expression library (21). It encodes three SH3 domains followed by one SH2 domain. Here, we establish that overexpression of *nck* results in transformation of 3Y1 rat fibroblasts. Nck-transformed cells display altered morphology, the ability to grow in soft agar, and the capacity to induce tumors in nude mice. However, these cells do not exhibit gross elevation of phosphotyrosine levels. This is in contrast to v-*crk*-induced transformation, which correlates with the extent of increased tyrosine phosphorylation. Hence, *nck* represents a novel SH2/SH3-containing oncogene whose mechanism of transformation is distinct from that of v-*crk*.

MATERIALS AND METHODS

Constructs and bacterial fusion proteins. The 2.1-kb *Eco*RI fragment encoding full-length human *nck* cDNA was excised from pUC19 (21) and inserted into the *Eco*RI site of the pMEXneo vector containing the neomycin resistance gene and the murine sarcoma virus long terminal repeat. To generate a glutathione-S-transferase (GST)-Nck fusion protein, the *Alw*NI-*Xba*I fragment, encoding the entire *nck*

* Corresponding author.

sequence except for the first SH3 domain, was subcloned into the *Eco*RI site of the pGEX-3X plasmid (Pharmacia). This construct is designated GST-Nck. For the GST-SH2 construct, the GST-Nck construct was partially digested with *Sma*I and *Stu*I and religated. Fusion proteins were prepared as described previously (40).

Cell culture. Rat 3Y1 fibroblasts were maintained in Dulbecco's modified Eagle medium (DEM) containing 5% bovine calf serum. Cell lines expressing *nck* were generated as follows. 3Y1 cells (2×10^6 per 10-cm plate) were transfected with 10 μg of the *nck*/pMEXneo construct by the calcium phosphate method. Cells were allowed to reach confluence, split 1:4, and placed under G418 selection (active concentration, 400 μg/ml). Individual colonies were picked and expanded.

Northern analysis. RNA was extracted by the guanidine hydrochloride method (37) and blotted onto Zetabind filters using the Vacublot system (Stratagene). [α-^{32}P]dCTP-labeled probe was prepared by using the random-primer oligolabeling kit (Pharmacia). As template, a gel-purified 750-bp *Hinc*II-*Nco*I fragment of *nck*/pMEXneo, containing solely *nck* and no plasmid sequences, was utilized. Filters were washed under high stringency (0.1 × SSC, 65°C [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]).

Immunoblotting of Nck from cell lines. Confluent 10-cm plates of the various cell lines were washed twice in phosphate-buffered saline (PBS) and then scraped with a rubber policeman and resuspended in RIPA (10 mM Tris [pH 7.4], 5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl) containing 0.1% sodium dodecyl sulfate (SDS) and 1% Trasylol–1 mM phenylmethylsulfonyl fluoride. Lysates were incubated on ice for 10 min and then microcentrifuged for 10 min at 4°C. Supernatants were collected, and protein concentrations were determined by the BioRad Bradford assay. For anti-Nck immunoblotting, whole-cell lysates (150 μg) of each cell line were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) blotted onto a polyvinylidene fluoride (PVDF) membrane in transfer buffer (18 mM Tris, 150 mM glycine, 20% methanol), and probed with anti-Nck antiserum. This polyclonal antibody was raised against a GST fusion with the SH2 and the second and third SH3 domains of Nck. Detection was via Enhanced Chemiluminescence (Amersham). For anti-phosphotyrosine blotting, 100 μg of total lysate was used per lane. Blots were probed with polyclonal anti-phosphotyrosine antibody and detected with ^{125}I-protein A (Amersham).

Colony formation assays. Cells were washed in PBS, trypsinized with 0.2% trypsin–1 mM EDTA, resuspended in 10 ml 5% DEM, and counted on a hemacytometer. Various dilutions (absolute numbers of cells, 10^3, 10^4, and 10^5) were resuspended in a total of 5 ml of 5% DEM containing 0.4% agarose and overlaid onto 6-cm plates containing a bottom layer of 0.8% agarose in 5% DEM (5 ml). The next day, plates were visually inspected, and those with uneven distributions of cells were appropriately noted or discarded to eliminate false positives. For the pooled colony assays in NIH 3T3 and 3Y1 cells, cells were transfected by the calcium phosphate method as described above. After an 18-day G418 selection period, colonies were trypsinized and pooled for plating in 0.4% soft agar as described above.

Injection of nude BALB/c mice. Cells were washed twice in sterile PBS and trypsinized. 10^6 cells were resuspended in 100 μl of PBS and injected subcutaneously into each flank of a nude BALB/c mouse with a 21-gauge needle. Two animals were used for each cell line.

Association of GST-Nck with phosphotyrosine-containing proteins. RIPA lysates of v-*crk*/3Y1 or v-*src*/3Y1 cells were prepared as described above. Lysates (100 μg) were incubated for 2 h at 4°C with glutathione Sepharose beads (Pharmacia) covalently linked (by dimethylpimelimidate [9]) to GST, GST-SH2, GST-Nck, or GST-v-Crk. Samples were washed three times in RIPA containing 300 mM NaCl, twice in RIPA containing 150 mM NaCl, and then once in RIPA containing 10 mM NaCl, followed by SDS-PAGE. The gel was then blotted and probed with anti-phosphotyrosine polyclonal antibody as described above.

In vitro kinase assays. GST-Nck (approximately 3 μg) was bound to glutathione Sepharose beads and incubated with 150 μg of RIPA lysate prepared from each of the cell lines. Beads were washed as described above and then washed twice in kinase buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], pH 7.0, 0.2% Triton X-100, 10 mM $MnCl_2$, 1 mM dithiothreitol, 5 mM β-glycerophosphate, 1 mM sodium vanadate). The kinase reaction was run in 30 μl of this buffer containing 10 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol) for 15 min at room temperature. Samples were washed twice in RIPA containing 10 mM NaCl, boiled in sample buffer, and subjected to SDS-PAGE. Phosphoamino acid analysis and partial *Staphylococcus aureus* V8 digestion were carried out as described previously (8).

Vanadate treatment of cell lines. Sterile-filtered sodium vanadate was added to subconfluent cells at a final concentration of 150 μM for 9 h. Cells were lysed and treated as described for immunoblotting above.

RESULTS

Generation of *nck*-overexpressing cell lines. If Nck is involved in regulation of cell growth, it is possible that its overexpression might result in transformation. To test the effects of overexpression of the Nck protein, 3Y1 rat fibroblasts were transfected with cDNA encoding the full-length human *nck* sequence (schematic diagram shown in Fig. 1A) inserted in the pMEXneo vector. Two weeks after initiation of G418 selection, resistant colonies were collected and screened for appropriate expression of *nck*. Four independent cell lines were selected for further characterization. The Northern blot analysis shown in Fig. 1B establishes expression of the human *nck* mRNA, which is 2.1 kb in length. The levels of *nck* expression vary, with the highest amount in the cell line designated Y2, followed by Y4, Y1, and Y18.

Overexpression of *nck* in these cell lines was confirmed by immunoblotting of whole-cell lysates with antiserum raised against a GST-Nck fusion protein which includes the second and third SH3 domains in addition to the SH2 region of Nck. This serum recognized the transfected Nck protein, with an apparent molecular mass of 45 kDa, as well as the endogenous rat homolog (Fig. 2, filled arrowhead). This band was completely abolished by preincubation of the antibody with an excess of purified bacterially expressed GST-Nck fusion protein (data not shown). The level of protein expression correlates with the amount of *nck* mRNA detected by Northern analysis, with *nck* expression highest in the Y2 cell line. In addition, a band of equal intensity in all cell lines appears at approximately 46 kDa (open arrowhead) that is also completely abolished in the presence of excess antigen (data not shown). The origin of this protein is presently unclear, but it may represent an additional isoform of Nck.

Overexpression of human *nck* induces transformation of 3Y1 cells. We next tested whether overexpression of Nck causes transformation, using standard assays. As shown in

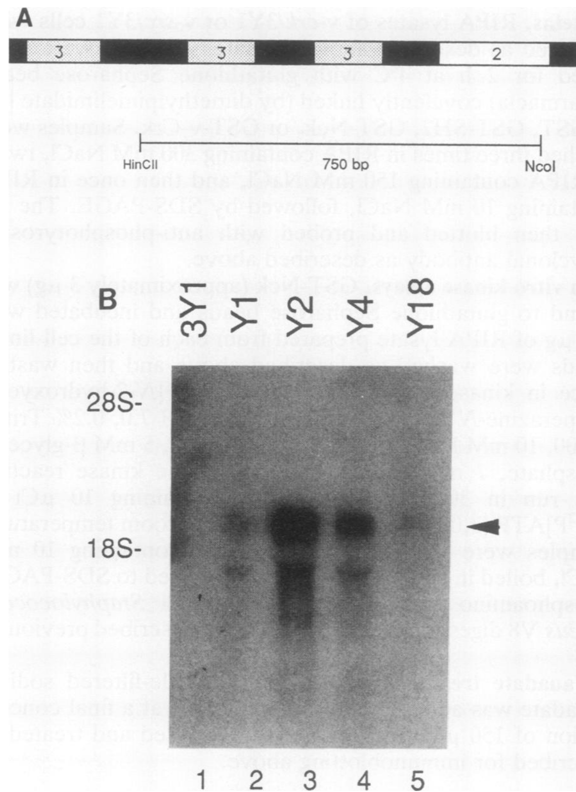

FIG. 1. Expression of human *nck* in rat fibroblasts. (A) Domain structure of the *nck* gene product. The human Nck protein comprises three SH3 domains (stippled bars), and one SH2 domain (white bar). The *Hin*cII-*Nco*I fragment represents the portion of *nck* used as a probe in the Northern blot analysis. a.a., amino acids. (B) Northern blot analysis of *nck*-expressing cell lines. RNA was extracted by the guanidine hydrochloride method. Total RNA (10 μg) was blotted onto Zetabind nylon filter and probed with a 750-bp *Hin*cII-*Nco*I *nck* fragment which was [^{32}P]dCTP labeled using the oligolabeling kit from Pharmacia. Arrowhead indicates the position of the transfected *nck* mRNA, which is 2.1 kb in length. Blots were washed under high stringency (0.1× SSC–1% SDS).

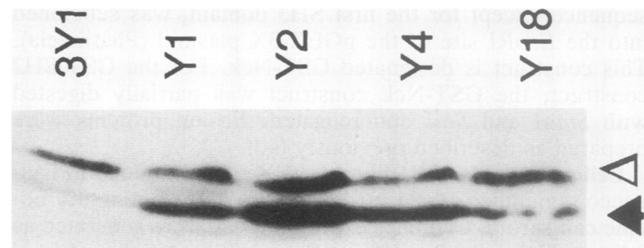

FIG. 2. Immunoblotting of Nck from cell lines. Whole-cell lysates (150 μg) of the parental and *nck*-transfected cell lines (indicated at the top of each lane) were subjected to SDS-PAGE, transferred to a PVDF membrane, and then probed with anti-Nck antiserum. Filled arrowhead indicates the position of the transfected human Nck, which comigrates with endogenous rat Nck. An additional protein of approximately 46 kDa (open arrowhead) is also recognized specifically by this antiserum.

Fig. 3, the *nck* transfectants show various degrees of morphological alteration. Cell line Y18 (panel E) exhibits the most striking difference from wild-type 3Y1, with the cells refractile, rounded up, and piling up into small foci at confluence. The Y1, Y2, and Y4 cell lines (panels B, C, and D, respectively) are less rounded up, but are also refractile and non-contact inhibited at confluence in liquid monolayer culture.

Another parameter typically used to assess transformation is anchorage-independent growth. Normal cells require a solid substrate on which to grow, and if suspended in soft agar they will not proliferate but become quiescent. Transformed cells, however, lack this requirement and will continue to grow when suspended in semisolid medium. All of the *nck* transfectant cell lines displayed this property, forming colonies at a slower rate and to a smaller size than v-*src*-transformed fibroblasts (Fig. 4). Macroscopically visible colonies appeared approximately 2 weeks after plating in all the *nck*-expressing cell lines. Parental 3Y1 cells yielded no colonies.

The ultimate test of oncogenicity is the ability to induce tumor formation in vivo. To address this, equal numbers of *nck*, v-*src*, or normal fibroblasts were injected into nude BALB/c mice. Mice injected with v-*src*/3Y1 cells rapidly developed tumors, which were first palpable at approximately 7 days after injection. As with the colony assay, the *nck* transformants lagged slightly behind v-*src*/3Y1. All four Nck-expressing cell lines formed tumors between 10 and 12 days after injection (data not shown). The size of the *nck*-induced tumors was comparable to that of those caused by v-*src*. No tumors were found in animals injected with the parental cells.

As further confirmation of the transforming ability of *nck*, we performed a pooled-transfectant colony assay. For this purpose we used the NIH 3T3 murine fibroblast cell line, which is more readily transformed than are 3Y1 cells. NIH 3T3 cells were transfected either with the pMEXneo vector alone or with pMEXneo containing the human *nck* sequence. G418-resistant colonies were then pooled (at least 30 colonies from either transfection) and resuspended in soft agar. Approximately 10% of the cells plated from the *nck*/pMEXneo transfection formed colonies, which were macroscopically visible 16 days after plating (data not shown). In contrast, the control pMEXneo transfectants exhibited no colonies even after 4 weeks. This demonstrates that overexpression of *nck* directly causes transformation in NIH 3T3 cells. This experiment was repeated with 3Y1 rat cells, but no colonies were obtained with the *nck*/pMEXneo over the control pMEXneo background. These results indicate that events in addition to *nck* overexpression may be required in 3Y1 cells to cause full transformation, but that Nck indeed contributes to this phenotype.

Nck-expressing cell lines do not exhibit detectable elevations in phosphotyrosine levels. As mentioned previously, overexpression of v-*crk* in CEF and in 3Y1 cells (25) results in increased tyrosine phosphorylation of proteins with molecular masses of 60, 70, 90, 110, and 130 to 155 kDa. It was therefore expected that overexpression of *nck* might similarly result in hyperphosphorylation of specific proteins on tyrosine. However, immunoblot analysis using anti-phosphotyrosine antibody revealed that there was no difference in overall levels of phosphotyrosine in the *nck*-transformed cell lines and in parental 3Y1 cells (Fig. 5). This was confirmed by labeling the various cell lines in vivo with $^{32}P_i$ and immunoprecipitating with anti-phosphotyrosine antibody. Again, no enhancement of tyrosine-phosphorylated species was seen in the *nck*-transformed cells relative to normal fibroblasts (data not shown). This absence of tyrosine hyperphosphorylation is apparently not due to the

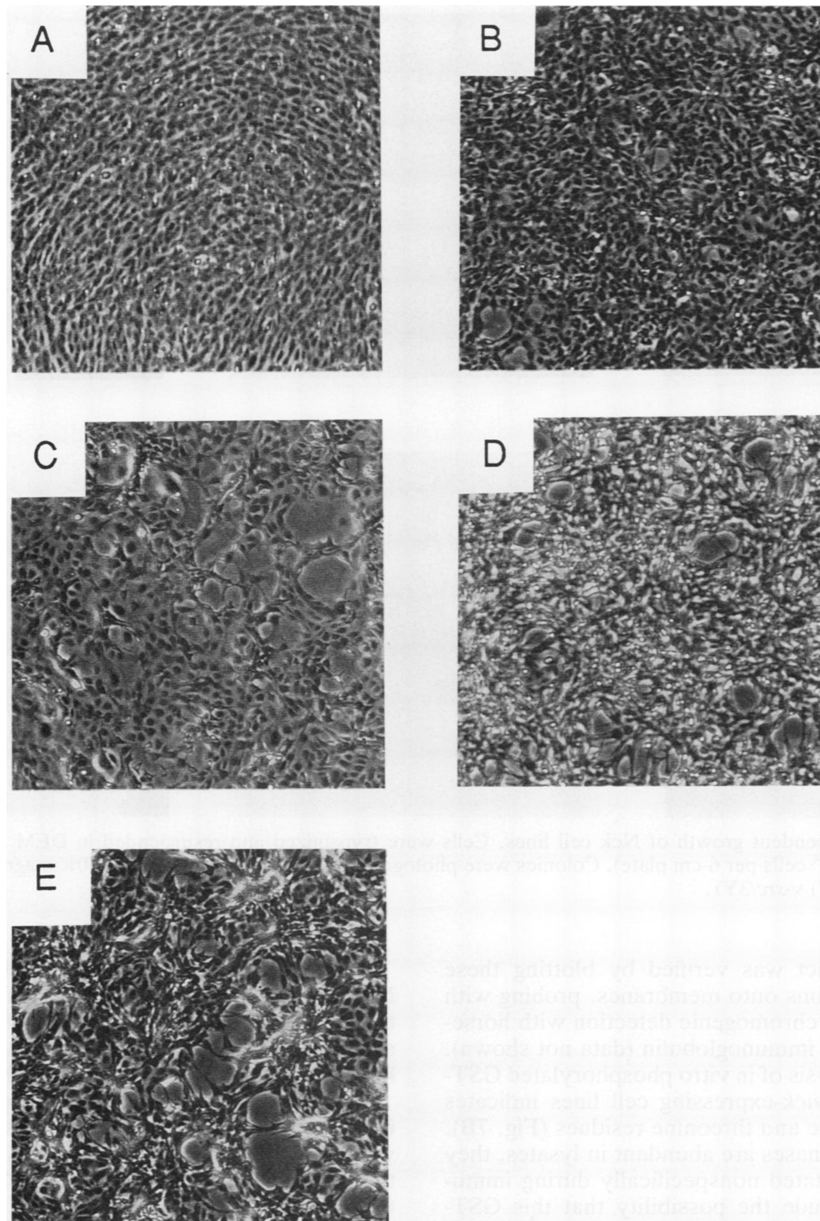

FIG. 3. Morphological alteration of *nck*-transfected 3Y1 cell lines. (A) 3Y1; (B) Y1; (C) Y2; (D) Y4; (E) Y18.

inability of Nck to bind phosphotyrosine-containing proteins. To see the binding capacity of Nck, GST-Nck beads (i.e., GST-Nck fusion protein bound to glutathione Sepharose beads) were incubated with v-*crk*/3Y1 or v-*src*/3Y1 lysates, washed, and then subjected to immunoblotting with anti-phosphotyrosine antibody. As shown in Fig. 6, several phosphoproteins were observed to bind to GST-Nck. Moreover, the isolated SH2 domain of Nck, expressed as a GST fusion protein, was capable of binding a profile of proteins similar to that of the GST-Nck construct (Fig. 6, lanes 3 and 7), with equal or higher affinity.

GST-Nck binds to p60$^{v\text{-}src}$ and serine/threonine kinases in vitro. Although phosphotyrosine levels were not elevated, it was nevertheless possible that Nck associates with a protein kinase, similarly to other SH2-containing proteins. Indeed, this was found to be the case. GST-Nck beads were mixed with lysates of the various cell lines and then washed to remove nonassociated proteins. These GST-Nck complexes were subsequently incubated with buffer containing [γ-^{32}P]ATP; boiled to elute the bound, labeled proteins; and subjected to SDS-PAGE (Fig. 7A). Several proteins were detected in this in vitro kinase assay with GST-Nck (Fig. 7A), whereas these were absent when GST was used alone (data not shown). The spectrum of phosphorylated proteins is similar in the parental 3Y1 cells and the 3Y1 cells expressing *nck* and v-*src*. However, the kinase activity associated with GST-Nck in v-*src*/3Y1 lysates is greatly elevated (Fig. 7A, lane 6). Since the anti-Nck antiserum was not suitable for immunoprecipitation, these in vitro kinase assays could not be performed on Nck immunoprecipitates from the cell lines.

The major band at approximately 60 kDa represents

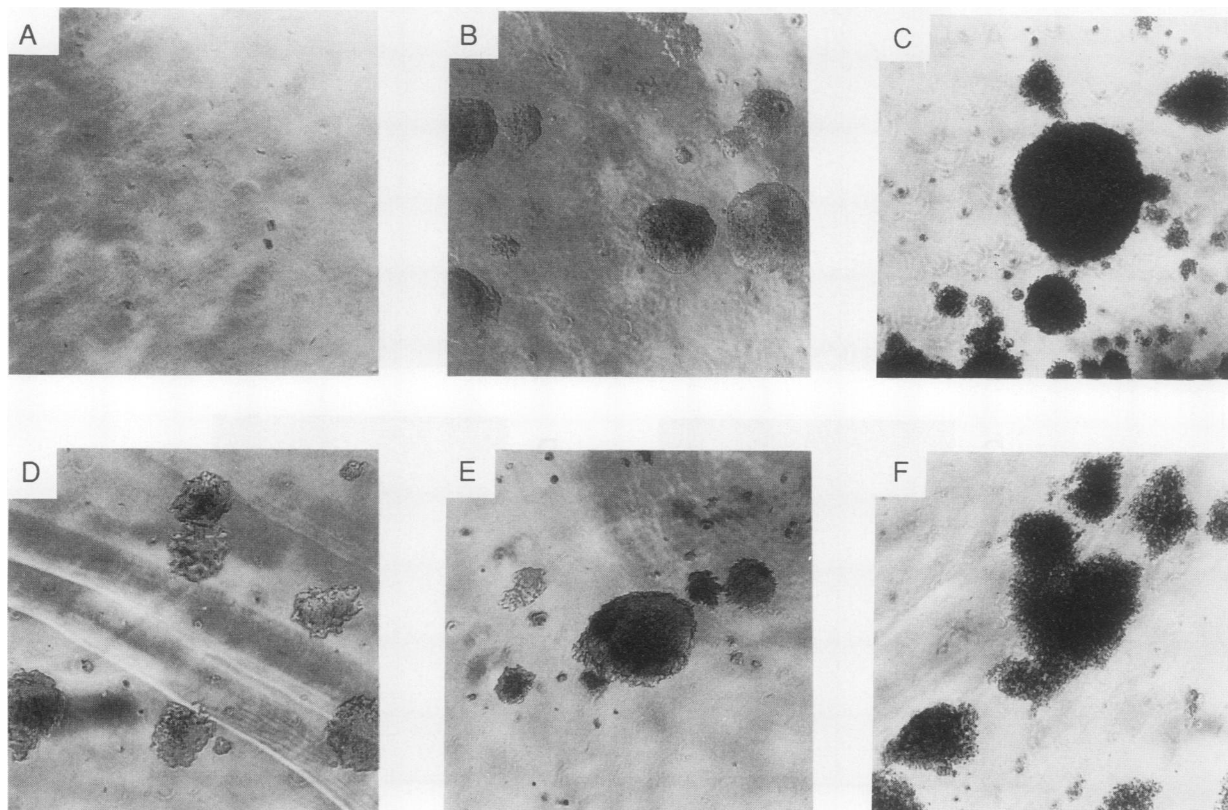

FIG. 4. Anchorage-independent growth of Nck cell lines. Cells were trypsinized and resuspended in DEM containing 5% bovine calf serum and 0.4% agarose (10^4 cells per 6-cm plate). Colonies were photographed 18 days after plating at 40× magnification. (A) 3Y1; (B) Y1; (C) Y2; (D) Y4; (E) Y18; (F) v-*src*/3Y1.

GST-Nck itself. This fact was verified by blotting these ^{32}P-labeled kinase reactions onto membranes, probing with anti-Nck antiserum, and chromogenic detection with horseradish peroxidase-linked immunoglobulin (data not shown). Phosphoamino acid analysis of in vitro phosphorylated GST-Nck from 3Y1 and the *nck*-expressing cell lines indicates phosphorylation on serine and threonine residues (Fig. 7B). Since serine/threonine kinases are abundant in lysates, they are commonly coprecipitated nonspecifically during immunoprecipitation. To exclude the possibility that this GST-Nck-associated kinase was nonspecific, beads bound to GST alone were incubated with lysates and washed. Purified GST-Nck was then added back to the reaction, and an in vitro kinase assay was performed. Under these conditions, no phosphorylation of GST-Nck was seen (data not shown), indicating that the serine/threonine kinase is specifically associated with Nck.

In contrast to the parental and *nck*-expressing cells, in vitro kinase assays of GST-Nck complexes from v-*src*/3Y1 lysates resulted in phosphorylation of GST-Nck mostly on tyrosine. When the GST-Nck beads were incubated with v-*src*/3Y1 lysates, washed, and subjected to immunoblotting with anti-Src antibody, p60^{v-src} was in fact detected in these complexes (data not shown). Comparison with whole lysates indicated that GST-Nck bound approximately 5 to 10% of the p60^{v-src} present (data not shown). V8 digestion of the 60-kDa band in Fig. 7A, lane 6, revealed a pattern that was distinct from that established for v-Src (data not shown), but this may be because GST-Nck is the preferred substrate in this assay.

In the accompanying papers (22, 30, 34), it is reported that Nck associates with various activated receptors, including the EGF, PDGF, and immunoglobulin E receptors. Thus, it appears that in addition to binding to receptor tyrosine kinases, Nck can also associate with p60^{v-src}.

Nck, p60^{v-src}, and v-Crk bind a common ligand of 130 kDa in vitro. One of the tyrosine-phosphorylated proteins in v-*src*-transformed cells is a 130-kDa species which binds to the SH2 domain of p60^{v-src} (14). A 130-kDa product is also the major phosphoprotein present in v-*crk*-transformed cells and is a candidate for the v-Crk-associated tyrosine kinase activity. Since the anti-phosphotyrosine blot in Fig. 6 indicated that GST-Nck can bind the 130-kDa phosphoprotein in v-*crk*-transformed 3Y1 cells, we explored the possibility that the 130-kDa band in the in vitro kinase assay (Fig. 7A, all lanes) might represent the same species. v-Crk was immunoprecipitated from v-*crk*/3Y1 cells (by using anti-gag antibody) and subjected to an in vitro kinase assay, and the 130-kDa band was subjected to partial V8 digestion. Figure 7C shows that the 130-kDa protein from v-Crk immunoprecipitates is identical to the 130-kDa protein present in the GST-Nck complexes. Moreover, they are phosphorylated in a similar manner. These results suggest that p60^{v-src}, Crk, and Nck signal in at least partially overlapping pathways, sharing a common 130-kDa ligand. To further characterize this protein, attempts have been made to determine whether it is a tyrosine kinase by filter kinase assays (6), but these have been unsuccessful thus far.

Vanadate treatment of *nck* cell lines indicates perturbation of endogenous tyrosine kinase pathways. It seemed perhaps

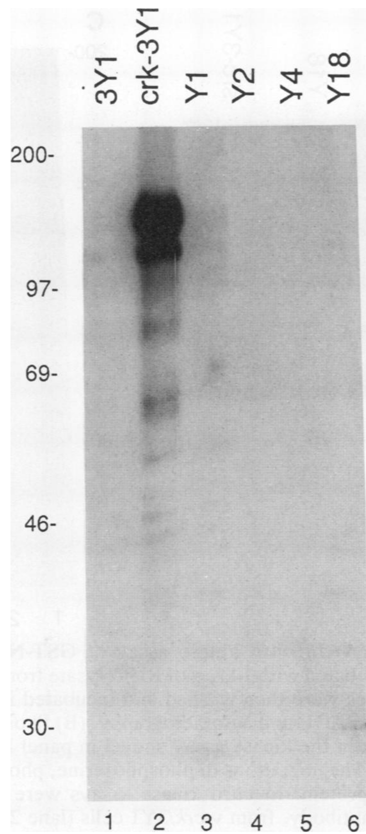

FIG. 5. Phosphotyrosine levels are not elevated in the *nck* cell lines. Whole-cell lysates (100 μg) were run on SDS-PAGE, blotted onto a PVDF membrane overnight, probed with polyclonal anti-phosphotyrosine antiserum, and detected with ^{125}I-protein A.

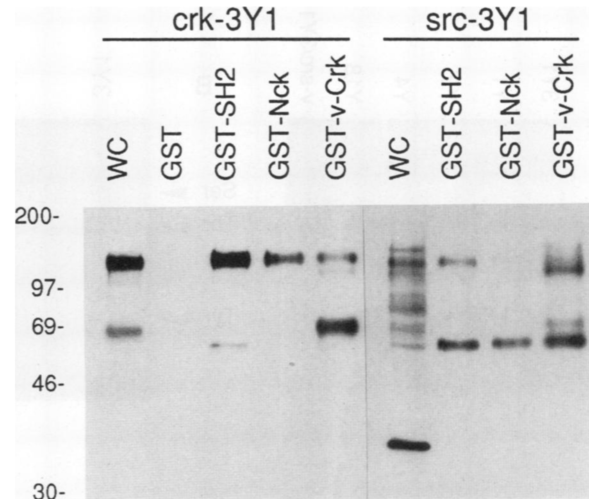

FIG. 6. The full-length Nck protein and its isolated SH2 domain are both capable of binding phosphotyrosine-containing proteins. Lysates of v-*src*/3Y1 or v-*crk*/3Y1 cells (100 μg) were incubated with purified GST fusion proteins covalently coupled to glutathione-Sepharose. In addition to the GST-Nck fusion, chimeric proteins containing the SH2 domain of Nck (GST-SH2) or Gag-v-Crk (GST-v-Crk) were tested. Complexes were subjected to SDS-PAGE, blotted onto PVDF membrane, and probed with anti-phosphotyrosine antibody. Whole-cell lysates (WC) of v-*crk*/3Y1 (35 μg) and v-*src*/3Y1 (25 μg) were also loaded.

contradictory that Nck has the capacity to associate with a number of tyrosine kinases yet no enhancement of tyrosine-phosphorylated species was seen in the *nck*-expressing cell lines relative to normal 3Y1 cells. One possibility was that changes in phosphorylation did occur, but that they were below the levels of detection. Alternatively, elevation of phosphotyrosine content per se might not be crucial to *nck*-induced transformation. Rather, expression of human Nck in rat cells might result in altered affinity or specificity of the protein for the normal substrates of the rat homolog. In the hope of unmasking such modulation of tyrosine kinase activity, cells were grown in the presence of sodium vanadate, a potent tyrosine phosphatase inhibitor. As shown in the anti-phosphotyrosine immunoblot in Fig. 8, *nck*-transformed cells treated with vanadate exhibit a pattern of tyrosine phosphorylation that is distinct from that observed in 3Y1 cells. Certain bands are hyperphosphorylated in the Nck cell lines (indicated by arrowheads), while others are hypophosphorylated relative to 3Y1 cells (indicated by asterisks). The *nck*-overexpressing cell lines, particularly the highest expressor Y2, were very sensitive to vanadate, rounding up and detaching from the plate after several hours of treatment. However, 3Y1 cells typically exhibited higher overall phosphotyrosine levels than the *nck*-transformed cells. Figure 8 is representative of a number of different experiments, demonstrating that the various *nck*-overexpressing cell lines demonstrate a similar pattern. Thus, although human Nck protein levels are lower in lines Y1 and Y18, a molecular or biochemical modification occurs in these cells similar to that seen in the Y2 cell line. These experiments indicate that although gross phosphotyrosine levels are not elevated in the *nck*-overexpressing cell lines, Nck does modulate tyrosine kinase, or phosphatase, pathways within the cell.

DISCUSSION

We have established the *nck* gene product as a new oncoprotein. Overexpression of human *nck* in rat fibroblasts results in adoption of characteristics typical of transformed cells. First, the *nck*-expressing 3Y1 cells exhibit altered morphology as well as loss of contact inhibition. The various clones demonstrate different morphologies, with the highest expressor, Y2, assuming the least-altered morphology. Cells of the Y18 line, which produces lower levels of Nck, are much more refractile and rounded in appearance; these cells even pile up to form small foci at confluence. This lack of correlation between the level of *nck* expression and morphological alteration may be due to differences in additional genetic alterations accumulated by the individual cell lines. Indeed, results of the pooled-transfectant colony assay imply that additional mutations may be required for full transformation in 3Y1 cells. Secondly, all of the Nck cell lines are capable of anchorage-independent growth, a hallmark of the transformed phenotype. Colony growth occurred at a rate slightly decreased relative to v-*src*/3Y1 cells, and the sizes of the colonies formed by the various cell lines were also diminished. Importantly, anchorage-independent growth could be induced in two different cell lines, NIH 3T3 and 3Y1, further confirming the role of *nck* in transformation. And finally, injection of all *nck*-overexpressing fibroblast lines resulted in tumor formation in nude BALB/c mice, at a rate only slightly lagging behind that of those induced by

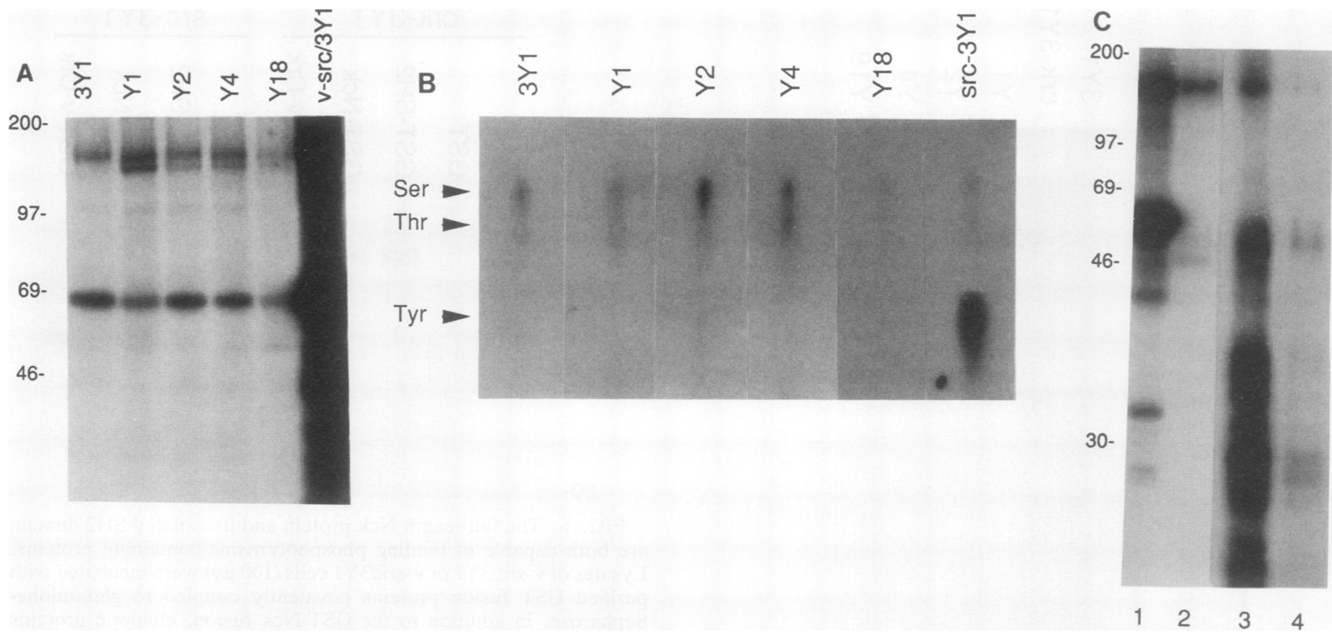

FIG. 7. Serine/threonine and tyrosine kinases associate with GST-Nck in vitro. (A) In vitro kinase assay of GST-Nck complexes. GST-Nck (approximately 3 μg of protein) bound to glutathione-Sepharose beads was incubated with 100 μg of RIPA lysate from parental 3Y1, v-*src*/3Y1, or Nck-overexpressing cells, as indicated at the top of each lane. Complexes were then washed and incubated in kinase buffer containing 10 μCi of [γ-^{32}P]ATP for 15 min. Samples were washed and subjected to SDS-PAGE and autoradiography. (B) Phosphoamino acid analysis of in vitro phosphorylated Nck. The 60-kDa bands corresponding to GST-Nck in the kinase assay shown in panel A were excised from the gel, trypsinized, acid hydrolyzed, and subjected to thin-layer chromatography. The migrations of phosphoserine, phosphothreonine, and phosphotyrosine are indicated. (C) Partial V8 digestion of the 130-kDa phosphoprotein. In vitro kinase assays were carried out on GST-Nck/v-*src*/3Y1 complexes (lane 1) and v-Crk immunoprecipitates, using anti-gag antibody, from v-*crk*/3Y1 cells (lane 2). The 130-kDa band from both was excised and subjected to partial V8 digestion as described previously. Lanes 3 and 4 represent digests of the 130-kDa protein from lanes 1 and 2, respectively.

v-*src*/3Y1-cells. By these criteria, *nck* can be classified as a new oncogene.

Several points warrant further discussion. First, it was observed that NIH 3T3 fibroblasts are directly transformed by *nck* in the pooled-transfectant colony assay, in contrast to 3Y1 cells. This may be due to a general species difference in susceptibility to transformation by the human sequence. Alternatively, it may be due to the fact that the NIH 3T3 line is already partially transformed (20).

Another point is that the degree of transformation does not correlate strictly with the amount of *nck* overexpression, since the cell lines expressing low levels induced a phenotype comparable to that of the highly expressing line, as judged by colony formation in soft agar and tumorigenesis. There are several reasons that may account for this observation. One possibility is that high levels of *nck* expression over the endogenous level are not required for transformation. The human Nck protein may have an affinity or specificity different from that of the endogenous protein for its normal substrates and associated proteins. This is suggested by the results of the experiments in which cells were grown in the presence of vanadate. A second possibility, as mentioned above, is that additional genetic changes occurred during establishment of the individual cell lines that contributed to transformation.

Surprisingly, none of the Nck cell lines displays gross elevations in phosphotyrosine levels, as determined by both anti-phosphotyrosine immunoblots of whole-cell lysates and anti-phosphotyrosine immunoprecipitation of ^{32}P$_i$-labeled cells. This was unexpected since, following the paradigm of v-*crk*-induced transformation, one might predict increased tyrosine phosphorylation of specific substrates. v-Crk, like Nck, is a composite of SH2 (one) and SH3 (one) domains, without juxtaposition of any recognizable catalytic sequences. Crk is postulated to induce elevated cellular steady-state phosphotyrosine, and thereby transformation, either by maintaining a target kinase in its activated state, by stabilizing tyrosine phosphorylated substrates, or by a combination thereof. This property of Crk is dependent on its SH2 domain and also partly on its SH3 domain: while deletion of the SH3 domain of v-Crk causes a mild decrease in its ability to elevate phosphotyrosine levels and to transform CEF, deletion of the SH2 domain completely abolishes these effects (29). We had therefore anticipated that overexpression of another SH2/SH3-containing protein might yield transformation by a similar mechanism. This does not appear to be the case with Nck. It is possible that small yet significant elevations in tyrosine phosphorylation of specific substrates may be beyond the level of detection. However, vast elevations are not a prerequisite for transformation.

Despite this finding, it appears that Nck does play a role in several tyrosine kinase-initiated pathways. Our results show that Nck can associate with p60^{v-src} in vitro. The work presented in the accompanying papers demonstrates binding of Nck to the activated EGF, PDGF, nerve growth factor, and surface immunoglobulin E receptors (22, 30, 34), as well as its subsequent phosphorylation. The lysis conditions used for our kinase assays are more stringent (RIPA containing 0.1% SDS) than those that are normally used to examine receptor complexes (19, 38). Thus, it appears that the

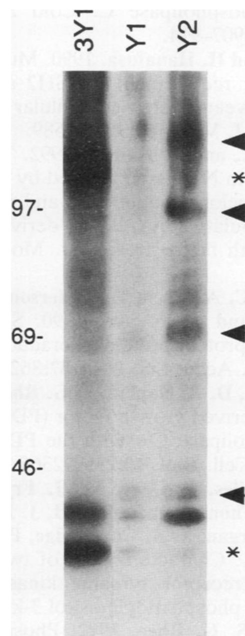

FIG. 8. Anti-phosphotyrosine Western blot (immunoblot) of vanadate-treated cell lines. Cells were grown in DEM containing 5% bovine calf serum and 150 μM sodium vanadate for 9 h. Whole-cell lysates (150 μg per lane) were prepared and subjected to SDS-PAGE, blotted onto a PVDF membrane, probed with anti-phosphotyrosine antibody, and then detected with ^{125}I-protein A. Arrowheads indicate bands that are hyperphosphorylated in vanadate-treated Nck cell lines, while asterisks indicate those that are hypophosphorylated relative to 3Y1 cells.

association of Nck with p60^{v-src} is more stable, or that v-Src is more resistant to denaturation.

Although phosphotyrosine levels are not elevated in the *nck*-transformed cell lines, it appears that Nck does affect tyrosine kinase pathways within these cells. Consistent with this observation, it was observed that vanadate treatment of Nck cell lines resulted in a tyrosine phosphorylation pattern that was altered from that of parental 3Y1 cells. Various bands appeared to be hypo- or hyperphosphorylated in the transformed lines, suggestive of a perturbation of endogenous tyrosine kinase/phosphatase pathways. While it is possible that additional genetic alterations occurred in the various cell lines to cause full transformation, it is extremely unlikely that they would have accumulated identical mutations that result in the same alteration of phosphotyrosine pattern from the parental cells. It is most likely that these common perturbations in tyrosine phosphorylation are due to Nck overexpression. As mentioned above, this deregulation may be due to altered affinity or specificity of Nck for its regulators or effectors. Additionally, overexpression of Nck may result in constitutive or enhanced activation of its targets. Thus, we propose that Nck causes transformation by enhanced or constitutive activation of its effector(s), because of its overexpression and/or altered affinity relative to the endogenous protein; this allows their activation and phosphorylation by upstream regulators of Nck, namely tyrosine kinases. These putative changes may be too subtle to see in the absence of vanadate.

Future studies include the identification of Nck effectors and the domains of Nck required for their regulation. This includes dissection of the three SH3 domains. Compared to the role of SH2 regions, the role of SH3 domains in tyrosine kinase signal transduction has remained nebulous. Thus far, mutations in the SH3 regions of v-Crk (35), c-Src (10, 11, 13), and c-Abl (7, 12) have implicated it as a negative modulator of kinase activity. Moreover, the presence of these sequences in cytoskeleton-associated proteins suggests a role in subcellular localization (18). Recent genetic studies of *Caenorhabditis elegans* have identified an SH2/SH3 adaptor protein, called Sem-5, that is required for vulval development (3). These experiments indicate that the SH3 domains may be required for downstream signalling after the initial SH2-mediated binding of Sem-5 to a tyrosine kinase receptor (presumably the product of the *let-23* gene) (1). Identification of Nck SH3 targets and the possible functional redundancy of its three SH3 domains will be the focus of future experiments.

ACKNOWLEDGMENTS

We thank Jurgen M. Lehmann and Judith P. Johnson for providing us with the human *nck* clone. Many thanks are owed to Gerd A. Blobel for invaluable discussions and helpful comments on this manuscript, and also to Glen Scholz and Heidi Greulich for critical reading of this manuscript. We also extend thanks to Joseph Schlessinger, Tony Hunter, and Sue Goo Rhee for discussion of results prior to publication.

This work was supported by grant VM2 from the American Cancer Society, grant CA44356 from the National Cancer Institute, grant 2517 from the Council for Tobacco Research, National Institutes of Health training grants A107233-15 and CA09673-16 (M.M.C.), and a Damon Runyon Walter Winchell Cancer Research Fund Fellowship, DRG 1049 (J.E.F.).

REFERENCES

1. **Aroian, R. V., M. Koga, J. E. Mendel, Y. Ohshima, and P. W. Sternberg.** 1990. The *let-23* gene necessary for *Caenorhabditis elegans* vulval induction encodes a tyrosine kinase of the EGF receptor subfamily. Nature (London) **348:**693–699.
2. **Bjorge, J. D., T. O. Chan, M. Antczak, H.-J. Kung, and D. J. Fujita.** 1990. Activated type I phosphatidylinositol kinase is associated with the epidermal growth factor (EGF) receptor following EGF stimulation. Proc. Natl. Acad. Sci. USA **87:** 3816–3820.
3. **Clark, S. G., M. J. Stern, and H. R. Horvitz.** 1992. C. elegans cell-signalling gene *sem-5* encodes a protein with SH2 and SH3 domains. Nature (London) **356:**340–344.
4. **Coppola, J., S. Bryant, T. Koda, D. Conway, and M. Barbacid.** 1991. Mechanism of activation of the *vav* protooncogene. Cell Growth Differ. **2:**95–105.
5. **Escobedo, J. A., S. Navankasattusas, W. M. Kavanaugh, D. Milfay, V. A. Fried, and L. T. Williams.** 1991. Complementary DNA cloning of a novel 85 kD protein that has SH2 domains and regulates binding of phosphatidylinositol 3-kinase to the PDGF β-receptor. Cell **65:**75–82.
6. **Ferrell, J. E., Jr., and G. S. Martin.** 1990. Identification of a 42-kilodalton phosphotyrosyl protein as a serine(threonine) protein kinase by renaturation. Mol. Cell. Biol. **10:**3020–3026.
7. **Franz, W. M., P. Berger, and J. Y. J. Wang.** 1989. Deletion of an N-terminal regulatory domain of the c-abl tyrosine kinase activates its oncogenic potential. EMBO J. **8:**137–147.
8. **Hamaguchi, M., C. Grandori, and H. Hanafusa.** 1988. Phosphorylation of cellular proteins in Rous sarcoma virus-infected cells: analysis by use of anti-phosphotyrosine antibodies. Mol. Cell. Biol. **8:**3035–3042.
9. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
10. **Hirai, H., and H. E. Varmus.** 1990. Mutations in src homology regions 2 and 3 of activated chicken c-src that result in preferential transformation of mouse or chicken cells. Proc. Natl. Acad. Sci. USA **87:**8592–8596.
11. **Hirai, H., and H. E. Varmus.** 1990. Site-directed mutagenesis of

the SH2- and SH3-coding domains of c-*src* produces varied phenotypes, including oncogenic activation of p60[c-src]. Mol. Cell. Biol. **10**:1307–1318.

12. **Jackson, P., and D. Baltimore.** 1989. N-terminal mutations activate the leukenic potential of the myristoylated form of c-*abl*. EMBO J. **8**:449–456.
13. **Jove, R., B. J. Mayer, H. Iba, D. Laugier, F. Poirier, G. Calothy, T. Hanafusa, and H. Hanafusa.** 1986. Genetic analysis of p60[v-src] domains involved in the induction of different cell transformation parameters. J. Virol. **60**:840–848.
14. **Kanner, S. B., A. B. Reynolds, H.-C. Wang, R. R. Vines, and J. T. Parsons.** 1991. The SH2 and SH3 domains of pp60src direct stable association with tyrosine phosphorylated proteins p130 and p110. EMBO J. **10**:1689–1698.
15. **Kaplan, D. R., M. D. K. Morrison, G. Wong, F. McCormick, and L. T. Williams.** 1990. PDGF β-receptor stimulates tyrosine phosphorylation of GAP and association of GAP with a signalling complex. Cell **61**:125–133.
16. **Kazlauskas, A., C. Ellis, T. Pawson, and J. A. Cooper.** 1990. Binding of GAP to activated PDGF receptor. Science **247**:1578–1581.
17. **Klippel, A., J. A. Escobedo, W. J. Fantl, and L. T. Williams.** 1992. The C-terminal SH2 domain of p85 accounts for the high affinity and specificity of the binding of phosphatidylinositol 3-kinase to phosphorylated platelet-derived growth factor β receptor. Mol. Cell. Biol. **12**:1451–1459.
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**:668–674.
19. **Kypta, R. M., Y. Goldberg, E. T. Ulug, and S. A. Courtneidge.** 1990. Association between the PDGF receptor and members of the *src* family of tyrosine kinases. Cell **62**:481–492.
20. **Land, H., L. F. Parada, and R. A. Weinberg.** 1983. Cellular oncogenes and multistep carcinogenesis. Science **222**:771–778.
21. **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.
22. **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.
23. **Margolis, B., N. Li, A. Koch, M. Mohammadi, D. R. Hurwitz, A. Zilberstein, A. Ullrich, T. Pawson, and J. Schlessinger.** 1990. The tyrosine phosphorylated carboxy terminus of the EGF receptor is a binding site for GAP and PLC-γ. EMBO J. **9**:4375–4380.
24. **Margolis, B., S. G. Rhee, S. Felder, M. Mervic, R. Lyall, A. Levitzki, A. Ullrich, A. Zilberstein, and J. Schlessinger.** 1989. EGF induces tyrosine phosphorylation of phospholipase-II: a potential mechanism for EGF receptor signaling. Cell **57**:1101–1107.
25. **Marshall, C. P., B. J. Mayer, and H. Hanafusa.** Unpublished data.
26. **Matsuda, M., B. J. Mayer, and H. Hanafusa.** 1991. Identification of domains of the v-*crk* oncogene product sufficient for association with phosphotyrosine-containing proteins. Mol. Cell. Biol. **11**:1607–1613.
27. **Mayer, B., M. Hamaguchi, and H. Hanafusa.** 1988. A novel viral oncogene with structural similarity to phospholipase C. Nature (London) **332**:272–275.
28. **Mayer, B. J., M. Hamaguchi, and H. Hanafusa.** 1989. Characterization of P47[gag-crk], a novel oncogene product with sequence similarity to a putative modulatory domain of protein-tyrosine kinases and phospholipase C. Cold Spring Harbor Symp. Quant. Biol. **53**:907–914.
29. **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.
30. **Meisenhelder, J., and T. Hunter.** 1992. The SH2/SH3 domain-containing protein Nck is recognized by certain anti-phospholipase C-γ1 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.
31. **Moran, M. F., C. A. Koch, D. Anderson, C. Ellis, L. England, G. S. Martin, and T. Pawson.** 1990. Src homology region 2 domains direct protein-protein interactions in signal transduction. Proc. Natl. Acad. Sci. USA **87**:8622–8626.
32. **Morrison, D. K., D. R. Kaplan, S. G. Rhee, and L. T. Williams.** 1990. Platelet-derived growth factor (PDGF)-dependent association of phospholipase C-γ with the PDGF receptor signaling complex. Mol. Cell. Biol. **10**:2359–2366.
33. **Otsu, M., I. Hiles, I. Gout, M. J. Fry, F. Ruiz-Larrea, G. Panayotou, A. Thompson, R. Dhand, J. Hsuan, N. Totty, A. D. Smith, S. J. Morgan, S. A. Cortneidge, P. J. Parker, and M. D. Waterfield.** 1991. Characterization of two 85 kD proteins that associate with receptor tyrosine kinases, middle-T/pp60[c-src] complexes, and phosphatidylinositol 3-kinase. Cell **65**:91–104.
34. **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.
35. **Reichman, C. T., B. J. Mayer, S. Keshav, and H. Hanafusa.** 1992. The product of the cellular *crk* gene consists primarily of SH2 and SH3 regions. Cell Growth Differ. **3**:451–460.
36. **Sadowski, I., J. C. Stone, and T. Pawson.** 1986. A noncatalytic domain conserved among cytoplasmic protein-tyrosine kinases modifies the kinase function and transforming activity of Fujinami sarcoma virus P130[gag-fps]. Mol. Cell. Biol. **6**:4396–4408.
37. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.
38. **Samelson, L. E., A. F. Phillips, E. T. Luong, and R. D. Klausner.** 1990. Association of the fyn protein tyrosine kinase with the T-cell antigen receptor. Proc. Natl. Acad. Sci. USA **87**:4358–4362.
39. **Skolnik, E. Y., B. Margolis, M. Mohammadi, E. Lowenstein, R. Fischer, A. Drepps, A. Ullrich, and J. Schlessinger.** 1991. Cloning of PI 3-kinase associated p85 utilizing a novel method for expression/cloning of target proteins for receptor tyrosine kinases. Cell **65**:83–90.
40. **Smith, D. B., and K. S. Johnson.** 1988. Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione-S-transferase. Gene **67**:31–40.
41. **Stahl, M. L., C. R. Ferenz, K. L. Kelleher, R. W. Kriz, and J. L. Knopf.** 1988. Sequence similarity of phospholipase C with the non-catalytic region of *src*. Nature (London) **332**:269–272.
42. **Suh, P.-J., S. H. Ryu, K. H. Moon, H. W. Suh, and S. G. Rhee.** 1988. Inositol phospholipase-specific PLC: complete cDNA and protein sequences and sequence homology to tyrosine kinase-related oncogene products. Proc. Natl. Acad. Sci. USA **85**: 5419–5423.
43. **Trahey, M., G. Wong, R. Halenbeck, B. Rubinfeld, G. A. Martin, M. Ladner, C. Long, W. J. Crosier, K. Watt, K. Koths, and F. McCormick.** 1988. Molecular cloning of two types of GAP complementary DNA from human placenta. Science **242**: 1697–1700.