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Phosphorylation of Nck in Response to a Variety of Receptors, Phorbol Myristate Acetate, and Cyclic AMP

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The 47-kDa protein coimmunoprecipitated with phospholipase C (PLC)-γ1 by anti-PLC-γ1 monoclonal antibodies is proved to be Nck, a protein composed almost exclusively of one SH2 and three SH3 domains. Nck and PLC-γ1 are recognized by certain anti-PLC-γ1 monoclonal antibodies because Nck and PLC-γ1 share an epitope that likely is located in their SH3 domains. Nck is widely distributed in rat tissues, with an especially high level of expression in testes. The expression levels of Nck remains unchanged during the development of rat brain, whereas PLC-γ1 decreases during the same developmental period. Stimulation of A431 cells with epidermal growth factor elicits the tight association of Nck with the epidermal growth factor receptor and phosphorylation of Nck on both serine and tyrosine residues. The phosphorylation of Nck is also enhanced in response to stimulation of the nerve growth factor receptor in PC12 cells, the T-cell receptor complex in Jurkat cells, the membrane immunoglobulin M in Daudi cells, and the low-affinity immunoglobulin G receptor (FcγRII) in U937 cells. The phosphorylation of Nck was also enhanced following treatment of A431 cells with phorbol 12-myristate 13-acetate or forskolin. These results suggest that Nck is a target for a variety of protein kinases that might modulate the postulated role of Nck as an adaptor for the physical and functional coordination of signalling proteins.

Treatment of a number of cell types with platelet-derived growth factor or epidermal growth factor (EGF) specifically activates PLC-γ, one of three phospholipase C (PLC) types identified in mammalian cells (18, 24, 42). PLC-γ, unlike the other two PLC types, PLC-β and PLC-δ, contains the so-called *src* homology (SH2 and SH3) domains (32). The SH2 domain (reviewed in reference 14) is a sequence of ~100 amino acids, originally identified as the regulatory region common to a variety of nonreceptor tyrosine kinases (29) and later found in many other signalling proteins such as PLC-γ (36, 38), Ras GTPase-activating protein (GAP) (39, 41), and an 85-kDa regulatory subunit of phosphatidylinositol 3-kinase (p85 PI3-kinase) (9, 35). It has been shown that SH2 domains regulate protein-protein interactions by recognizing amino acid sequences that encompass phosphorylated tyrosine residues (1, 19, 22, 25). Most SH2-containing proteins also possess a distinct motif of ~45 amino acids termed the SH3 domain (14, 29). SH3-like sequences have been identified in a variety of proteins that either comprise or associate with cytoskeletal and membrane proteins (33).

The binding of a growth factor to its receptor induces the receptor to dimerize and to autophosphorylate at specific tyrosine residues within its intracellular region (40). The SH2 domain of PLC-γ binds the autophosphorylated tyrosine, resulting in the tight association of PLC-γ with the receptor and subsequent phosphorylation of PLC-γ by the receptor tyrosine kinase (31). Tyrosine phosphorylation was shown to be a critical step for the activation of PLC-γ1, one of two known isozymes classified as a PLC-γ (12).

During the course of studies on the activation of PLC-γ1 by tyrosine kinases, several groups noticed that immunoprecipitates obtained with a mixture of six monoclonal antibodies (MAbs) to PLC-γ1 contain the growth factor receptor as well as three phosphoproteins of 100, 84, and 47 kDa (24, 43, 44). Further studies led Meisenhelder et al. (24) to conclude that the 47-kDa protein contains an epitope recognized by one of the anti PLC-γ MAbs but is not a fragment of PLC-γ. Meisenhelder et al. also obtained evidence that the two larger proteins of 100 and 84 kDa are complexed with PLC-γ1.

We purified the 47-kDa protein by using an affinity gel with immobilized MAb to PLC-γ1. Here we describe evidence that the 47-kDa protein is Nck. Our studies also suggest that the function of Nck is likely to be modulated by phosphorylation on its serine/threonine and tyrosine residues.

MATERIALS AND METHODS

Materials. MAbs to PLC-γ1 were prepared as described previously (37). Rabbit antisera #57 and #65 to Nck were gifts from Wei Li and Joseph Schlessinger at New York University Medical Center, New York. The antiserum #57 was generated against a peptide corresponding to amino acid residues 80 to 100 of Nck and used in immunoblots, and antiserum #65 was raised to the GST-Nck fusion protein containing the carboxyl-terminal tail (amino acids 199 to 377) of Nck and used for immunoprecipitation (see accompanying paper by Li et al. [16]). Rabbit antiserum to the EGF receptor (EGFR) and monoclonal antiphosphotyrosine antibody (anti-PY MAb) were purchased from Upstate Biotechnology, Inc., Lake Placid, N.Y. Phorbol 12-myristate 13-acetate (PMA) and 7β-deacetyl-7β-(γ-*N*-methylpiperazino) butyryl forskolin dihydrochloride (forskolin) were purchased from Calbiochem.

Immunoaffinity chromatography of rat brain proteins. The purified MAb to PLC-γ1, B16-5, was coupled to Affigel 10 (Bio-Rad) at 5 mg of MAb per ml of gel according to procedures recommended by the manufacturer. For the purification of PLC-γ1 and coimmunoprecipitating proteins, 200 g of rat brains was homogenized in 1.5 liter of 10 mM Tris-HCl (pH 7.4)–1 mM EDTA–1 mM phenylmethylsulfonyl fluoride (PMSF)–5 μg of leupeptin per ml, and 5 μg of aprotinin per ml with a Polytron PT-10 homogenizer (Brink-

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mann Instrument). Homogenates were centrifuged at 1,000 × *g* for 12 min. Supernatants were further centrifuged at 27,000 × *g* for 1 h. The resulting supernatants (1.2 liter) were then applied to a B16-5 column (5 ml) at a flow rate of 40 ml/h. The column was washed with 600 ml of 10 mM KH_2PO_4 (pH 7.4)–500 mM NaCl–2 mM Mg acetate–0.1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid]–0.3 mM PMSF and with 100 ml of 20 mM KH_2PO_4 (pH 7.4)–2 mM Mg acetate–0.1 mM EGTA–0.3 mM PMSF. The column-bound proteins were eluted with 50 ml of 50 mM Na citrate (pH 3.0)–1 mM EDTA–0.3 mM PMSF. To neutralize the eluted proteins, each 3-ml fraction was collected into tubes containing 1 ml of 1 M Tris-HCl (pH 8.0). To determine which proteins bound nonspecifically to the column, the column flowthrough was applied to a second MAb B16-5 column (5 ml) which was then washed and eluted as described above. Aliquots of fractions from the first and second columns were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining and then compared to identify proteins that were present in the first column eluant but not in the second column eluant. Columns were regenerated by sequential washings with 500 ml of 50 mM Tris-HCl (pH 7.4)–1 M NaCl and 100 ml of 50 mM Tris-HCl (pH 7.4)–0.1% NaN_3, and were then stored at 4°C.

Amino acid sequence determination of 47-kDa protein. The immunoaffinity-purified proteins were concentrated by using Centriprep 30 (Amicon), fractionated by SDS-PAGE (10% polyacrylamide), and stained with Coomassie blue R250. The band containing the 47-kDa protein was excised. The gel piece was digested with *Staphylococcus aureus* V8 protease, and the resulting digestion mixture was separated by SDS-PAGE (16% polyacrylamide) according to the method of Cleveland et al. (7). Following SDS-PAGE, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) and stained with Coomassie Blue R250 (20). The pieces of PVDF membranes containing protein bands were excised and subjected to amino acid sequence analysis on an Applied Biosystems model 477A sequencer (Foster City, Calif.) at the Macromolecular Structure Facility, Michigan State University, East Lansing.

Cell culture and labeling. A431 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, and PC12 cells were grown in DMEM containing 6.5% horse serum and 6.5% fetal bovine serum. A431 cells and PC12 cells were seeded in 10-cm-diameter dishes and grown until about 70% confluent, at which point they were switched into DMEM containing 0.5% dialyzed fetal calf serum for 36 to 48 h before the addition of 1.25 mCi of $^{32}P_i$ (ICN Radiochemicals, Irvine, Calif.) per dish. Fifteen hours after addition of radioactive label, cells were treated with 30 nM EGF or 1.5 nM nerve growth factor (NGF) (7S-NGF; Calbiochem) for the indicated time intervals or with 1 μM PMA or 100 μM forskolin for 10 min. Following treatments, cells were rinsed three times with ice-cold phosphate-buffered saline and lysed in 1 ml of cold RIPA buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, 50 mM NaF) per dish. Lysates were clarified at 14,000 × *g* for 30 min at 4°C. The resulting supernatants were treated for 4 h at 4°C with 3 μg of anti-PLC-γ1 MAb or 5 μl of antiserum to Nck. Then the immune complexes of anti-PLC-γ MAb were precipitated by a 2-h incubation with 60 μl of a 33% solution of protein A-Sepharose (Boehringer Mannheim) precoated with rabbit anti-mouse immunoglobulin G (IgG), and the immune complexes of anti-Nck antibody were precipitated by a 2-h incubation with 60 μl of a 33% solution of protein A-Sepharose alone. The precipitated immune complexes were washed five times with 1 ml of RIPA buffer. Proteins were released by the addition of 100 μl of Laemmli sample buffer and subjected to electrophoresis on an SDS–8% polyacrylamide gel. U937, Daudi, and Jurkat cells were maintained in RPMI1640 (Biofluid) supplemented with 10% fetal bovine serum. Prior to stimulation, U937 cells were fasted and at the same time radiolabeled by incubating for 18 h in RPMI 1640 containing 0.25% fetal bovine serum and 1.25 mCi of $^{32}P_i$. Daudi and Jurkat cells were labeled and stimulated without prior fasting. Cells were harvested by centrifugation, washed once with culture medium in which serum was replaced by 1 mg of bovine serum albumin per ml, and resuspended in the washing medium at the cell densities of 5×10^7 cells per ml for U937, 4×10^7 cells per ml for Daudi, and 1×10^7 cells per ml for Jurkat. U937 cells were preincubated with mouse anti-human FcγRII antibody (10 μg/ml) for 10 min and then stimulated by adding rabbit anti-mouse IgG $F(ab')_2$ (100 μg/ml) for 30 sec. Daudi cells were stimulated with goat $F(ab')_2$ anti-human IgM (20 μg/ml) for 5 min; Jurkat cells were stimulated with OKT3 (1:500 dilution of ascites fluid) for 1 min. Stimulation was terminated by the addition of 0.25 volume of ice-cold 5× RIPA buffer. Nck was immunoprecipitated as described for A431 and PC12 cells.

Tryptic phosphopeptide mapping. Immunoprecipitated ^{32}P-labeled proteins were separated on an SDS–8% polyacrylamide gel. The gel was dried and exposed to Kodak XAR film for autoradiography. The Nck band visualized by autoradiogram was excised from the gel, and protein was extracted and subjected to tryptic digestion as described (24). The resulting phosphopeptides were separated in two dimensions on a 100-μm thin-layer cellulose plate (EM Science) by electrophoresis for 30 min at 1 kV with pH 1.9 buffer (acetic acid–88% formic acid–water [156:50:1794, vol/vol]) followed by chromatography in *n*-butanol-pyridine-acetic acid-water (75:50:15:60, vol/vol).

RESULTS

Copurification of 47-kDa protein with PLC-γ1 on anti-PLC-γ1 MAb affinity column. Having learned that the anti-PLC-γ1 MAb B16-5 precipitates a 47-kDa protein as well as PLC-γ1, we prepared the immunoadsorbent of B16-5 and applied rat brain homogenate. After extensive washings, the column-bound proteins were eluted with Na citrate buffer (pH 3.0). Aliquots of each fraction were analyzed by SDS-PAGE and silver staining (Fig. 1A). A number of proteins were eluted along with the 145-kDa PLC-γ1. Bands indicating 66-, 56-, 47-, 43-, and 39-kDa proteins were apparent. To determine whether these proteins bound the column specifically, the flowthrough fractions from the B16-5 column were reapplied to another B16-5 column, which was then washed and eluted as before. Aliquots of fractions from the second column were analyzed by SDS-PAGE and silver staining (Fig. 1B). Intensities of the protein bands due to 66-, 56-, 43-, and 39-kDa proteins are nearly the same in Fig. 1A and Fig. 1B, whereas the 145- and 47-kDa bands are absent in Fig. 1B. This result indicates that PLC-γ1 and the 47-kDa protein bound the antibody column specifically, in contrast to the other four proteins that were retained by the column through nonspecific interaction. The absence of PLC-γ1 and the 47-kDa proteins in the flowthrough fractions indicates that the binding capacity of the antibody column was not a limiting factor.

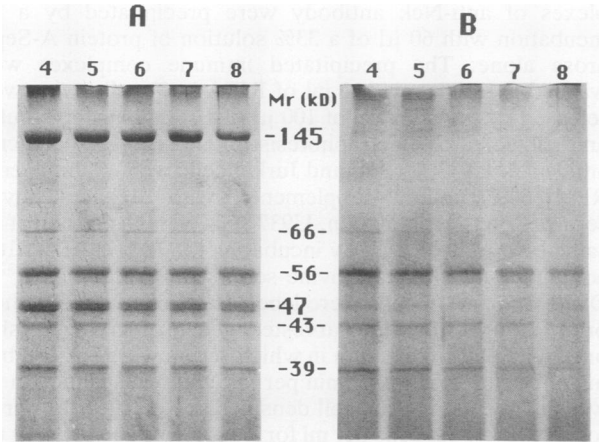

FIG. 1. SDS-PAGE analysis of fractions from the immunoaffinity column. Rat brain cytosolic proteins were applied to the B16-5 affinity column, and the column-bound proteins were eluted as described in Materials and Methods (A). The flowthrough fractions were reapplied to another B16-5 column, and the column was eluted as before (B). Aliquots (80 μl) of each fraction were separated on 10% gels, and proteins were visualized with silver staining. The molecular masses (Mr) of visualized protein bands are indicated in kilodaltons (kD). The numbers above each lane refer to the column fraction numbers.

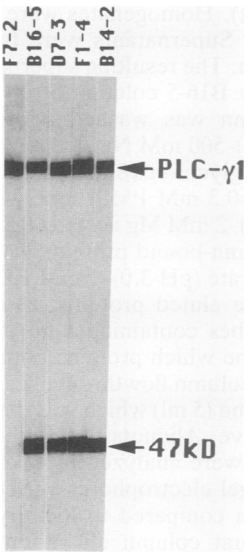

FIG. 2. Immunoreactivity of various anti-PLC-γ1 MAbs to PLC-γ1 and the 47-kDa protein. Fractions 4 through 8 (Fig. 1A) were pooled and concentrated. The concentrated proteins were analyzed on a SDS–10% polyacrylamide gel and transferred to nitrocellulose paper. Strips of nitrocellulose were probed with anti-PLC-γ1 MAbs. Lanes: 1, F7-2; 2, B16-5; 3, D7-3; 4, F1-4; 5, B14-2.

Fractions containing the 47-kDa protein were pooled and used for further experiments. The total amount of the 47-kDa protein in the pooled fractions obtained from 200 g of rat brain tissue was estimated to be ~40 μg on the basis of silver staining intensity. Inclusion of 1% Triton X-100 in homogenation buffer did not increase the recovery yield, suggesting that the 47-kDa protein is a mainly cytoplasmic protein. The immunoaffinity-purified proteins were subjected to immunoblot analysis with the use of several different anti-PLC-γ1 MAbs (Fig. 2). Previously, anti-PLC-γ1 MAbs were divided into six groups that recognize different epitopes on PLC-γ1 (37). The MAbs that belong to group IV (represented by B16-5 and D7-3 in lanes 2 and 3, respectively, of Fig. 2) and group V (represented by F1-4 and B14-2 in lanes 4 and 5, respectively, of Fig. 2) reacted with the 47-kDa protein as well as with PLC-γ1, whereas the MAbs of groups I, II, III, and VI (represented by F7-2 in lane 1) recognized only PLC-γ1. These results substantiate an earlier suggestion that the 47-kDa protein and PLC-γ1 share an epitope in common.

The 47-kDa protein is NCK. In an effort to characterize the 47-kDa protein, we obtained partial sequences of the protein. The affinity-purified proteins were separated by SDS-PAGE, and the band corresponding to the 47-kDa protein was cut and digested with V8 protease. After separation of the digestion mixture by SDS-PAGE, proteolytic fragments were transferred to a nitrocellulose membrane and partial sequences of five peptides (Fig. 3A) were obtained directly by using nitrocellulose strips. None of these sequences were found in PLC-γ1. However, a search of GenBank revealed that all five sequences could be found in a human protein called Nck. A cDNA corresponding to Nck has been cloned from a human melanoma library and sequenced by Lehmann et al. (15). As shown in Fig. 3B, Nck is a protein composed almost entirely of one SH2 domain and three SH3 domains (designated in order beginning at the amino terminus as SH3-1, SH3-2, and SH3-3). Of a total 36 amino acid residues identified for five peptides, only methionine, the first amino acid of peptide 2, was different from the residue (leucine) in the corresponding position of Nck. This minor difference can be attributed to species difference: the peptide sequences were from a rat protein whereas the Nck sequence was deduced from human cDNA. The SH3 sequence of PLC-γ1 shows amino acid sequence identities of 41, 54, and 28% with SH3-1, SH3-2, and SH3-3, respectively, of Nck. The carboxyl-terminal SH2 domain of PLC-γ1 is 36% identical to the Nck SH2. Immunoblots of *Escherichia coli*-expressed fusion proteins that contain SH2/SH3 domains of PLC-γ1 suggest that the binding site of B16-5 is located in the SH3 domain (data not shown). Identical stretches consisting of five to seven residues are shared by the SH3 domains of PLC-γ1 and Nck: Nck residues 52 to 57 (PSNYVE in SH3-1), 121 to 125 (REDEL in SH3-2), 142 to 146 (GWWRG in SH3-2), and 154 to 160 (WFPSNYV in SH3-2) perfectly match corresponding residues in PLC-γ1. However, none of the synthetic peptides corresponding to Nck residues 11 to 18 (FDYVAQQE) in SH3-1, 51 to 57, 119 to 126, 141 to 148, and 155 to 161 was able to block the binding of B16-5 to Nck or PLC-γ1 in immunoblots (data not shown). These results suggest that the binding site for B16-5 might consist of more than one stretch of amino acids in SH3.

Nck is widely distributed in tissues. The distribution of Nck in a variety of rat tissues was examined by immunoblot with B16-5. Figure 4A shows that Nck is widely expressed and that testes contain significantly more Nck than the other six tissues examined. We have also studied the changes in Nck and PLC-γ1 during development of the rat brain. PLC-γ1 decreased gradually between 4 days before and 27 days after birth. However, during the same period Nck levels remained unchanged (Fig. 4B).

Phosphorylation of Nck occurs rapidly following EGF treatment of A431 cells. To confirm that the 47-kDa protein recognized by B16-5 is Nck, we have examined the cross-reactivity of the 47-kDa protein with anti-Nck antibodies.

A.

peptide #1: LDIKKNE

peptide #2: MLWLLDDS

peptide #3: LSLIKGTK

peptide #4: KGDVMDVI

peptide #5: MALNE

B.

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          *  *** ** *:* * : *       :::  :***    :  *  :
  1 MAEEVVVVAK FDYVAQQEQE LDIKKNERLW LLDDSKSWWR VRNSMNKTGF
      SH3-1              #1       #2
     ******
 51 VPSNYVERKN SARKASIVKN LKDTLGIGKV KRKPSVPDSA SPADDSFVDP

                  *:* *: ***** : *:   :  :**   :***** * *
101 GERLYDLNMP AYVKFNYMAE REDELSLIKG TKVIVMEKCS DGWWRGSYNG
              SH3-2          #3
    *******                                          :
151 QVGWFPSNYV TEEGDSPLGD HVGSLSEKLA AVVNNLNTGQ VLHVVQALYP

    :   ::**  * *: ::: : **   * :** :          : * ***
201 FSSSNDEELN FEKGDVMDVI EKPENDPEWW KCRKINGMVG LVPKNYVTVM
    SH3-3          #4
                                      ** : :**  ***  *
251 QNNPLTSGLE PSPPQCDYIR PSLTGKFAGN PWYYGKVTRH QAEMALNERG
                                      SH2          #5
    ::* **:*   :  ** : :* ::*:** **  :**  :     :*   *  :
301 HEGDFLIRDS ESSPNDFSVS LKAQGKNKHF KVQLKETVYC IGQRKFSTME

    :*:  * *
351 ELVEHYKKAP IFTSEQGEKL YLVKHLS
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FIG. 3. Sequence comparison of the 47-kDa protein-derived peptides, Nck, and PLC-γ1 SH domains. (A) Partial sequences of the peptides derived from the 47-kDa protein. The 47-kDa protein band shown in Fig. 1A was cut, digested with V8 protease, and separated according to the method of Cleveland et al. (7). Partial sequences of five peptides were determined as described in Materials and Methods. (B) Comparison of the sequences of the five V8 peptides listed above and the SH (SH2 and SH3) domains of PLC-γ1 with the sequence of Nck. The amino acid sequence of Nck deduced from human cDNA has been reported (15). The sequence corresponding to those of the V8 peptides are double underlined. Numbers under each double underline identify the V8 peptides listed above. Nck contains three SH3 domains and one SH2, which are underlined and sequentially labeled as SH3-1, SH3-2, SH3-3, and SH2. The sequence of the PLC-γ1 SH3 domain is compared to those three Nck SH3 domains. Also, the sequence of the carboxy-terminal SH2 of PLC-γ1 is compared to that of the Nck SH2. Identical and conservatively changed amino acids are indicated by stars and colons, respectively.

Since the 47-kDa protein coimmunoprecipitated with PLC-γ1 was shown to be a phosphoprotein (24, 43, 44), studies also included the effect of growth factor. A431 cells were treated with or without EGF, and the cell lysates were immunoprecipitated with B16-5 or anti-Nck antibodies. The precipitated proteins were analyzed on SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-Nck antibodies, B16-5, and anti-PY MAb. Figure 5 shows that the mobility of the 47-kDa protein precipitated by B16-5 is the same as that of the 47-kDa protein precipitated by anti-Nck antibodies (left and middle panels). Furthermore, the B16-5-precipitated 47-kDa protein was recognized by anti-Nck antibodies and Nck was likewise recognized by B16-5. When the B16-5-precipitated proteins were blotted with B16-5, a doublet of the 84-kDa protein was visible in addition to PLC-γ1 and Nck (first and second lanes of middle panel). This suggests that the 84-kDa protein might be a SH3-containing phosphoprotein. PLC-γ1 and the 84-kDa protein were neither precipitated nor blotted by anti-Nck

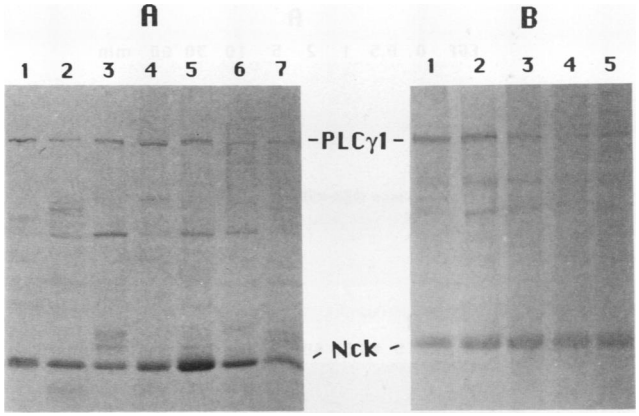

FIG. 4. Tissue distribution and developmental changes of PLC-γ1 and Nck. Tissues from Sprague-Dawley rats were homogenized in 50 mM Tris-HCl–1 mM EDTA–1 mM dithiothreitol–1 mM PMSF, and 10 μg each of leupeptin and aprotinin per ml. The homogenates were centrifuged at 1,000 × *g* for 10 min to remove nuclei and tissue debris. The resulting supernatants (50 μg of protein per lane) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with B16-5. (A) Distribution in adult rat tissues. Lanes: 1, brain; 2, heart; 3, lung; 4, liver; 5, testis; 6, pancreas; 7, kidney. (B) Developmental changes in rat brain Nck. Lanes: 1, 4 days before birth; 2, 1 day old; 3, 4 days old; 4, 14 days old; 5, 27 days old.

antibodies. Nck from EGF-stimulated but not from unstimulated cells was recognized by anti-PY MAb (right panel). The anti-PY MAb also reacted with PLC-γ1 and EGFR in the B16-5-precipitated proteins from the EGF-treated cells

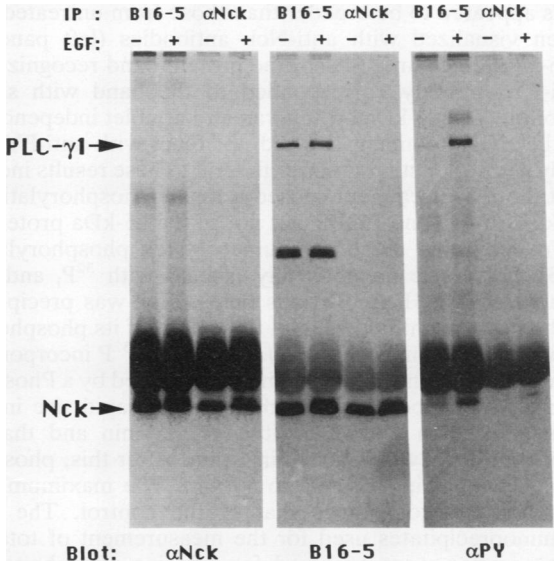

FIG. 5. Immunoblot analysis of proteins precipitated from A431 cells with B16-5 and anti-Nck antibodies. Lysates were prepared from cells treated with EGF for 1 min (+) or untreated cells (−). As indicated at the top of the figure, immunoprecipitation was performed with B16-5 or anti-Nck antibodies (αNck). Immunoprecipitates from three 10-cm dishes were pooled, divided into three identical sets, analyzed by SDS-PAGE, and immunoblotted with anti-Nck antibodies (left panel), B16-5 (middle panel), or anti-PY MAb (right panel), as indicated at the bottom.

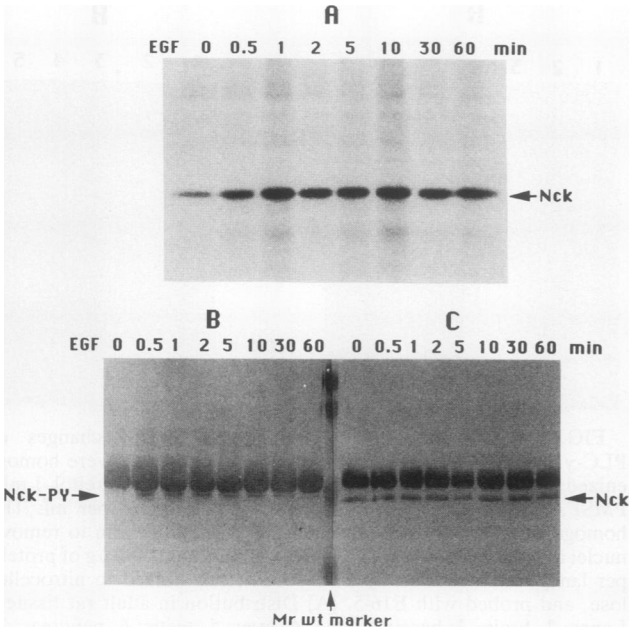

FIG. 6. Time course of EGF-stimulated Nck phosphorylation in A431 cells. Cells were incubated with 1.25 mCi of $^{32}P_i$ per 10-cm dish for 15 h prior to treatment with 30 nM EGF for the times indicated at the top of the figure. Immunoprecipitates obtained with anti-Nck antibodies from the cell lysates were divided into three sets. After SDS-PAGE analysis, one set was autoradiographed (panel A, 2-h exposure at −70°C) and the other two sets were immunoblotted with anti-PY MAb (panel B) or anti-Nck antibodies (panel C). Mr wt, molecular weight.

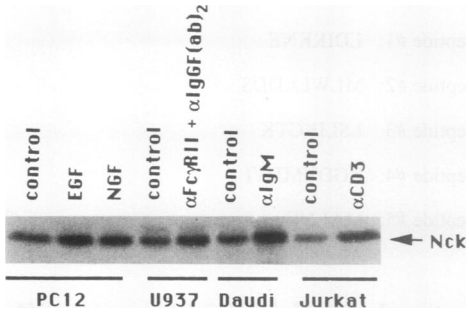

FIG. 7. Phosphorylation of Nck in response to various receptors. PC12, U937, Daudi, and Jurkat cells were each incubated with 1.25 mCi of $^{32}P_i$, and their respective cell surface receptors were stimulated as described in Materials and Methods with corresponding ligands as indicated at the top of the figure. The cell lysates were immunoprecipitated with B16-5, and the immunoprecipitates were analyzed by SDS-PAGE and autoradiography (4-h exposure at −70°C).

(lane 2 of right panel). The Nck bands from the EGF-treated cells appeared to be broader than those from untreated cells when visualized with anti-Nck antibodies (left panel) or B16-5 (middle panel). Also, the protein band recognized by anti-PY antibody corresponded to the band with slower mobility. The 84-kDa protein ran as a doublet independently of the EGF treatment and did not react with anti-PY antibody even after stimulation with EGF. These results indicate that the EGF treatment caused tyrosine phosphorylation of Nck, PLC-γ1, and EGFR but not of the 85-kDa protein.

To determine the time course of Nck phosphorylation, A431 cells were metabolically labeled with $^{32}P_i$ and then treated with EGF for various times. Nck was precipitated with anti-Nck antibodies, and the extent of its phosphorylation was determined (Fig. 6A). From total ^{32}P incorporation into Nck following EGF treatment, measured by a PhosphorImager, it can be seen that there was a rapid rise in Nck phosphorylation within as little as 0.5 min and that the maximum level was reached in 1 min. After this, phosphorylation was maintained up to 60 min. The maximum level was three to four times that of the control. The same immunoprecipitates used for the measurement of total ^{32}P incorporation were analyzed for tyrosine phosphorylation (Fig. 6B). The time course of tyrosine phosphorylation of Nck was similar to that of total ^{32}P incorporation, which mostly reflects serine phosphorylation according to the phosphoamino acid analysis in our laboratory (data not shown) and in the accompanying papers by Meisenhelder and Hunter (23) and Li et al. (16). An immunoblot obtained with anti-Nck antibodies (Fig. 6C) revealed that each lane received similar amounts of Nck. Comparison of the mobilities of Nck in Fig. 6B with those in Fig. 6C shows that tyrosine-phosphorylated Nck migrated more slowly in the SDS gel.

Phosphorylation of Nck occurs in response to stimulation of a variety of receptors. The receptor for NGF is a protein tyrosine kinase, and its occupancy by NGF elicits the activation of the receptor tyrosine kinase as well as several serine/threonine kinases in the pheochromocytoma cell line PC12 (11, 13). PC12 cells also contain EGFR. Figure 7 shows that stimulation of PC12 cells by EGF or NGF caused the phosphorylation of Nck as evidenced by the enhanced ^{32}P radioactivity in the immunoprecipitated Nck band.

Several leukocyte receptors were also examined for effects on Nck phosphorylation. The T-cell-receptor complex (TCR), low-affinity IgG receptor (FcγRII), and membrane IgM (mIgM) are not protein kinases but are functionally linked to the activation of a number of protein kinases, including certain nonreceptor tyrosine kinases, and elicit phosphorylation of PLC-γ1 on both serine and tyrosine residues (5, 8, 17, 28, 34). Ligation or oligomerization of FcγRII in a monocytic cell line (U937), membrane IgM in a B-cell line (Daudi), and TCR in a T-cell line (Jurkat) all caused the stimulation of Nck phosphorylation (Fig. 7).

EGF elicits association between Nck and EGF receptor. Recently it has been observed that some SH2-containing proteins are coprecipitated with autophosphorylated receptor kinases (3, 9, 10, 14, 19, 22). Therefore, we immunoprecipitated Nck from lysates of EGF-stimulated as well as unstimulated A431 cells by using anti-Nck antibodies to see whether the Nck is physically associated with EGFR. The immune complexes were subjected to SDS-PAGE analysis, transferred to nitrocellulose membrane, and immunoblotted with anti-EGFR or anti-PY MAb. Figure 8 shows that EGFR coimmunoprecipitated with Nck and that the coprecipitation was dependent on EGF. The EGFR precipitated from EGF-stimulated cells was recognized by anti-PY MAb. The presence of similar amounts of Nck in the lanes for EGF-stimulated and unstimulated cells was verified by immunoblotting with anti-Nck antibodies (data not shown).

Phorbol ester and forskolin enhance the phosphorylation of Nck. It has been shown that PMA and cyclic AMP (cAMP)-elevating agents attenuate receptor-coupled PLC activity and that the attenuation seen in a number of cell lines can, at least in part, be attributed to the phosphorylation of PLC-γ1

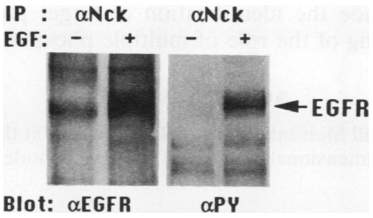

FIG. 8. Coimmunoprecipitation of Nck and EGFR with anti-Nck antibodies. A431 cells were lysed after stimulation with (+) or without (−) EGF for 5 min. As indicated at the top of the figure, immunoprecipitation was then performed with anti-Nck antibodies (αNck). The immunoprecipitates were analyzed by SDS-PAGE and immunoblotted with either anti-EGFR antibodies (αEGFR) or anti-PY MAb (αPY) as indicated at the bottom.

by either protein kinase C or cAMP-dependent protein kinase (13, 27). During the course of this study, we observed that the ^{32}P radioactivity associated with several proteins in the immunoprecipitate with anti-PLC-γ1 MAbs increased following treatment with PMA or a cAMP-elevating agent such as forskolin or 8-bromo-cAMP.

We extended this investigation by using A431 cells labeled with $^{32}P_i$. The labeled cells were treated with either PMA or forskolin for 10 min, washed, solubilized, and subjected to immunoprecipitation with B16-5, followed by SDS-PAGE and autoradiography. Both treatments resulted in a four- to fivefold increase in phosphorylation (Fig. 9). Phosphorylation of PLC-γ1 and two proteins of 84 and 100 kDa was also enhanced. Two-dimensional analysis of phosphopeptides was undertaken to compare maps of Nck from untreated cells with those from cells treated with either PMA or forskolin (Fig. 10). Nck isolated from untreated cells gave rise to at least five tryptic phosphopeptides designated as peptides 1 through 5, as shown in Fig. 10, top panel. Treatment with PMA or forskolin resulted in increased phosphorylation of these five peptides, but no new phosphopeptide was apparent. It was also clear that the increase in the phosphorylation of peptide 3 was more pronounced in PMA-treated cells than in forskolin-treated cells (Fig. 10, middle and bottom panels). This result suggests that while multiple phosphorylation of Nck occurs at the same sites whether triggered by PMA or cAMP, the protein kinases thus activated exhibit different activities toward each of those sites.

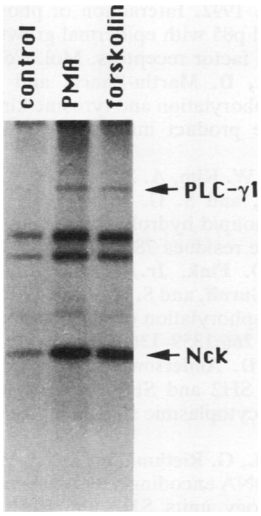

FIG. 9. Phosphorylation of Nck by PMA or forskolin treatment in A431 cells. A 10-cm dish of cells was incubated with 3 mCi of $^{32}P_i$ for 15 h prior to treatment with 1 μM PMA or 100 μM forskolin for 10 min. Cell lysates were immunoprecipitated with B16-5 and analyzed by SDS-PAGE and autoradiography (2-h exposure at −70°C).

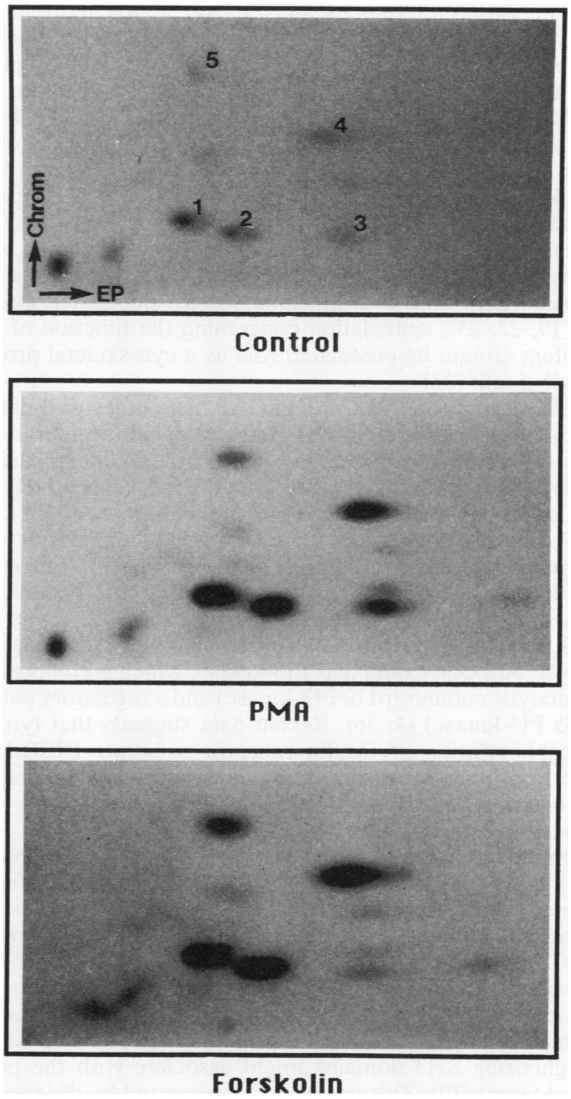

FIG. 10. Tryptic phosphopeptide maps of Nck phosphorylated by PMA and forskolin. Nck extracted from gel bands in Fig. 9 was digested with trypsin, separated in two dimensions, and autoradiographed (exposure time, 7 days), as described in Materials and Methods.

DISCUSSION

Nck is a SH protein (a protein that contains either the SH2 or SH3 domain or both domains) consisting of little other than one SH2 and three SH3 domains linked in the order SH3-SH3-SH3-SH2 beginning at the amino terminus. We have shown here that Nck is the 47-kDa protein consistently seen in the SDS-PAGE of the proteins that were precipitated

from a variety of cells by anti-PLC-γ1 MAbs and that Nck and PLC-γ1 share a high amino acid sequence identity between their respective SH3 domains, where the epitope for the Nck-precipitating anti-PLC-γ1 MAb is located.

Although most SH proteins contain both SH2 and SH3 domains, there are many which have one without the other. For example, both the SH domains found in GAP are SH2, while only the SH3 is found in myosin-1B (33). The separate appearances of SH2 and SH3 domains suggests that these domains could have independent functions. Indeed, while convincing evidence has shown that the function of the SH2 domain is to bind in a phosphotyrosine-dependent manner (1, 19, 22, 25), speculation concerning the function of SH3 centers around its postulated role as a cytoskeletal protein-binding unit (33).

SH proteins like PLC-γ and GAP have other well-defined domains in addition to SH domains which confer to the proteins their characteristic functional abilities, such as hydrolyzing PI and activating ras GTPase, respectively. On the other hand, SH proteins like p85 PI3-kinase (9, 35), Crk (21), Sem-5 (6), and Nck (15) carry no additional functional domains. Recent data suggest that one possible role of the latter class of proteins is to act as adaptors that couple tyrosine-phosphorylated proteins to catalytic targets that lack their own tyrosine phosphate-binding SH2 domain (6, 14, 22, 30). One example is PI3-kinase, which is composed of a catalytic subunit (p110 PI3-kinase) and a regulatory subunit (p85 PI3-kinase) (4, 26). Recent data suggests that tyrosine phosphorylation of insulin receptor substrate-1(IRS-1) by the insulin receptor tyrosine kinase and the subsequent association of IRS-1 with p85 PI3-kinase activate the catalytic activity of p110 PI3-kinase (2).

Another example of the adaptor role that SH proteins might play is found in Sem-5, a *Caenorhabditis elegans* protein consisting exclusively of one SH2 and two SH3 domains. Genetic evidence suggests that Sem-5 couples Let-23 receptor tyrosine kinase (a *C. elegans* analog of EGF receptor) directly or indirectly to Let-60 (*C. elegans* analog of ras) (6). It was further envisaged that while the SH2 domain of Sem-5 binds to the autophosphorylated Let-23, neighboring SH3 domains might associate with the postulated target (30). This notion is corroborated by the fact that a single mutation in the SH3 domain of Sem-5 is critical for the normal functioning of Sem-5 (6).

The example of p85 PI3-kinase and Sem-5 presents a case for sketching a similar adaptor model for Nck. Our data suggest that Nck tightly associates with autophosphorylated EGFR and is subsequently phosphorylated on tyrosine and serine residues. In addition, studies with TCR, FcγRII, and membrane IgM suggest that Nck is a target for protein kinases coupled to these leukocyte receptors. It is well established that stimulation of a variety of leukocyte receptors, including TCR, FcγRII, and membrane IgM, elicits the activation of nonreceptor tyrosine kinases, members of the src family (8). Thus, possible candidates for the target of the Nck SH2 domain include a variety of growth factor receptor kinases and nonreceptor tyrosine kinases. However, at the present time, there are no experimental clues to guide speculation on the other target to which the SH3 domains of Nck bind.

The adaptor role, in which Nck coordinates these target molecules, may be modulated by multiple phosphorylation, as Nck appears to be a substrate for a variety of serine/threonine kinases activated by growth factors, PMA, and cAMP, as well as for protein tyrosine kinases linked to various cell surface receptors. Thus, future research on Nck should include the identification of target proteins and an understanding of the role of multiple phosphorylation.

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