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Binding of NCK to SOS and Activation of *ras*-Dependent Gene Expression

QIANJIN HU, DALE MILFAY, AND LEWIS T. WILLIAMS*

Daiichi Research Center, Cardiovascular Research Institute, University of California at San Francisco, San Francisco, California 94143

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NCK, an SH2- and SH3 domain-containing protein, becomes phosphorylated and associated with tyrosine kinase receptors upon growth factor stimulation. The sequence of NCK suggests that NCK functions as a linker between receptors and a downstream signaling molecule. To determine if NCK can mediate growth factor-stimulated responses, we measured the ability of NCK to activate the *fos* promoter. We found that in NIH 3T3 cells, NCK strongly activates this promoter. The effect of NCK on the *fos* promoter is enhanced by c-*ras* and blocked by dominant negative *ras*. We also found that NCK binds directly to the guanine nucleotide exchange factor SOS. This interaction is mediated by the SH3 domains of NCK. These findings suggest that NCK can regulate p21^{ras}-dependent gene transcription through interaction with SOS protein.

NCK, a 45-kDa protein consisting of one SH2 domain and three SH3 domains, is expressed in a wide range of tissues and cell lines (20). After stimulation of cells by growth factors, NCK becomes phosphorylated on Ser, Thr, and Tyr residues and associates with tyrosine kinase receptors (5, 17, 21, 25, 28). In this way, NCK resembles other proteins that have SH2 domains and associate with receptors through interaction between SH2 domains and specific phosphotyrosines (13, 19, 24, 29). Overexpression of NCK transforms mammalian fibroblasts, suggesting that its SH2 and SH3 domains may play important roles in cell growth regulation (5, 21). However, the mechanism of action of NCK is not understood.

Recent studies of SH2- and SH3 domain-containing signaling molecules that associate with tyrosine-phosphorylated receptors have shown that signaling molecules are of two general types. The first type, typified by phospholipase C-γ, is an enzyme itself. The p85 subunit of phosphatidylinositol 3-kinase (10, 27, 34) represents a second type of receptor-associated molecule, in that it probably has no defined intrinsic enzyme activity but serves as a linker that connects an enzyme, in this case the catalytic subunit of phosphatidylinositol 3-kinase, to a receptor. Another example of the second type of linking molecule is the Sem5 gene product in *Caenorhabditis elegans* and its mammalian counterpart, GRB2. The SH2 domain of Sem5/GRB2 binds to tyrosine kinase receptors (22, 26, 33). The SH3 region of Sem5/GRB2, like the SH3 region of the Abelson protein (6, 7, 30), binds to a proline-rich motif that is a common feature of a number of molecules implicated in growth regulation (9, 16, 26, 32, 33).

The absence of a recognizable catalytic domain of NCK suggests that NCK serves as a linker molecule connecting tyrosine-phosphorylated proteins to enzymes which might lack their own SH2 domains. It has been shown that NCK forms a physical complex with the platelet-derived growth factor (PDGF) receptor and the epidermal growth factor receptor in a ligand-dependent manner (17, 21). To determine if NCK can mediate growth factor-stimulated early cellular responses, we tested the ability of NCK to activate transcription of the *fos* gene, a well-characterized immediate-early response gene. We found that NCK strongly activates the *fos* promoter in a *ras*-dependent manner. In investigating how NCK interacts with the *ras* pathway, we found that NCK binds directly to the guanine nucleotide exchange factor SOS.

MATERIALS AND METHODS

Cell culture. CHO cells were grown in Ham's F12 medium supplemented with 10% calf serum and with penicillin and streptomycin (50 mg/ml each). Stable CHO cell lines transfected with pSV-PDGF-r or pBJ-NCK-HI were cultured in the same medium containing 400 mg of G418 per ml.

Immunoprecipitation. The *NCK* gene was cloned from human K562 cDNA by PCR. After confirmation by sequencing, the fragment was tagged with an influenza virus hemagglutinin (HI) epitope (NCK-HI) and cloned in a mammalian expression vector pBJ (36) (pBJ-NCK-HI). After stimulation with or without PDGF-BB (2 nM) for 5 min, the stable CHO cells were lysed in 150 mM NaCl–20 mM Tris (pH 7.5)–10% glycerol–1% Triton–1 mM phenylmethylsulfonyl fluoride–aprotinin and leupeptin (1 mg/ml each)–10 mM Na_3VO_3–10 mM NaF. The lysates (1 ml from one 15-cm-diameter plate) were immunoprecipitated with anti-HI antibody 12CA5 (at 1:2,000 dilution) (13) at 4°C for 4 h and then washed with the same lysis buffer. The proteins were separated by polyacrylamide gel electrophoresis (PAGE) (6% gel) and transferred to nitrocellulose filters. The filters were probed with anti-SOS rabbit antiserum (1:2,000 dilution), raised against a synthetic peptide spanning residues 40 to 62 of the mouse SOS-1 (mSOS-1) amino acid sequence (2).

Far-Western blotting. For assay of direct binding of the SH3 regions of NCK to SOS in vitro, the CHO cell lysates were precipitated with preimmune or anti-SOS antiserum (1:1,000 dilution). The SOS proteins were separated by sodium dodecyl sulfate (SDS)-PAGE (6% gel) and transferred to a nitrocellulose filter. The three SH3 domains of NCK (amino acids [aa] 1 to 249) were cloned into a pGEX derivative vector pG-IKS (kindly proved by D. Mirda) containing a glutathione *S*-transferase (GST) sequence and a heart muscle kinase site (RRASV). The GST-NCKSH3 (three SH3 domains of NCK protein) fusion protein was purified on glutathione-Sepharose beads and ^{32}P labeled as described by Kaelin et al. (18). The filter was incubated with ^{32}P-labeled GST-NCKSH3 or ^{32}P-labeled GST-p85SH3 (SH3 domain of p85 protein; kindly provided by A. Klippel) protein, with or without SOS peptides, in 22 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-KOH (pH 7.7)–75 mM KCl–0.1 mM EDTA–2.5 mM $MgCl_2$–1% milk–0.05% Nonidet P-40–1 mM dithiothreitol (1, 18). The purified recombinant NCK (wild type), NCK-332 (aa 110 to 375), and NCK-32 (aa 210 to 375) proteins were kindly provided by X. Wu and J. Kuriyan.

Two-hybrid system. The SH3 regions of NCK (residues 1 to 249), full-length GRB2, the SH3 domain of p85, the SH3 domain of GTPase-activating protein (GAP; residues 273 to 351), and SNF1 cDNA were fused to the C terminus of the GAL4 DNA binding domain (4) in yeast expression vector pGTB9, which has a Trp^+ selection marker; the C-terminal portion of the mSOS-1 sequence (residues 986 to the C-terminal end) or SNF4 cDNA was fused to the N terminus of the GAL4 activation domain (4) in yeast expression vector pGAD GH, which contains a Leu^+ selection marker. Each pair of these plasmid constructs was cotransfected into *Saccharomyces cerevisiae* YGHI (Trp^- Leu^- His^-) by the

* Corresponding author. Mailing address: University of California, 505 Parnassus Ave., Box 0130, San Francisco, CA 94143-0130. Phone: (415) 476-4402. Fax: (415) 476-0429.

polyethylene glycol-lithium acetate method (31), and the cells were then plated on Trp^-, Leu^-, and His^- selection medium and incubated at 30°C for 3 to 4 days.

c-*fos* promoter-luciferase assay. The c-*fos* promoter-driven luciferase plasmid (pFL700) was constructed by replacing the *cat* sequence (cut with *Xba*I and *Bam*HI) in plasmid pFC700 (15) with the luciferase gene (cut with *Nhe*I and *Bam*HI) from pGL2 (Promega). pBJ-c-ras was constructed by digestion of pV-IKS-c-ras (kindly provided by A. Kikuchi) with *Eco*RI and *Bag*II. The c-*ras* insert was then cloned into the pBJ vector. The dominant negative *ras* (DN-*ras*) sequence was isolated from pBSK-DN-ras (kindly provided by A. Kikuchi) by *Xho*I and *Not*I digestion and ligated into *Xho*I and *Not*I sites of the pBJ vector.

Transfection was carried out as described by Wagner et al. (37). Briefly, NIH 3T3 cells were plated at 10^5 cells per well (12-well plate) 12 to 24 h before transfection; 0.5 mg of each plasmid DNA in 100 ml of 0.25 M $CaCl_2$ was added dropwise to 100 ml of 2× HBBS (50 mM HEPES [pH 7.05], 280 mM NaCl, 1.5 mM Na_2HPO_4) to form precipitates. The DNA precipitates were added into each well and incubated at 37°C for 20 min. Dulbecco modified eagle (DME) medium with 10% calf serum was then added to each well. After incubation at 37°C for 4 h, the medium was replaced with fresh DME medium–0.5% calf serum. After another 48 h of incubation at 37°C in 5% CO_2, the cells were lysed in 100 ml of lysis buffer (Promega), the luciferase activity assay was performed by adding luciferase substrate (Luciferin; Molecular Probes), and activity was measured with a luminometer (Monolight 2221; Analytical Luminescence Laboratory).

RESULTS

Activation of the c-*fos* promoter by NCK through the *ras* pathway. The c-*fos* gene has been shown to be one of the immediate-early genes responsive to PDGF and many other growth stimuli (8, 37). To explore the possibility that NCK mediates growth factor-stimulated early cellular responses, we used a detection system in which the c-*fos* promoter drives expression of luciferase. Plasmids containing NCK cDNA and the *fos*-luciferase reporter were cotransfected into NIH 3T3 cells, and luciferase activity was measured as described in Materials and Methods. The luciferase activity was increased upon NCK transfection, and this activity was further enhanced by cotransfection of a c-*ras* cDNA construct, although c-*ras* alone had little effect on luciferase activity (Fig. 1A). In contrast, the NCK-induced activity was completely blocked by cotransfection of a DN-*ras*, which has been shown to be a potent inhibitor of endogenous c-*ras* activities (3, 12). To further confirm that this activation is dependent on the *ras* signaling pathway, a dominant negative *raf* mutant construct (NAF) (23) was cotransfected with NCK. The activation of the *fos* promoter by NCK was inhibited by NAF (Fig. 1B). The expression level of NCK was not affected by cotransfection with c-*ras*, DN-*ras*, or NAF (Fig. 1C). These results strongly suggest that NCK is capable of activating the early-response gene, *fos*, in a *ras*-dependent manner.

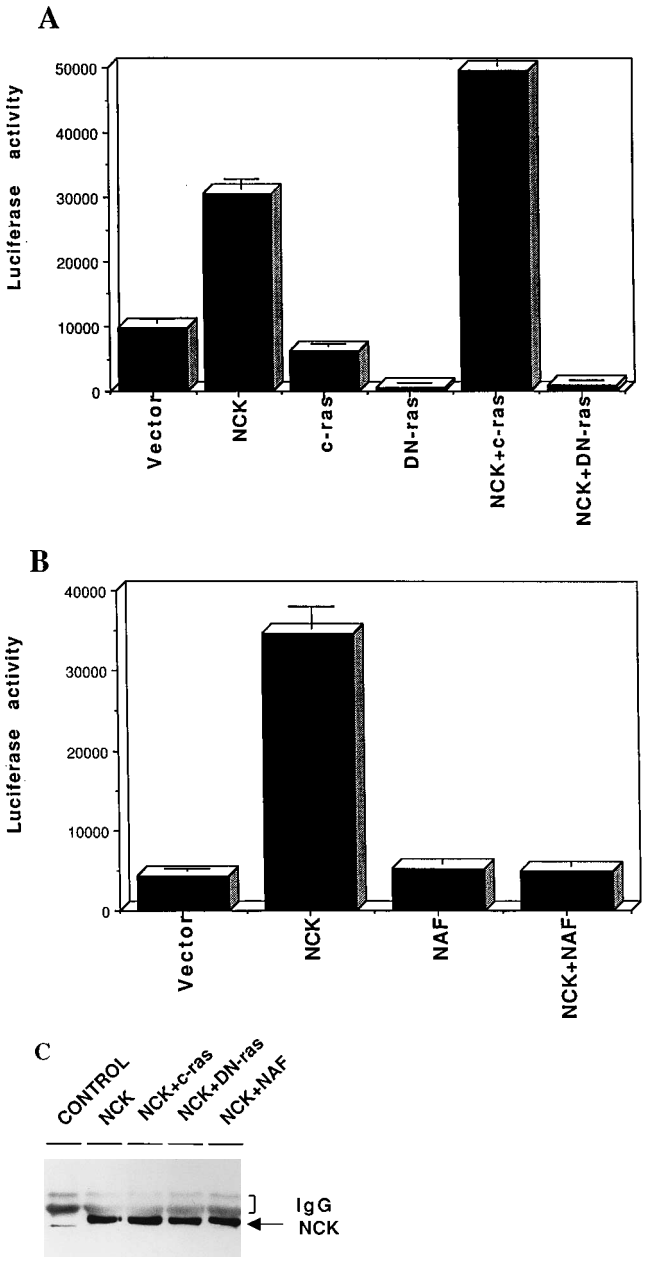

FIG. 1. Activation of a c-*fos* promoter by NCK. The indicated plasmids were transfected into NIH 3T3 cells along with a c-*fos* promoter-driven luciferase plasmid, pFL700, as described in Materials and Methods. After 48 h in 0.5% calf serum–DME medium, the cells were lysed and the luciferase activities were measured (A and B) or the cell lysate was immunoprecipitated and Western blotted with an anti-HI tag antibody, 12CA5 (C). Each bar in panels A and B represents the mean of triplicate samples. IgG, immunoglobulin G.

NCK associates with SOS in vitro and in vivo. To investigate the mechanism of NCK interaction with the *ras* pathway, we searched for NCK-associated proteins. NCK-HI was stably transfected into CHO cells, and immunoprecipitations were then carried out with an anti-HI monoclonal antibody, 12CA5, from the lysates of these cells. The immunoprecipitates were separated by SDS-PAGE and immunoblotted with various candidate antibodies. The SOS protein, a guanine nucleotide exchange factor, was found to be coprecipitated with the NCK-HI protein in the NCK-HI-transfected cells (Fig. 2A, lane 2). There was no detectable SOS protein in anti-HI immunoprecipitation of nontransfected cells (Fig. 2A, lane 1), although they had the same amount of SOS protein as the transfected cells (Fig. 2A, lanes 3 and 4). To address whether this association was caused by overexpression of NCK-HI, we examined the expression levels of endogenous NCK and NCK-HI. The lysates from NCK-HI-transfected and untransfected CHO cells were Western blotted with an anti-NCK antibody (Transduction Laboratories) that recognizes denatured forms of NCK. It appeared that the amount of NCK-HI (the slower-migration form due to the HI tag) was lower than the amount of endogenous NCK (Fig. 2B).

The association of NCK and tyrosine kinase receptors appears to be ligand dependent; therefore, the effects of PDGF stimulation on the interaction of NCK and SOS were examined. CHO cells stably transfected with the PDGF receptor and NCK-HI cDNA were treated with or without PDGF for 5 min, and the cell lysates were analyzed as described above, using anti-SOS antiserum. In response to PDGF stimulation, the mobility of SOS was shifted (Fig. 2C, lanes 1 to 4) as a

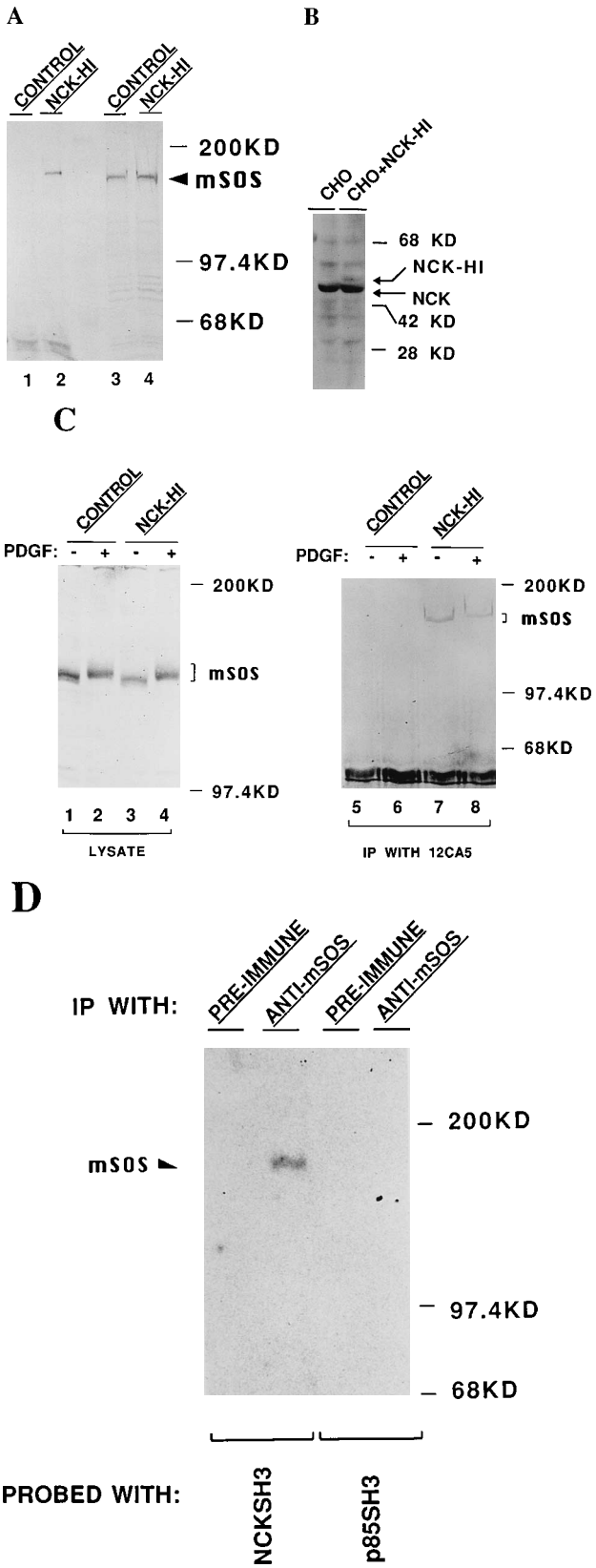

FIG. 2. NCK associates with SOS proteins in vitro and in vivo. (A) CHO cells stably transfected with NCK-HI were lysed and immunoprecipitated with an anti-HI tag monoclonal antibody, 12CA5. The immunoprecipitates (lanes 1 and 2) and the total lysate (lanes 3 and 4) were separated on an SDS–6% gel, transfected to a nitrocellulose filter, and probed with anti-mSOS antiserum. (B) Lysates from NCK-HI-transfected and untransfected CHO cells were Western blotted with an anti-NCK antibody (Transduction Laboratories). (C) CHO cells stably transfected with PDGF receptor and NCK were treated with or without PDGF (2 nM) for 5 min, and Western blot analysis was performed as described for panel A. IP, immunoprecipitation. (D) CHO cell lysates were precipitated with either rabbit preimmune (lanes 1 and 3) or anti-mSOS (lanes 2 and 4) serum. The proteins were separated on an SDS-gel, and the blots were probed with either ^{32}P-labeled GST-NCKSH3 (lanes 1 and 2) or ^{32}P-labeled GST-p85SH3 (lanes 3 and 4).

result of phosphorylation of SOS (17, 21). This mobility shift, however, did not appear to affect the interaction of SOS with NCK (Fig. 2C, lanes 7 and 8). This experiment shows that the fraction of SOS that is associated with NCK becomes modified as a consequence of PDGF stimulation.

Proline-rich sequences, such as XPXXPPPXXP, are known to bind SH3 domains (9, 30). The C terminus of SOS (2) contains those motifs, suggesting that this region of SOS may bind directly to the SH3 domains of NCK. To test whether NCK can directly interact with SOS, we performed a far-Western blot analysis. CHO cell lysates were immunoprecipitated with anti-SOS or preimmune serum. After transfer to nitrocellulose, the filters were probed with ^{32}P-labeled GST-NCKSH3 and ^{32}P-labeled GST-p85SH3 proteins, respectively. SOS was detected only by the NCKSH3 probe (Fig. 2D), indicating that the interaction between the SH3 domains of NCK and the SOS is not only specific but also independent of other cellular proteins.

The immunoprecipitation and far-Western experiments demonstrated that NCK and SOS can form a stable complex in vitro. To further confirm that the interaction between SOS and NCK occurs in living cells and to demonstrate the specificity of this interaction, a yeast two-hybrid system which has been used by others to detect protein-protein interactions (4, 14) was adapted. The three SH3 regions (aa 1 to 249) of NCK (NCKSH3) were fused to the C-terminal end of the GAL4 DNA binding domain. Human Sem5, also known as GRB2 (22), and the SH3 domains of p85 and GAP (p85SH3 and GAPSH3) were also fused to the GAL4 DNA binding domain. The C-terminal half of SOS (aa 986 to 1336) was fused to the N-terminal-end GAL4 activation domain. As a positive control, SNF1 and SNF4 were fused to the GAL4 DNA binding and activation domains, respectively, since the interaction of SNF1 and SNF4 in this system has been well documented (4, 14). The GAL4 DNA binding plasmid contained a Trp^+ selection marker, whereas the plasmid containing the activation domain had a Leu^+ selection marker. Each pair of the DNA binding and activation plasmids was cotransfected into *S. cerevisiae* YGH1 (Leu^- Trp^- His^-), containing a *his*$^+$ gene driven by a GAL4 promoter. Transfected cells were plated on Leu^-, Trp^-, and His^- selection plates. If the fusion proteins interacted, the *his*$^+$ gene was expressed and the cells grew on the selection medium. The results of this experiment clearly demonstrated the interaction of NCKSH3 with SOS in intact cells (Fig. 3), supporting the coimmunoprecipitation results in CHO cells (Fig. 2). In contrast, cells cotransfected with the SOS fusion protein and fusion proteins containing other SH3 domains, such as the SH3 domain of p85 or GAP, failed to grow on His^- plates, showing the specificity of the interaction between the SH3 domains of NCK and SOS. As expected, the control experiments showed that SNF1 and SNF4 interacted with each other but not with SOS and NCK (Fig. 3). Our studies in intact cells confirmed that SOS also interacts with GRB2, consistent with recent reports that Drk, a *Drosophila* GRB2 homolog, forms a complex with SOS protein in vitro (26, 33) and that GRB2 binds to SOS in vivo (9, 16, 32).

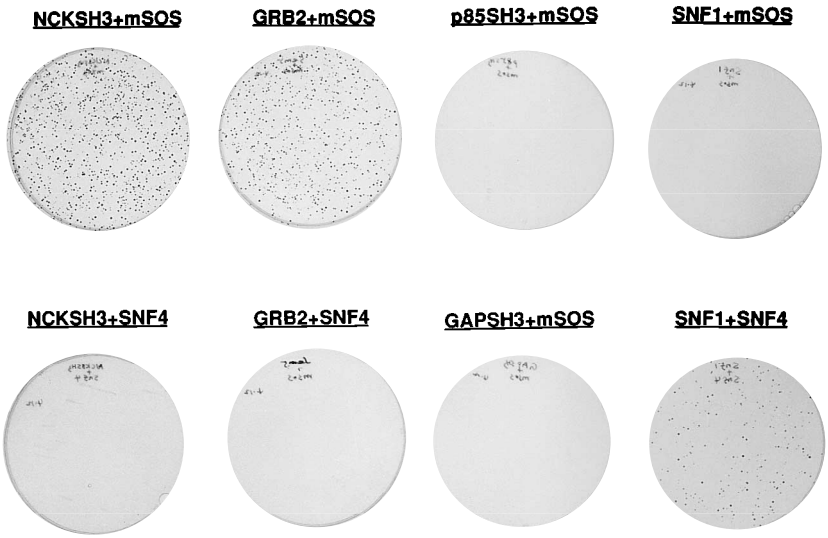

FIG. 3. NCK interacts with SOS in intact cells. The SH3 region of NCK, full-length GRB2, the SH3 domain of p85, the SH3 domain of GAP (aa 273 to 351), and SNF1 cDNA were fused to the C terminus of the GAL4 DNA binding domain in plasmid pGTB9, which has a Trp^+ selection marker; the C-terminal tail of the mSOS sequence (residue 986 to the C terminus of mSOS-1) or full-length SNF4 cDNA was fused to the N terminus of the GAL4 activation domain in plasmid pGAD GH, which has a Leu^+ selection marker. Each pair of constructs was cotransfected into *S. cerevisiae* YGH1 (Trp^- Leu^- His^-), and the cells were plated on Trp^-, Leu^-, and His^- selection medium and incubated at 30°C for 3 days. Each spot represents a colony that survived in the selection medium.

Mapping the binding sites on SOS and NCK. SOS protein has several proline-rich motifs in its carboxyl-terminal end. To identify which of these proline-rich motifs are responsible for the NCK-SOS interaction, a series of peptides corresponding to these motifs was synthesized. SOS proteins were immunoprecipitated from CHO cells and transferred to nitrocellulose. The filters were then probed with ^{32}P-labeled NCKSH3 protein in the presence of various concentrations of the SOS peptides. The SOS P1 ($_{1145}$KGTDEVPVPPPVPPRR RPES$_{1164}$) and its short version, SOS P6 ($_{1149}$EVPVPPPVP PRR$_{1160}$), appeared to completely inhibit the interaction of SOS and NCKSH3 at 40 mM (Fig. 4A). In contrast, peptides SOS P2 ($_{1176}$SKHLDSPPAIPPRQPTSKAY$_{1195}$), SOS P3 ($_{1203}$DRTSISDPPESPPLLPPREPVR$_{1224}$), and SOS P4 ($_{1249}$NAFFPNSPSPFTPPPQTPSPH$_{1270}$) did not inhibit the interaction at all, even at concentrations as high as 200 mM. SOS P7 ($_{1288}$HSIAGPPVPPRQ$_{1299}$) inhibited the interaction but less efficiently than SOS P1 and SOS P6 did. These peptides also seem to inhibit GRB2 binding to SOS in a similar fashion (data not shown). The half-maximal inhibition concentration of SOS P1 to NCK (Fig. 4B) and to GRB2 (Fig. 4C) is approximately 10 mM, which is comparable to the inhibitory concentration of phosphotyrosine peptides to SH2 domain-containing proteins (11).

NCK consists of three SH3 domains. It is unclear which SH3 domains are involved in the interaction with SOS. To address this question, a far-Western experiment was carried out as described above except that the wild-type or a truncated version of NCK protein was added into the binding reaction mixture. Both NCK (wild type) and NCK-332 (deleted of the first SH3 domain) were able to inhibit completely the binding of ^{32}P-labeled NCK to SOS at 50 nM (Fig. 4D). In contrast, NCK-32 (deleted of the first and second SH3 domains) had no inhibitory activity even at 100 nM. These results indicate that the second, not the first, SH3 domain is essential for the NCK-SOS interaction.

DISCUSSION

In this study, we have demonstrated that in NIH 3T3 cells, NCK strongly activates the *fos* promoter and that this activation is further enhanced by c-*ras* and blocked by DN-*ras* and dominant negative *raf*. We also found that NCK binds directly to SOS, a guanine nucleotide exchange factor of *ras*. This interaction is mediated primarily by the second SH3 domain of NCK and one of proline-rich regions at the C-terminal end of SOS protein.

NCK is associated with phosphotyrosine kinase receptors and is phosphorylated after exposure to many growth stimuli. Furthermore, overexpression of NCK transforms cells. All of these features suggest that NCK may be involved in growth factor-mediated signal transduction. The activation of the *fos* promoter is the first evidence, to our knowledge, that NCK is capable of mediating early cellular responses induced by growth factors. This activation is inhibited by the DN-*ras* mutant and the dominant negative *raf* mutant, suggesting that NCK interacts with the *ras* pathway.

There are two ways to regulate *ras* activity. One is by interacting with GAP, the *ras*-GTPase activator, therefore down-regulating *ras* activity. The other way is by increasing the guanine nucleotide exchange factor activity, therefore shifting the Ras protein from the GDP-bound form (inactive) to the GTP-bound form (active). The finding of the association of NCK and SOS helps to elucidate the mechanism of the interaction between NCK and the *ras* pathway. By binding to SOS, NCK may bring SOS to cell membrane where the Ras protein is located.

In addition to NCK, another SH2- and SH3 domain-containing protein, GRB2, has been reported to associate with SOS protein. Evidence from both genetic and cell biology studies (7, 22, 26, 33) have shown that GRB2 is involved in regulating the *ras* signaling pathway. In all experiments that we have performed so far, including the *fos* promoter assay, far-Western analysis, and assays with the yeast two-hybrid system, NCK and GRB2 behave similarly and appear to have compa-

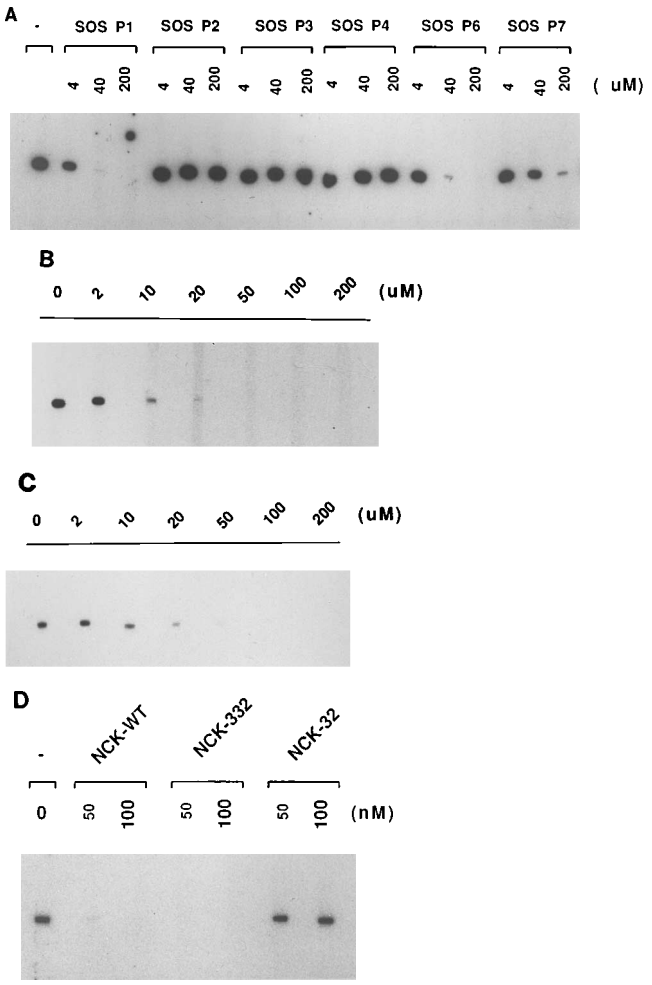

FIG. 4. Mapping the binding sites of NCK and SOS. (A) SOS proteins were immunoprecipitated with anti-SOS antiserum and transferred to nitrocellulose filters. The filters were probed with ^{32}P-labeled GST-NCKSH3 in the presence of various concentrations of SOS peptides. (B and C) The filters were probed with either ^{32}P-labeled GST-NCKSH3 (B) or ^{32}P-labeled GST-GRB2 (C) in the presence of various concentrations of SOS P1 peptide. (D) The filters were probed with ^{32}P-labeled GST-NCKSH3 in the presence of various concentrations of wild-type or truncated NCK protein. NCK-WT, wild-type NCK protein; NCK-332, NCK protein without the first SH3 domain; NCK-32, NCK protein without the first and second SH3 domains.

rable affinities for SOS, indicating they may have similar functions. Why mammalian cells use both NCK and GRB2 to link SOS protein to tyrosine kinase growth factor receptors is not clear. However, SH2 domains of NCK and GRB2 appear to bind to different phosphotyrosine motifs (35); their SH3 domains, on other hand, may target some other cellular proteins in addition to SOS. The combination of those differences, not just differences in the SH2 domain, may thereby trigger different phenotypic changes in response to different stimuli at different stage of cell growth and differentiation. Our findings suggest that a signaling enzyme, in this case SOS, can interact with a distinct linker protein (NCK or GRB2), which, in turn, may associate with distinct tyrosine-phosphorylated proteins. Therefore, diverse stimuli may use a common intracellular signaling pathway. Further experiments will be required to assess the relative roles of NCK, GRB2, and other SH2- and SH3 domain-containing proteins in the PDGF receptor-mediated signal transduction.

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