The NCK adapter protein is comprised of three consecutive Src homology 3 (SH3)-protein-protein interaction domains and a C-terminal SH2 domain. Although the association of NCK with activated receptor protein-tyrosine kinases, via its SH2 domain, implicates NCK as a mediator of growth factor-induced signal transduction, little is known about the pathway(s) downstream of NCK recruitment. To identify potential downstream effectors of NCK we screened a bacterial expression library to isolate proteins that bind its SH3 domains. Two molecules were isolated, the Wiskott-Aldrich syndrome protein (WASP, a putative CDC42 effector) and a serine/threonine protein kinase (PRK2, closely related to the putative Rho effector PKN). Using interspecific backcross analysis the Prk2 gene was mapped to mouse chromosome 3. Unlike WASP, which bound the SH3 domains of several signaling proteins, PRK2 specifically bound to the middle SH3 domain of NCK and (weakly) that of phospholipase Cγ. PRK2 also specifically bound to Rho in a GDP-dependent manner and cooperated with Rho family proteins to induce transcriptional activation via the serum response factor. These data suggest that PRK2 may coordinately mediate signal transduction from activated receptor protein-tyrosine kinases and Rho and that NCK may function as an adapter to connect receptor-mediated events to Rho protein signaling.

Upon association with ligand, receptor-protein-tyrosine kinases (PTKs)3 dimerize and transphosphorylate on tyrosine residues. These phosphotyrosine-containing sequences then provide docking sites for SH2 and PTB domains, modular protein-protein interaction domains that bind to phosphotyrosine in the context of adjacent C- or N-terminal peptide sequences, respectively (1). In addition to recruiting signaling enzymes such as phosphoinositide 3-kinase, phospholipase Cγ, and p120 GAP, adapter molecules including Grb2, Crk, and NCK also bind to PTKs via their SH2 domains. While lacking enzymatic activities, these adaptor proteins possess SH3 domains that bind to proline-rich sequences, minimally containing a consensus PXPF motif, in other signaling molecules (1–3). Possibly the best characterized adapter molecule is Grb2 that binds to PXPF motifs in the C terminus of the Ras guanine nucleotide exchange factor, Sos, recruiting it to the plasma membrane where it promotes Ras activation (4).

The role of the adapter molecule NCK is less clearly understood. NCK is made up of three consecutive SH3 domains and a C-terminal SH2 domain (5). While the SH2 domain binds to a number of receptor-PTKs, including the epidermal, platelet-derided, and vascular endothelial cell growth factor receptors as well as the insulin receptor substrate, IRS-1 (6–8), little is known about the putative effector proteins that bind to its three SH3 domains. NCK's SH3 domains have been shown to bind to Cbl (9), a major PTK substrate and possible inhibitor of cellular signaling (10), as well as Sos1 (11), two molecules that also associate with Grb2 (12). However, unlike Grb2 (13, 2), NCK has also been found to induce cellular transformation and to coprecipitate with serine/threonine protein kinases (7, 14, 15), suggesting that these adapter proteins have divergent functions.

To gain further insight into NCK function, we have used bacterial expression cloning to identify two additional NCK SH3-binding proteins. One of these proteins is the gene product of the autosomal recessive immune deficiency Wiskott-Aldrich syndrome (WASP) (16). The other is a serine-threonine kinase, PRK2, sharing significant homology to PKN/PRK1, a lipid-regulated protein kinase recently isolated by virtue of its homology to protein kinase C (17, 18) and shown to be activated by the Rho GTPase (19, 20). The interaction of NCK with WASP, PRK2, and PAK (21), three proteins that are all putative effectors of Rho family GTPases (19–22), suggests that NCK may play a role in coupling PTK activation to Rho family signaling. We show here that cotransfection of NIH 3T3 cells with plasmids encoding PRK2 and Rho family GTPases results in cell transformation. A unifying feature of these adapter proteins is that they are all SH3 ligands that associate with the SH3 domains of NCK and select for SH3 ligands containing a proline-rich motif. The data support a role for NCK as an adapter that associates with both PTKs and Rho family GTPases to couple growth factor signaling to these downstream effectors.

*Abbreviations used: PTK, protein-tyrosine kinase; GST, glutathione S-transferase; PKN, protein kinase N(ovel); PRK, protein kinase C-related kinase; SH, Src homology domain; WASP, Wiskott-Aldrich syndrome protein; RFLP, restriction fragment length polymorphism; kb, kilobase pair(s); bp, base pair(s); SRF, serum response factor.

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sulted in enhancement of Rho-stimulated transcriptional activation by the c-fos serum response factor. Therefore, PRK2 may be a downstream effector of Rho that mediates its ability to induce gene expression.

MATERIALS AND METHODS

Plasmid Constructs—Individual NCK SH3 domains (first SH3, codons 1–68; second SH3, codons 101–166; third SH3, codons 191–257) were generated by polymerase chain reaction using the human NCK cDNA (5) as template and subcloned into pGEX-XL (a pGEX-2T derivative encoding the A kinase-phosphorylatable RRASV sequence 5‘ of the BamHI site). These and other GST-fusion proteins were generated in XL-1 blue as described (3). For expression in mammalian cells, the NCK cDNA was cloned into the pZIPNeoSVxI retroviral vector (23) or inserted in frame with the hemagglutinin epitope tag of the pCGN vector (24).

Library Screening—A AEXox 16-day mouse embryo cDNA library (Novagen) was plated (720,000 plaques) and recombinant protein expression was induced overnight by overlaying 10 mM isopropyl β-D-thiogalactoside-impregnated nitrocellulose filters. Filters were washed in Tris-salt buffer (pH 7.4), containing 10% Tween 20 and 0.1% Tween 20, 1.0 mM EDTA, 1 mM dithiothreitol, and 3% bovine serum albumin. The GST-NCKSH3/SH3 probe was labeled by incubation of 100 μl of bead-immobilized fusion protein in 20 mM Tris-HCl, 100 mM NaCl, 12 mM MgCl2, 1 mM dithiothreitol, 4 μl of [γ-32P]ATP (3000 Ci/mmol), and 10 units of heart muscle kinase (Sigma). After a total volume. Beads were extensively washed and labeled glutathione. Filters were probed overnight in blocking solution supplemented with 32P-labeled GST-NCK- and Rho-mediated Signaling

RESULTS AND DISCUSSION

WASP and PRK2 Bind to the SH3 Domains of NCK—Little is known about the signaling pathways that are mediated by NCK association with activated PTKs. Therefore, to identify NCK binding molecules that might interact with its SH3 domains to couple PTKs with novel downstream effectors, we generated a GST-fusion protein containing the second and third SH3 domains of NCK (codons 101–257) plus a consensus phosphorylation site (RRASV) to facilitate labeling of the protein with [γ-32P]ATP and A kinase.

The labeled probe was then used to screen a λEXox 16-day mouse embryo cDNA expression library. Of the unique cDNAs isolated, one encoded the C-terminal (residues 230–502) portion of the mouse Wiskott-Aldrich syndrome protein was expressed in Escherichia coli strain BL21 (lysE) from the pEXox bacterial expression vector. Bacterial proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane prior to incubation with 20 μg of the indicated GST-SH3 proteins. Binding of SH3 domains to WASP was detected using anti-GST antibody. The WASP C terminus bound to the SH3 domains of NCK, Src, Grb2, phospholipase Cγ, and (very weakly) Abl but not to the other indicated SH3 domains or to GST. A, a T7–10 fusion protein containing codons 555–736 of mouse PRK2 was expressed and blotted as described above. The PRK2 fragment bound to the middle SH3 domain of NCK and weakly to the SH3 domain of rat phospholipase Cγ but not to the other SH3 domains tested.
Src, Grb2, phospholipase Cγ and (very weakly) Abl as well as the 3rd SH3 domain of NCK interact with WASP in vitro (Fig. 1A). A similar observation has recently been made by Rivero-Lezcano et al. (35). Thus, WASP may be a site of convergence for signaling from multiple SH3-containing proteins. The T cells of WASP patients have been reported to exhibit cell surface defects consistent with cytoskeletal abnormalities (36). It is therefore not surprising that WASP has recently been found to associate with the Rho family GTPase, CDC42, that regulates the actin skeleton associated with cell surface projections/filopodia (22, 37).

A second clone derived from our library screen shares significant homology with the catalytic domain of protein kinase C. Using this 500-bp cDNA as a probe to screen a human fibroblast cDNA library (25) we isolated a full-length clone encoding a 984-amino acid protein kinase. This protein is >99% identical to PRK2 (18) and shares considerable identity with PRK1/PKN, a lipid-regulated serine/threonine kinase (17, 18) that has recently been demonstrated to bind to and be activated by Rho-GTP (19, 20).

Northern analysis of adult mouse tissue mRNA using the original 500-bp clone (encoding mouse PRK2 residues 555–706) as a probe indicated that like NCK, PRK2 is ubiquitously expressed. A ~6-kb message was present in all tissues with a smaller less abundant message being expressed most strongly in testis (Fig. 2A). In contrast, WASP is only expressed in cells of hematopoietic origin (16). To determine the mouse chromosomal location of Prk2, interspecific backcross analysis using progeny derived from matings of [(C57BL/6J × M. spretus)F1 × C57BL/6J] mice was performed (27). C57BL/6J and M. spretus DNAs were digested with several enzymes and analyzed by Southern blot hybridization for informative RFLPs using a mouse cDNA Prk2 probe. The 6.2-kb HincII M. spretus RFLP (see "Materials and Methods") was used to follow the segregation of the Prk2 locus in backcross mice to the distal region of mouse chromosome 3, 5.2 cm distal of Nfibb1 and 8.2 cm proximal of Rpe65 (Fig. 2B). Several genetic defects in mice map close to this site (38, 39), and it will be of interest to determine if they are due to PRK2 mutation. The distal region of mouse chromosome 3 shares regions of homology with human chromosomes 4q and 1p (summarized in Fig. 2B), suggesting that Prk2 will reside on 4q or 1p, as well.

Based on the sequence of the initial 500-bp cDNA isolate (encoding residues 555–706), the NCK-binding motif of PRK2 was predicted to reside within a unique proline-rich motif, located just N-terminal to the kinase domain that is absent from PKN. Consistently, using a phage-displayed random peptide library containing the sequence XnPXPPXn (3) we determined the consensus binding sequence for the second NCK SH3 domain to be very similar to the putative NCK-binding domain of PRK2 (Table 1). Furthermore, only this second SH3 domain of NCK specifically bound to the residues 555–706 PRK2 fragment (Fig. 1B). While PRK2 weakly interacted with the SH3 domain of phospholipase Cγ, it did not bind to the SH3 domains of other adapter molecules, protein kinases, cytoskeletal proteins (actin, fodrin),2 neutrophil NADPH oxidase p67, or p120 Ras-GAP, suggesting a specific role for PRK2 in NCK-mediated signaling.

**NCK May Couple PTK Signaling to Rho-activated Pathways**—In addition to WASP and PRK2, NCK has also been reported to bind to the p21 Rac- and CDC42-activated kinase, PAK3 (21).3 Indeed, the N-terminal sequences of PAKs share considerable homology with the NCK SH3-binding sequences of PRK2, including a negatively charged sequence N-terminal to the PXXP motif (Table 1). These findings suggested that NCK mediates signal transduction from activated PTKs to the Rho family (Rho, Rac, and CDC42) of GTPases that regulate cytoskeletal and/or transcriptional events via multiple effectors.

The PRK2-related kinase, PKN, is activated following association of an α-helical N-terminal domain (located between residues 34 and 103 of PKN) with Rho-GTP (19). Since this region of PKN is well conserved (53% identity, 84% homology) with PRK2, we investigated whether it too might interact with Rho family GTPases. As shown in Fig. 3, incubation of GST-fusion proteins of RhoA, Rac1, and CDC42 or the Ras-related Rap1A with cytosol from COS cells transiently overexpressing recombinant PRK2 resulted in the specific association of RhoA

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**Fig. 2. Tissue and chromosomal localization of mouse Prk2**. A, Prk2 message is ubiquitously expressed. A mouse multigain poly(A)+ RNA Northern blot (Clontech) was probed with a random-primed Prk2 fragment consisting of bases 690-1507 of the mouse cDNA as described under "Materials and Methods." A message of ~6 kb was detected in all tissues with a smaller (<4 kb) message being most abundant in testis. B, Prk2 maps in the distal region of mouse chromosome 3. Prk2 was placed on mouse chromosome 3 by interspecific backcross analysis. The segregation patterns of Prk2 and flanking genes in 139 backcross animals that were typed for all loci are shown at the top of the figure. For individual pairs of loci, more than 139 animals were typed. Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J × M. spretus)F1 parent. The black boxes represent the presence of a C57BL/6J allele, and white boxes represent the presence of a M. spretus allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. A partial chromosome 3 linkage map showing the location of Prk2 in relation to linked genes is shown at the bottom of the figure. The number of recombinant N2 animals is presented over the total number of N2 animals typed, to the left of the chromosome map. The recombination frequencies, expressed as genetic distance in centimorgans (±1 S.E.), are also shown. The positions of loci in human chromosomes, where known, are shown to the right. References for the human map positions of loci cited in this study can be obtained from GDB (Genome Data Base), a computerized database of human linkage information maintained by The William H. Welch Medical Library of The Johns Hopkins University (Baltimore, MD).
TABLE I
Comparison of peptide sequences that bind to the second
NCK-SH3 domain

| PXPPRXSL | Random peptide consensus sequence |
|-----------|-------------------------------|
| DLEPPAPPAPPSSSL | PRK2 codons 569-584 |
| DNEKPPARLPSNN | PAK3 codons 6-21 |

Fig. 3. PRK2 binds to Rho in a GTP-dependent manner. COS monkey kidney cells were transfected with an expression plasmid encoding FLAG-tagged PRK2. After 48 h, cells were lysed as described under “Materials and Methods,” and cytosol was incubated with glutathione-agarose plus 10 mg of the indicated GST-fusion proteins of under “Materials and Methods,” and cytosol was incubated with glutathione-agarose plus 10 mg of the indicated GST-fusion proteins of GST-Rho-GTP, Rac1-GTP, CDC42-GTP, or Rap1A-GTP. After 3 h at 4 °C, the beads were washed and GTPase-associated FLAG-tagged PRK2 was detected by Western blot using the M5 anti-FLAG antibody (Kodak). No association of PRK2 with GST, Rac1, CDC42, or Rap1A was detectable even after extending this 2-min chemiluminescence exposure for 3 h.2

with PRK2 in a GTP-dependent manner. Thus, similarly to PKN, PRK2 is likely to be a Rho protein effector. In contrast to studies on PKN where GST-Rho-GTP·S was shown to activate its kinase activity 2–6-fold in vitro (19, 20), we did not observe any significant effect of Rho or Rac on the ability of FLAG-PRK2 to phosphorylate itself or a protein kinase C α pseudosubstrate peptide.2 This may have been due to the presence of the epitope tag, which was required to immunoprecipitate the enzyme from cell lysates, constitutively activating the kinase. Indeed a similar problem was also recently encountered upon epitope-tagging PAK1 (40).

Rho family proteins have been reported to induce transcriptional activation from the c-fos serum response element via the serum response factor (SRF) (32). However, the downstream effectors(s) of Rho that mediate SRF activation has yet to be identified. To determine if PRK2 might mediate Rho protein-induced transcriptional activation we looked at its ability to cooperate with Rho family GTPTases to induce gene expression from the c-fos serum response element. PRK2 induced transcriptional activation from a SRF-luciferase reporter construct and cooperated with the Rho family GTPTases RhoA and CDC42 (Fig. 4) as well as Rac1 and the Rho family guanine nucleotide exchange factor, Dbl.2 Since PRK2 specifically interacts with GST-Rho-GTP·S, GST-NCK inhibited the ability of PRK2 to phosphorylate the PCKα pseudosubstrate peptide by 47.4 ± 10.0% (mean ± S.E., from four independent experiments). However, as noted above, FLAG-PRK2 kinase activity may have been artificially elevated by its epitope tag. Similarly to Grb2-mediated regulation of Sos (4), the role of NCK may be to target effectors such as PRK2, WASP, and PAK to sites of PTK activation, e.g. plasma membrane or focal adhesion complexes. Alternatively, the kinase experiments indicate that NCK may also be a negative regulator of PRK2 function.

Although it is becoming apparent that Rho family GTPTases, and their subsequent induction of transcriptional activation via Jun NH2-terminal kinase/stress-activated protein kinase and SRF, are important downstream components of many cellular signaling pathways (41), little is known about how extracellular stimuli modulate Rho protein function. Our observation that NCK, which associates with activated PTKs, can bind to two putative Rho protein effectors suggests that NCK acts as an adapter to connect receptor-mediated events to Rho protein signaling. Taken together with the previous observation that NCK also binds to PAKs (21),3 these data suggest that NCK may modulate multiple Rho protein-mediated signaling events.

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Fig. 4. PRK2 cooperates with Rho family proteins to induce transcriptional activation via the c-fos serum response factor. NIH 3T3 cells were cotransfected with 0.5 μg of pZIP plasmids encoding RhoA(63L) or CDC42(12V) and PRK2 as indicated along with 2.5 μg of the pSP63/2-luciferase reporter plasmid. Cells were serum-starved (0.5%) for 48 h, and luciferase activity was determined on cell lysates as described (31). PRK2 cooperated with these activated mutants of RhoA and CDC42 to enhance transcriptional activation. Results are the mean ± S.E. for two experiments performed in duplicate and are representative of at least six experiments.

pathway in Saccharomyces cerevisiae, where binding of Rho-GTP to PKC1 leads to activation of a mitogen-activated protein kinase cascade via SCK, MKK, and MPK to induce cell wall biosynthesis (42). Since the catalytic domains of PRK2 and PKN show strong identity with those of protein kinases C, they may represent functional mammalian homologs of the yeast PKC1. While PRK2 and PKN share significant identity, the ability of PRK2 to bind to NCK may promote differential regulation and/or subcellular localization of these two kinases. Indeed, unlike GST or GST-Rho-GTP·S, GST-NCK inhibited the ability of PRK2 to phosphorylate the PCKα pseudosubstrate peptide by 47.4 ± 10.0% (mean ± S.E., from four independent experiments). However, as noted above, FLAG-PRK2 kinase activity may have been artificially elevated by its epitope tag. Similarly to Grb2-mediated regulation of Sos (4), the role of NCK may be to target effectors such as PRK2, WASP, and PAK to sites of PTK activation, e.g. plasma membrane or focal adhesion complexes. Alternatively, the kinase experiments indicate that NCK may also be a negative regulator of PRK2 function.
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