R-Ras Contains a Proline-rich Site That Binds to SH3 Domains and Is Required for Integrin Activation by R-Ras*

(Received for publication, August 19, 1999, and in revised form September 27, 1999)

Bingcheng Wang‡, June X. Zou, Barbro Ek-Rylander§, and Erkki Ruoslahti¶

From the Cancer Research Center, The Burnham Institute, La Jolla, California 92037

R-Ras contains a proline-rich motif that resembles SH3 domain-binding sites but that has escaped notice previously. We show here that this site in R-Ras is capable of binding SH3 domains and that the SH3 domain binding may be important for R-Ras function. A fusion protein containing the SH3 domains of the adaptor protein Nck interacted strongly with the R-Ras proline-rich sequence and with the intact protein. The binding was independent of whether R-Ras was in its GDP or GTP form. The Nck binding, which was mediated by the second of the three SH3 domains of Nck, was obliterated by mutations in the proline-rich sequence of R-Ras. The interaction of Nck with R-Ras could also be shown in yeast two-hybrid assays and by co-immunoprecipitation in human cells transfected with Nck and R-Ras. Previous results have shown that the expression of a constitutively active R-Ras mutant, R-Ras(38V), converts mouse 32D monocyctic cells into highly adherent cells. Introducing the proline mutations into R-Ras(38V) suppressed the effect of R-Ras on 32D cell adhesion while not affecting GTP binding. These results reveal an unexpected regulatory pathway that controls R-Ras through an SH3 domain interaction. This pathway appears to be important for the ability of R-Ras to control cell adhesion.

Integrins mediate cell adhesion to extracellular matrices and, in some cases, to other cells. Their activity also helps regulate cell survival, growth, differentiation, and migration. Integrin activity is, in turn, regulated by the cell. The most striking examples of regulated integrin activity are the activation of the platelet αIIbβ3 integrin in blood clotting and the activation of the leukocyte β2 integrins in inflammation (1, 2). The molecular mechanisms of integrin regulation are not well understood. There appear to be two ways of enhancing the cell attachment-promoting activity of integrins; clustering of integrins at the cell surface can enhance the avidity of integrins in cell attachment, whereas conformational changes can increase the affinity of individual integrin molecules (3–6). Cytoskeletal connections of integrins are likely to be important in the avidity regulation (7), whereas the affinity of integrins may be regulated by other types of proteins. Cytosheins are phosphatidylinositol-binding proteins, at least one of which binds to the cytoplasmic tail of the β2 integrin subunit and can up-regulate the activity of β2 integrins in leukocytic cells (8). Endonexin is a β3-binding protein that increases the activity of the αIIbβ3 integrin when overexpressed in cells (9). ILK is a protein kinase capable of interacting with β1, β2, and β3 integrin subunit cytoplasmic tails; it can reduce the activity of integrins containing these subunits (10). The calcium/calmodulin-dependent protein kinase II also down-regulates integrin activity (11).

Certain oncogenes can change integrin activity, generally by lowering it. Thus, c-Src phosphorylates a tyrosine residue in the β1 integrin cytoplasmic domain, and this phosphorylation reduces the ligand binding activity of β1 integrins and changes their subcellular localization (12, 13). The small GTPases of the Ras and Rho families are intimately involved in integrin and cytoskeletal regulation. The activation of Rho induces reorganization of the actin cytoskeleton, with consequent cell spreading and effects on integrins (14, 15). The related proteins Rac and Cdc42 alter the cytoskeleton differentially than Rho, inducing membrane ruffling and microspike formation, respectively. The oncogenic p21ras (Ras) reduces integrin activity (16).

R-Ras, a small GTPase with a poorly understood function, regulates integrin activity (17–21). Unlike Ras, R-Ras promotes integrin activity and converts cells that normally grow in suspension into highly adhesive cells (17). Moreover, a dominant negative R-Ras (R-Ras43N), when transfected into adherent cells, causes the cells to round up, suggesting that R-Ras is necessary for the maintenance of integrin activity (17). Unlike dominant negative mutants of the oncogenic Ras proteins, R-Ras(43N) is not growth inhibitory (20). Thus, R-Ras may be primarily a regulator of cell adhesion, and it is important to understand how this regulation functions.

Examining R-Ras for sequence features that might be responsible for its integrin activating function, we noticed that R-Ras differs from Ras and most other members of Ras superfamily small GTPases in that it possesses a distinct proline-rich site. We show here that this site can bind SH3 domains and that the adaptor protein Nck is one of the SH3 domain proteins that interact with R-Ras in cells. We also show that the SH3-binding site is required for integrin activating function of R-Ras. These results reveal an unexpected regulatory interaction for R-Ras that appears to be important for the ability of R-Ras to control cell adhesion. This interaction represents a novel form of cross-talk between a small GTPase and other cellular signaling pathways.

EXPERIMENTAL PROCEDURES

Cells, Antibodies, and Reagents—Mouse monocytic cell line 32D.3 was maintained essentially as described (17) in RPMI 1640 medium supplemented with 1 mM glutamine, 100 units/ml penicillin G, 100 μg/ml streptomycin, 10% heat-inactivated fetal calf serum, and 20% conditioned medium from the interleukin-3-producing cell line WEHI-3B. Human embryonic kidney 293 cells were maintained in Dulbecco's
modified Eagle's medium plus 10% fetal calf serum. Polyclonal rabbit antibody against the N-terminal 26-amino acid peptide of R-Ras was a gift from Dr. John Reid (21). Mouse monoclonal antibody against hemagglutinin (HA)1 12CA5 was provided by Dr. J.-L. Guan. Mouse monoclonal antibody against the N-terminal 26-amino acid peptide of R-Ras was a gift from Dr. John Reed (21). Mouse monoclonal antibody against the N-terminal 26-amino acid peptide of R-Ras was a gift from Dr. John Reed (21). Mouse monoclonal antibody against the N-terminal 26-amino acid peptide of R-Ras was a gift from Dr. John Reed (21). Mouse monoclonal antibody against the N-terminal 26-amino acid peptide of R-Ras was a gift from Dr. John Reed (21).

**Plasmid Construction and Recombinant Protein Purification** —To create the maltose-binding protein (MBP) fusion plasmid pMBP-RRC, two oligonucleotides spanning the coding sequence for amino acids 1-188 (5’-CTAGACTACGGCCGCCTTCTCCTGTGTCGCTTCCT-3’) of R-Ras were used to amplify the R-Ras coding sequence into pXbo1 and EcoRI overhangs (sense strand: 5’-ATTCAGAATATCCAGGAAACAGAGCTCCTCCAGGAACTGCCCCAGGAAAGGCGGGGTTGAATG-3’) (underlined letters indicate codons for proline 202 and 203; antisense strand: 5’-CTAGACTACGGCCGCCTTCTCCTGTGTCGCTTCCT-3’). The oligonucleotides were annealed, phosphorylated by using polynucleotide kinases, and ligated in frame in the pPR997 maltose fusion protein vector (New England Biolab) digested with Xbo1 and EcoRI. Mutants were derived by changing the codon for proline to alanine at amino acids 202 (P202A), 203 (P203A), or both 202 and 203 (P202A,P203A). The plasmids were transformed into Escherichia coli strain XL-1 blue (Strategene), and MBP fusion proteins were purified from the lysates by affinity chromatography on MBP-Sepharose.[12]

To introduce double proline to alanine (P202A,P203A) mutations into the R-Ras mammalian expression vector, R-Ras was first amplified by polymerase chain reaction using a 3’ antisense primer containing P202A,P203A mutations (5’-GCTCTAGACGTCGACGAGAACGAGGTGCAGGAGG-3’) and a 5’ primer at the beginning of R-Ras coding sequence (5’-ATAGAGCTCTGTGC-3’). The polymerase chain reaction product was digested with Xbo1 and Stu1, which cuts near nucleotides coding for amino acids 174 of R-Ras. This fragment was then cloned into pCDNA3-HA-R-Ras( wt) or pCDNA3-HA-R-Ras(38V) to obtain pHR-Ras (wt) (P202A,P203A) and pHR38V (P202A,P203A) respectively. The polymerase chain reaction product was also cloned into pYES-Myc-R-Ras (pRR- Myc) to create pMRR-(P202A,P203A). All constructs were verified by dideoxynucleotide sequencing (U. S. Biochemical Corp.). pEBB-Nck was a generous gift from Dr. B. Mayer (22).

Preparation of glutathione S-transferase (GST) fusion proteins with SH3 domains from Src and Crk (N) (23), p53-a (24), Ab5 (25), Grb2 (C), Fgr, spectrin, and phospholipase Cy (26) have been described. Nck deletion mutants were constructed by removing internal or terminal fragments from Nck cDNA (27), and were symbolized by their termini from C to N termini by polymerase chain reaction. The polymerase chain reaction fragments were then cloned into PGEX-4T1 and expressed as GST fusion proteins. The plasmid for GST fusion protein of the second SH3 domain was obtained from Dr. Sakaue.

Expression vectors for GST-R-Ras and GST-R-Ras-( P202A,P203A) were constructed by digesting the plasmids pMRR-(My c-R-Ras) and pMRR-(P202A,P203A) with EcoRI and XhoI. The released inserts were then cloned into the vector pGEX4T1 (Amersham Pharmacia Biotech). All of the fusion proteins were expressed in bacteria and purified on glutathione-Sepharose (Amersham Pharmacia Biotech) as described (28).

**In Vitro Binding of MBP-R-Ras Fusion Proteins to GST-SH3 Fusion Proteins**—About 10 μg of MBP alone, MBP-RRC, or its mutants were labeled with Na2[35S] using Iodogen (Pierce) as described previously (29). In a typical binding assay, 10 μg of a GST-SH3 domain fusion proteins in phosphate-buffered saline (PBS) was immobilized on glutathione-Sepharose. The beads were washed twice with PBS and once with the binding buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40, 0.05% SDS, 1% BSA, and 0.2 mM phenylmethylsulfonyl fluoride, and resuspended in 20 μl of the same buffer at 4 °C. About 1 × 106 cpm of [35S]labeled MBP-RRC fusion protein in PBS containing 1% BSA was added to each tube and incubated for 30 min at 4 °C. The beads were washed four times with binding buffer without BSA. The bound materials were eluted with 50 μl of SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer, separated on 4–20% gradient SDS-PAGE minigel (Novex, San Diego), and exposed to x-ray films.

**Cell Adhesion Assay** —Serial dilutions of fibronectin in PBS was coated on 96-well microtiter plates starting at 10 μg/ml. Nonspecific binding sites were blocked with 1% BSA in PBS. 32D cells and their transfectants were collected by pipetting or by using brief treatment with 1 mm EDTA in PBS. The suspended cells were washed and plated in duplicate onto fibronectin-coated wells in serum-free medium at 1 × 104 cells/well and allowed to adhere for 1 h at 37 °C. Adherent cells were then trypsinized and counted. The results were expressed as the number of adherent cells for each condition.

**Stable Transfection of 32D Cells** —Log phase 32D.3 cells were transfected with indicated plasmids by as described (16). One day after transfection, 0.7 μg/ml Geneticin (G418) (Life Technologies, Inc.) was added. Individual clones were obtained by limited dilution. Levels of expression in each clone was examined by immunoblot with an anti-HA epitope monoclonal antibody 12CA5.

1 The abbreviations used are: HA, hemagglutinin; MBP, maltose-binding protein; GST, glutathione S-transferase; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; GTP- S, guanosine 5’-O-(3-thio)triphosphate.
R-Ras Contains a Proline-rich Region That Resembles SH3 Domain-binding Sites—In a search for potential mediators for the integrin activating function of R-Ras, we examined sequence features of R-Ras outside the effector domain. As shown in Fig. 1 a proline-rich region toward the C terminus of R-Ras contains three possible PXXP minimal SH3 domain recognition sequences, one of which conforms to the consensus features of a preferred binding site for type II SH3 domains (33, 34). No PXXP motif is present in other members of Ras superfamily small GTPases with the exception of TC21 and CDC42Hs. TC21 is most closely related to R-Ras (35) and contains a single PPSP motif (Fig. 1B). CDC42Hs belongs to the Rho subfamily of small GTPases (36), its PPEPKK site also conforms to the consensus type II SH3-binding motif (Fig. 1B). Interestingly, the brain-specific version of CDC42Hs, G25K, (37) lacks the potential SH3-binding site.

Proline-rich Region of R-Ras Selectively Interacts with SH3 Domains in Vitro—To determine whether R-Ras can indeed bind to SH3 domain-containing proteins, we fused the C-terminal portion of R-Ras (amino acids 192–212) to MBP and screened a panel of GST-SH3 domain fusion proteins for binding to this fusion protein, MBP-RRC. A recombinant protein encompassing the three SH3 domains of Nck bound avidly to MBP-RRC (Fig. 2A). By counting the excised band, it was estimated that about 12% of 125I-labeled MBP-RRC could be bound by the Nck SH3 domain fragment. None of the other SH3 domains tested exhibited binding above the background level of GST alone. As a control, MBP alone was tested and did not bind to any SH3 domain fusion proteins under the same conditions (Fig. 2A, lower panel). These results show that the proline-rich domain of R-Ras can serve as selective binding site for SH3 domain-containing proteins, Nck in particular.

SH3 Domain Binding Requires Proline Residues 202 and 203 of R-Ras—To demonstrate that the interaction between the C-terminal portion of R-Ras and Nck is mediated by the proline-rich region, proline to alanine point mutations were introduced to alter the PXXP motifs in R-Ras. Fig. 2B shows that mutation of proline 202 to alanine (P202A), which eliminates the first PXXP site (Fig. 1A), greatly reduced the binding to Nck SH3 domains. Disrupting the second and third PXXP sites with the P203A mutation similarly reduced Nck binding. Mutating both prolines (P202A,P203A) resulted in further reduction of the Nck binding. These results show that the proline-rich domain is required for binding to Nck SH3 domains. Testing with fusion proteins containing various combinations of the three Nck SH3 domains showed that only the middle SH3 domain of Nck binds R-Ras (Fig. 2C).

FIG. 1. Proline-rich R-Ras sequence. A, the putative SH3 domain-binding site for Nck at residues 199–206 of R-Ras. Indicated are three possible PXXP motifs. The third motif conforms to the consensus sequence for type II SH3-binding sites. B, Ha-Ras does not have any polyproline sites. Only two other members of the Ras superfamily small GTPases (TC21 and CDC42Hs) contain a PXXP motif.

μl of elution buffer (1% SDS and 20 mM EDTA) for 5 min at 65 °C. Radioactivity of a 20-μl sample was quantified by scintillation counting.

RESULTS

R-Ras Contains a Proline-rich Region That Resembles SH3 Domain-binding Sites—In a search for potential mediators for the integrin activating function of R-Ras, we examined sequence features of R-Ras outside the effector domain. As shown in Fig. 1 a proline-rich region toward the C terminus of R-Ras contains three possible PXXP minimal SH3 domain recognition sequences, one of which conforms to the consensus features of a preferred binding site for type II SH3 domains (33, 34). No PXXP motif is present in other members of Ras superfamily small GTPases with the exception of TC21 and CDC42Hs. TC21 is most closely related to R-Ras (35) and contains a single PPSP motif (Fig. 1B). CDC42Hs belongs to the Rho subfamily of small GTPases (36), its PPEPKK site also conforms to the consensus type II SH3-binding motif (Fig. 1B). Interestingly, the brain-specific version of CDC42Hs, G25K, (37) lacks the potential SH3-binding site.

Proline-rich Region of R-Ras Selectively Interacts with SH3 Domains in Vitro—To determine whether R-Ras can indeed bind to SH3 domain-containing proteins, we fused the C-terminal portion of R-Ras (amino acids 192–212) to MBP and screened a panel of GST-SH3 domain fusion proteins for binding to this fusion protein, MBP-RRC. A recombinant protein encompassing the three SH3 domains of Nck bound avidly to MBP-RRC (Fig. 2A). By counting the excised band, it was estimated that about 12% of 125I-labeled MBP-RRC could be bound by the Nck SH3 domain fragment. None of the other SH3 domains tested exhibited binding above the background level of GST alone. As a control, MBP alone was tested and did not bind to any SH3 domain fusion proteins under the same conditions (Fig. 2A, lower panel). These results show that the proline-rich domain of R-Ras can serve as selective binding site for SH3 domain-containing proteins, Nck in particular.

SH3 Domain Binding Requires Proline Residues 202 and 203 of R-Ras—To demonstrate that the interaction between the C-terminal portion of R-Ras and Nck is mediated by the proline-rich region, proline to alanine point mutations were introduced to alter the PXXP motifs in R-Ras. Fig. 2B shows that mutation of proline 202 to alanine (P202A), which eliminates the first PXXP site (Fig. 1A), greatly reduced the binding to Nck SH3 domains. Disrupting the second and third PXXP sites with the P203A mutation similarly reduced Nck binding. Mutating both prolines (P202A,P203A) resulted in further reduction of the Nck binding. These results show that the proline-rich domain is required for binding to Nck SH3 domains. Testing with fusion proteins containing various combinations of the three Nck SH3 domains showed that only the middle SH3 domain of Nck binds R-Ras (Fig. 2C).

FIG. 2. Binding of SH3 domains to R-Ras proline-rich site. A and B, the C-terminal region of R-Ras containing the proline-rich sequence (residues 119–212) was fused to MBP in pPr997 vector. This fusion protein (MBP-RRC) and its proline to alanine mutant derivatives (P202A, P203A, and double mutant P202A,P203A) were labeled with 125I, and equal amounts of each protein were tested for binding to SH3 domains from various proteins, produced as GST fusion proteins in bacteria, and immobilized on glutathione-Sepharose. Bound materials were eluted with SDS-PAGE sample loading buffer and separated on a 4–20% gradient gel. A, MBP-RRC but not MBP binds strongly to Nck. B, the proline to alanine R-Ras mutants show reduced binding to the Nck SH3 domain fragment. C, R-Ras interacts with the second SH3 domain of Nck. The full-length of GST-Nck fusion protein and GST fusion proteins of Nck mutants in which the second or third SH3 domain had been deleted, as well as a GST fusion protein of the second SH3 domain, were immobilized on glutathione-Sepharose beads. The beads were incubated with equal amounts of cell lysates from Myc-Ras transfected 293T cells at 4 °C for 1 h. After washing, the bound proteins (including the GST fusion protein) were eluted with SDS-PAGE sample buffer and blotted with anti-R-Ras antibody (Santa Cruz Biotechnology). Protein staining (lower panel) shows that equal amounts of the fusion proteins had bound to the beads. D, both GTP and GDP forms of R-Ras bind Nck, GST-R-Ras immobilized on glutathione-Sepharose beads was prelabeled with GTPγS or GDPγS. The beads were incubated with lysates prepared from cells expressing HA-Nck. Bound proteins were eluted with SDS-PAGE sample buffer, separated on a 4–20% gradient gel and transferred to a polyvinylidene difluoride membrane. Immunoblotting of the membrane with anti-HA showed Nck binding to GST-R-Ras loaded with either GTP or GDP but not to GST.

Nck SH3 Domain Fragment Binds to Both GTP-bound and GDP-bound R-Ras in Vitro—To investigate the role of GTP binding in the R-Ras interaction with Nck, binding of Nck to
GTP-bound and GDP-bound R-Ras-GST fusion protein was examined. Nck was found to bind both the GTP and GDP forms of R-Ras, indicating that the Nck interaction is not regulated by the R-Ras nucleotide binding (Fig. 2D).

**R-Ras Associates with Nck in Yeast Cells—**The interaction between R-Ras and Nck in cells was examined initially in a yeast two-hybrid assay (38, 39). A Nck fragment encoding the SH3 domain was the “prey,” and R-Ras with a deleted CAA box (R-Ras-CAAX) was the “bait.” R-Ras and Nck interacted strongly in this growth assay (Fig. 3) as well as in an assay based on β-galactosidase activity (not shown). As expected (40), R-Ras interacted also with Raf-1 (not shown). A negative control using lamin as bait showed no signal with the Nck prey. Thus, the SH3 domain is active in yeast cells, and R-Ras can associate specifically with Nck in these cells.

**R-Ras and Nck Form a Complex in Mammalian Cells—**To study R-Ras-SH3 domain interaction in mammalian cells, we transiently transfected Nck into 293 cells together with HA-tagged R-Ras or R-Ras-(P202A,P203A). Immunoprecipitation and immunoblot shows that wild-type R-Ras co-immunoprecipitated with Nck and that P202A,P203A mutation significantly reduced that association (Fig. 4A, left half). Similar results were obtained when cells were transfected with HA-tagged Nck and Myc-tagged R-Ras or R-Ras-(P202A,P203A) (Fig. 4A right half). These results indicate that R-Ras and Nck can associate in intact cells and that proline-rich domain is necessary for the association.

**The Proline-Rich Domain of R-Ras Is Required for Integrin Regulation by R-Ras—**We have previously demonstrated that an activated form of R-Ras, R-Ras(38V), can induce substrate attachment of 32D mouse monocytic cells, which normally grow in suspension, and that the increased adhesiveness is caused by integrin activation (17). We next used this assay to determine whether the proline-rich site contributes to the integrin-regulating activity of R-Ras. A major proportion of transfected 32D cells expressing the activated R-Ras(38V) became adherent and spread on tissue culture dishes (Fig. 5A), whereas 32D cells expressing an R-Ras-(P202A,P203A) mutant (left two lanes). Right lanes show cells transfected with HA-tagged Nck together with Myc-tagged R-Ras or R-Ras-(P202A,P203A) mutant. Immunoblots were performed on whole cell lysates to demonstrate similar levels of expression of wild type and mutant R-Ras. B, about 200 ng of cell lysates were used for immunoprecipitation of Nck with anti-Nck for Nck-transfected cells or with anti-HA for HA-Nck-transfected cells. The associated R-Ras was detected with an anti-HA or anti-Myc antibody.

R-Ras(38V)-transfected cells adhered to fibronectin, but clones expressing the R-Ras(38V)-(P202A,P203A) mutant either did not adhere to fibronectin or adhered poorly to it (Fig. 5D). That the various transfected proteins expressed equivalent levels of the transfected R-Ras was confirmed by immunoblotting cell extracts with anti-HA antibody (not shown). No adhesion was observed with R-Ras(wt)- or vector-transfected cells. Similar results were obtained when another adhesive protein, vitronectin, was used to coat the substrate. The lack of cell attachment-promoting activity by the R-Ras(38V)-(P202A,P203A) mutant was unlikely to be due to a loss of GTP binding capability, because controls showed the P202A,P203A mutation to have no effect on GTP binding by R-Ras (Fig. 6). These results indicate that the proline-rich domain contributes to the integrin activating function of R-Ras, possibly by interacting with the SH3 domains of Nck.

**DISCUSSION**

Our results reveal an unexpected regulatory interaction of R-Ras that is mediated through binding to SH3 domains. The results single out the adaptor protein Nck as a candidate cellular protein for SH3 domain-dependent binding of R-Ras and show that the integrity of SH3 domain binding is necessary for the ability of R-Ras to regulate integrin-mediated cell attachment.

The interactions of the R-Ras proline-rich domain have the hallmarks of an SH3 domain interaction. Three lines of evidence: direct binding assays with fusion proteins, two-hybrid analysis, and coimmunoprecipitation from cells, demonstrate the specificity and high avidity of the R-Ras binding to an SH3 domain of Nck. Nck is an adaptor protein comprising three consecutive SH3 domains and a C-terminal SH2 domain (41). Our use of SH3 domain fusion proteins in the binding assays...
places the interaction site in Nck to the SH3 domains, specifically to the middle SH3 domain. Moreover, our mutational analysis showed that the proline-rich segment of R-Ras, which includes three adjacent copies of the SH3 domain-binding consensus sequence PXXP, is the binding site on the R-Ras side. To our knowledge, this is the first demonstration of an SH3 domain interaction by a small GTPase. The independence of the SH3 domain binding on the GTP/GDP regulation of R-Ras suggests that Nck (and other SH3 domain proteins that might bind to this site) functions as an adaptor, rather than as downstream effector, of R-Ras.

The Nck SH3 domain fragment was the only efficient binder of the R-Ras C-terminal proline-rich sequence; fusion proteins of SH3 domains from nine other proteins showed no significant binding. The specificity of the SH3 domain binding was further underscored by the fact that only one of the three SH3 domains of Nck bound the R-Ras fragment. Moreover, we were also able to show that the Nck-R-Ras interaction takes place in cells. Specific binding of these proteins was seen in yeast two-hybrid tests, and we were also able to co-immunoprecipitate R-Ras and Nck from human cell extracts. The co-immunoprecipitation of Nck and R-Ras was only seen after transfection of the two proteins into the test cells, and only a small fraction of the transfected R-Ras appeared to be associated with Nck. This is not surprising, given the many other interactions of Nck with cellular signaling proteins. The interaction may also be regulated in such a way that only a subfraction of R-Ras is bound to Nck at any given time. Although we cannot exclude the possibility that some other SH3 domain proteins might also interact with R-Ras, these results suggest that R-Ras may be a physiological ligand of Nck.

The SH3 domain-binding site of R-Ras identified in this study may serve to target R-Ras to appropriate subcellular locations. The site is located in the 20 amino acids preceding the conserved C-terminal tetrapeptide motif, the CAAX box. This stretch of amino acids is highly variable among the various Ras proteins (42). However, it is conserved in the same Ras protein among species. The C-terminal regions of the only two R-Ras proteins for which the sequences are available, human and mouse, differ by one amino acid, and the proline-rich sequences are identical. This conservation suggests that the C-terminal region plays an important role in defining the divergent functions of the individual Ras proteins. In R-Ras, one such function appears to be SH3 domain binding.

Nck, a candidate binder of R-Ras in cells, interacts through its SH2 domain with p130Cas (43) and with activated receptor protein tyrosine kinases (44), including Eph receptors (45). p130Cas is a docking protein that accumulates in focal adhesions, which also contain clustered integrins (46, 47). Nck also associates with the focal adhesion kinase (48). Many tyrosine kinase receptors, including the insulin, platelet-derived growth factor and vascular endothelial growth factor receptors are also associated with integrins (49). Thus, Nck could, by binding to these molecules through an SH2 domain interaction and to R-Ras through an SH3 domain, bring R-Ras close to integrins. In addition, Nck may be able to bind R-Ras with its middle SH3 domain while simultaneously binding another protein with its other two SH3 domains. The cytokinin-associated proteins WASP and dynamin are possible candidates for such binding, because they interact with a different Nck SH3 domain (the C-terminal one) than R-Ras (50). How such interactions might contribute to cell adhesion regulation by R-Ras remains to be determined.

Acknowledgments—We thank Dr. B. Mayer for pEBB-Nck construct, Dr. J.-L. Guan for anti-HA antibody, Dr. M. Sakaue for the GSC-Nck-S43(2) construct, and Drs. Eva Engvall and Kristiina Vuori for comments on the manuscript.

REFERENCES
1. Haas, T. A., and Plow, E. F. (1994) Curr. Opin. Cell Biol. 6, 656–662
2. Springer, T. A. (1994) Cell 76, 301–314
3. Hynes, R. O. (1992) Annu. Rev. Cell Dev. Biol. 11, 541–570
4. Schwartz, M. A., Schaller, M. D., and Ginsberg, M. H. (1999) Annu. Rev. Cell Dev. Biol. 15, 549–599
5. Kao, D. P., Dustin, M. L., Miller, J. M., and Brown, E. J. (1996) J. Clin. Invest. 97, 2139–2144
6. Youwen, R. L., Felsenfeld, D. P., Kraeft, S. K., Chen, L. B., Sheetz, M. P., and Hemler, M. E. (1997) J. Exp. Med. 186, 1347–1355
7. Burridge, K., and Chrzanowska-Wodnicka, M. (1996) Annu. Rev. Cell Dev. Biol. 12, 463–518
8. Kolamus, W., Nagel, W., Schiller, B., Zeitlmann, L., Godar, S., Stockinger, H., and Seed, B. (1996) Cell 86, 253–262
9. Kashiwagi, H., Schwartz, M. A., Eigenthaler, M., Davis, K. A., Ginsberg, M. H., and Shattil, S. J. (1997) J. Cell Biol. 137, 1433–1443
10. Dedhar, S., and Hanaigou, G. E. (1996) Curr. Opin. Cell Biol. 8, 657–669
11. Bourd, D., Molla, A., and Block, M. R. (1998) J. Cell Sci. 111, 657–665
12. Hirst, R., Horwitz, A., Buck, C., and Rohrschneider, L. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 6470–6474
13. Johansson, M., Larsson, E., Luxing, B., Pasquale, E. B., and Ruoslahti, E. (1998) J. Cell Biol. 111, 657–665
14. Hirst, R., Horwitz, A., Buck, C., and Rohrschneider, L. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 6470–6474
15. Johansson, M., Larsson, E., Luxing, B., Pasquale, E. B., and Ruoslahti, E. (1998) J. Cell Biol. 111, 657–665
16. Hirst, R., Horwitz, A., Buck, C., and Rohrschneider, L. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 6470–6474
17. Zhang, Z., Vuori, K., Wang, H., Reed, J. C., and Ruoslahti, E. (1996) Cell 85, 61–69
18. Ramos, J. W., Hughes, P. E., Renshaw, M. W., Pfaff, M., Forsyth, J., Keivens, V. M., Schwartz, M. A., and Ginsberg, M. H. (1997) Cell 88, 521–530
19. Wang, B., Dickinson, L. A., Koivunen, E., Ruoslahti, E., and Kohwi-Shigematsu, T. (1995) J. Biol. Chem. 270, 23239–23242
20. Keely, P. J., Rusyn, E. V., Cox, A. D., and Parise, L. V. (1999) J. Cell Biol. 145, 1077–1088
21. Huff, S. Y., Quilliam, L. A., Cox, A. D., and Der, C. J. (1997) Oncogene 14, 133–143
22. Wang, H. G., Millan, J. A., Cox, A. D., Der, C. J., Rapp, U. R., Beck, T., Zha, H., and Reed, J. C. (1995) J. Cell Biol. 129, 1103–1114
23. Tanaka, M., Gupta, R., and Mayer, B. J. (1995) Mol. Cell Biol. 15, 6829–6837
24. Taylor, S. J., Anafi, M., Pawson, T., and Shalloway, D. (1995) J. Biol. Chem. 270, 10120–10124
25. Liu, X., Marengere, L. E., Koch, C. A., and Pawson, T. (1995) Mol. Cell Biol. 15, 5225–5232
26. Cicchetti, P., Mayer, B. J., Thiel, G., and Baltimore, D. (1992) Science 257, 803–806
27. Gout, I., Dhand, R., Hiles, I. D., Fry, M. J., Panayotou, G., Das, P., Truong, O., Totty, N. F., Huan, J., Booker, G. W., et al. (1993) Cell 75, 25–36
28. Kitamura, Y., Kitamura, T., Sakaue, H., Maeda, T., Ueno, H., Nishio, S., Ohno, S., Osada, S., Sakae, M., Ogawa, H., and Kasuga, M. (1997) Biochem. J. 322, 873–878
29. Wang, B., Dickinson, L. A., Koivunen, E., Ruoslahti, E., and Kohwi-Shigematsu, T. (1995) J. Biol. Chem. 270, 23239–23242
30. Mora, A., and Ruoslahti, E. (1992) J. Cell Biol. 118, 421–429
31. Voitek, A. B., and Ruoslahti, E. (1992) J. Cell Biol. 118, 421–429
32. Vojtek, A. B., and Hollenberg, S. M. (1995) Methods Enzymol. 235, 331–342
33. Schiestl, R. H., and Gietz, R. (1993) Curr. Genet. 16, 339–346
34. Alexandropoulos, K., Cheng, G., and Baltimore, D. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 3110–3114
35. Sparks, A. B., Rider, J. E., Hoffman, N. G., Fowlkes, D. M., Quilliam, L. A., and Kay, B. E. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1540–1544
36. Drivas, G. T., Shih, A., Coutavas, E., Rush, M. G., and D'Eustachio, P. (1990) Mol. Cell Biol. 10, 1793–1798
37. Shinjo, K., Konomi, J., Hart, M. J., Narasimhan, V., Johnson, D. I., Evans, T., and Cerione, R. A. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 9853–9857
38. Munemitsu, S., Innis, M. A., Clark, R., McCormick, P., Ulrich, A., and Polakis, P. (1990) Mol. Cell Biol. 10, 5977–5982
39. Soane, C., Holash, J. A., Pavlova, Y., and Pasquale, E. B. (1996) J. Cell Biol. 135, 781–795
40. Winning, R. S., Scales, J. B., and Sargent, T. D. (1996) Dev. Biol. 179, 309–319
41. Spaargaren, M., Martin, G. A., McCormick, F., Fernandez-Sarbia, M. J., and Bischoff, J. R. (1994) Biochem. J. 300, 303–307
42. Lehmann, J. M., Rietthumler, G., and Johnson, J. P. (1990) Nucleic Acids Res. 18, 1048
43. Barbe, M. (1987) Annu. Rev. Biochem. 56, 779–827
44. Schlaepfer, D. D., Broome, M. A., and Hunter, T. (1997) Mol. Cell Biol. 17, 1702–1713
45. Li, W., Hu, P., Skolnik, E. Y., Ulrich, A., and Schlessinger, J. (1992) Mol. Cell Biol. 12, 5824–5833
46. Holland, S. J., Peles, E., Pawson, T., and Schlessinger, J. (1998) Curr. Opin. Neurobiol. 8, 117–127
47. Hanks, S. K., and Polte, T. R. (1996) BioEssays 19, 137–145
48. Choudhury, G. G., Marra, F., and Abboud, H. E. (1995) Am. J. Physiol. 270, F295–F300
49. Giancotti, F. G., and Ruoslahti, E. (1999) Science 285, 1028–1032
50. McCarthy, J. H. (1998) BioEssays 20, 913–921
R-Ras Contains a Proline-rich Site That Binds to SH3 Domains and Is Required for Integrin Activation by R-Ras

Bingcheng Wang, June X. Zou, Barbro Ek-Rylander and Erkki Ruoslahti

J. Biol. Chem. 2000, 275:5222-5227.
doi: 10.1074/jbc.275.7.5222

Access the most updated version of this article at http://www.jbc.org/content/275/7/5222

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 50 references, 27 of which can be accessed free at http://www.jbc.org/content/275/7/5222.full.html#ref-list-1