

# Nischarin, a Novel Protein That Interacts with the Integrin $\alpha 5$ Subunit and Inhibits Cell Migration

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**Abstract.** Integrins have been implicated in key cellular functions, including cytoskeletal organization, motility, growth, survival, and control of gene expression. The plethora of integrin  $\alpha$  and  $\beta$  subunits suggests that individual integrins have unique biological roles, implying specific molecular connections between integrins and intracellular signaling or regulatory pathways. Here, we have used a yeast two-hybrid screen to identify a novel protein, termed Nischarin, that binds preferentially to the cytoplasmic domain of the integrin  $\alpha 5$  subunit, inhibits cell motility, and alters actin filament organization. Nischarin is primarily a cytosolic protein, but clearly associates with  $\alpha 5\beta 1$ , as demonstrated by coimmunoprecipitation. Overexpression of Nischarin markedly reduces  $\alpha 5\beta 1$ -dependent cell migration in

several cell types. Rat embryo fibroblasts transfected with Nischarin constructs have “basket-like” networks of peripheral actin filaments, rather than typical stress fibers. These observations suggest that Nischarin might affect signaling to the cytoskeleton regulated by Rho-family GTPases. In support of this, Nischarin expression reverses the effect of Rac on lamellipodia formation and selectively inhibits Rac-mediated activation of the c-fos promoter. Thus, Nischarin may play a negative role in cell migration by antagonizing the actions of Rac on cytoskeletal organization and cell movement.

**Key words:** integrin • Rac • cell migration • cytoskeleton • two hybrid

## Introduction

The integrin family of cell surface glycoproteins plays a major role in the interaction of cells with the extracellular matrix (Aplin et al., 1998; Giancotti and Ruoslahti, 1999). Integrins exist as  $\alpha/\beta$  heterodimers and each subunit has a large extracellular domain, a single helical transmembrane domain, and, typically, a relatively short cytoplasmic domain. At specialized sites of cell–matrix adhesion, termed focal contacts, integrin cytoplasmic domains articulate, directly or indirectly, with various proteins, including talin,  $\alpha$ -actinin, vinculin, paxillin, tensin, and focal adhesion kinase (FAK),<sup>1</sup> that are involved in coupling between integrins and the actin cytoskeleton (Burrige and Chrzanowska-Wodnicka, 1996). Integrin–cytoskeletal linkages play a critical role in cell adhesion, determination of cell shape, and cell motility (Burrige and Chrzanowska-Wodnicka, 1996; Miyamoto et al., 1998). Integrins also play an important role in signal transduction processes, either by directly generating signals or by modulating signals generated by other receptors (Clark and Brugge, 1995;

Schwartz, 1997; Aplin et al., 1998, 1999a; Giancotti and Ruoslahti, 1999). Integrin modulation of signaling affects control of the cell cycle (Assoian, 1997) and regulation of programmed cell death (Frisch and Ruoslahti, 1997).

The cytoplasmic domains of integrins play a key role in their function. Thus, the  $\beta$  chain cytoplasmic tail has been implicated in the recruitment of integrins to focal contacts (Reszka et al., 1992), activation of FAK (Akiyama et al., 1994), and determining the affinity of integrins for their ligands (Wang et al., 1997). Similarly, the  $\alpha$  subunit cytoplasmic tail has been implicated in regulation of integrin affinity (O’Toole et al., 1994) and control of cell motility (Chan et al., 1992; Bauer et al., 1993).

Integrins can interact with a variety of partner proteins, including various membrane receptors that bind to the extracellular and transmembrane domains of integrins (Hemler, 1998; Porter and Hogg, 1998), as well as intracellular proteins that associate with integrin cytoplasmic tails (Aplin et al., 1998). Yeast two-hybrid techniques have been used to identify several proteins that interact with integrin  $\beta$  subunit cytoplasmic domains and that have interesting and important biological functions (Kolanus et al., 1996; Biffo et al., 1997; Chang et al., 1997; Kashiwagi et al., 1997; Delcommenne et al., 1998; Li et al., 1999; Zhang and Hemler, 1999). Fewer proteins have been reported to interact with  $\alpha$  chain cytoplasmic domains. Thus, calreticulin

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<sup>1</sup>Abbreviations used in this paper: FAK, focal adhesion kinase; GFP, green fluorescent protein; ORF, open reading frame; REF, rat embryonic fibroblast; RIE, rat intestinal epithelial.

has been reported to bind the conserved GFFKR motif found in all  $\alpha$  chains and to modulate integrin affinity (Coppolino et al., 1997), whereas calcein integrin binding protein (CIB) is a calcium-binding protein that associates specifically with the cytoplasmic domain of  $\alpha$ IIB, possibly playing a role in activation of the  $\alpha$ IIB $\beta$ 3 integrin (Naik et al., 1997).

The  $\alpha$ 5 $\beta$ 1 integrin, a receptor for fibronectin, seems to play a special role in regulating growth and survival in some cell types. Thus, high expression of  $\alpha$ 5 $\beta$ 1 has been linked with reductions in tumor cell growth rates both in vitro and in vivo (Giancotti and Ruoslahti, 1990; Schreiner et al., 1991; Varner et al., 1995). Surprisingly,  $\alpha$ 5 $\beta$ 1 also plays a unique role in protecting cells against apoptosis triggered by mitogen deprivation (Zhang et al., 1995; O'Brien et al., 1996; Lee and Juliano, 2000). In addition,  $\alpha$ 5 $\beta$ 1 has been reported to regulate muscle cell growth and differentiation (Sastry et al., 1999). These data suggest that certain effects of  $\alpha$ 5 $\beta$ 1 on growth or apoptosis may be  $\alpha$ 5 specific, and thus, there may be intracellular proteins that selectively interact with the  $\alpha$ 5 cytoplasmic tail to mediate these events. Accordingly, we have made use of the yeast two-hybrid system to identify proteins that bind to the  $\alpha$ 5 cytoplasmic domain. We have identified a novel protein that associates with the cytoplasmic tail of the  $\alpha$ 5 subunit, and, to a minor degree, with cytoplasmic domains of other  $\alpha$  subunits, and that strongly affects cell migration and influences cytoskeletal organization. We named this novel protein Nischarin, which is derived from a term in classic Sanskrit that connotes slowness of motion. This designation is based on the finding, shown below, that overexpression of Nischarin dramatically impairs cell migration.

## Materials and Methods

### Yeast Two-Hybrid Screening

Here, L40 (Mata his3 $\Delta$ 200 trp1-901, 112 ade2 LYS2::(*lexAop*)<sub>4</sub>-HIS3 URA3(*lexAop*)<sub>8</sub>-LacZ Gal4) and AMR70 (Mata his3 lys2 trp1 leu2 URA3::(*lexAop*)<sub>8</sub>-LacZ Gal4) yeast strains were used (gifts from Dr. Stan Hollenberg, Vollum Institute, Oregon Health Sciences University, Portland, OR). Yeast two-hybrid screening was conducted as previously described (Vojtek et al., 1993). The pBTM  $\alpha$ 5 plasmid, which has the  $\alpha$ 5 cytoplasmic domain fused to the LexA DNA-binding domain and with a tryptophan marker, was transformed into yeast strain L40 and selected for tryptophan prototrophy. Plasmids (pVp16) containing mouse embryonic cDNA libraries of 9.5 and 10.5 d fused to the VP16-transactivating domain and a leucine marker were transformed into the L40 strain containing the bait plasmid and screened for leucine, tryptophan, and histidine prototrophy. A total of  $1.7 \times 10^7$  transformants were screened for positives. The libraries and vectors were gifts from Dr. Stan Hollenberg. Histidine-positive colonies were further tested for LacZ activation. Dual positives were further confirmed for specificity of the interaction using various baits and included integrin  $\beta$ 1,  $\alpha$ 2, and  $\alpha$ v cytoplasmic domains, as well as lamin, an irrelevant protein in this context. Specificity of the interaction was confirmed by mating experiments. The pBTM bait plasmids were "cured" from dual-positive clones by growth in nonselective medium. The presence of the library plasmids with inserts in the "cured" clones was confirmed by PCR using vector-specific primers. AMR 70 strain cells were transformed separately with pBTM  $\alpha$ 5, pBTM  $\beta$ 1, pBTM  $\alpha$ v, pBTM  $\alpha$ 2, pBTM lamin, or pBTM vector alone. These transformed cells were mated with the "cured" L40 cells that contained positive pVP16 library plasmids.

### Cloning of Full-Length Nischarin

To clone full-length Nischarin, we screened a mouse brain library in the lambda Zap II vector (Stratagene). Using a colony hybridization technique, ~30,000 plaques were screened with a <sup>32</sup>P-labeled PCR product consisting of 0.45 kb of the integrin-binding region of Nischarin. From this screen, one strong positive plaque was identified and confirmed in two

further rounds of screening. Sequence analysis of this clone (A3.1) indicated that the sequence was incomplete at the 3' end. Using a different PCR probe, the lambda Zap library was screened again to obtain the remainder of the Nischarin cDNA. This screen gave several positives, and the longest clone (clone 14.2) was picked. Clones A3.1 and 14.2 provided the complete open reading frame (ORF) of Nischarin.

### DNA Constructions and Transfection

The construction of two-hybrid bait plasmids, GST chimeras, and partial- and full-length myc-tagged Nischarin mammalian expression constructs followed standard recombinant DNA procedures. Clones A3.1 and 14.2, mentioned above, were used to make full-length expression constructs. Full details are available upon request. A chimera comprised of full-length Nischarin and GFP was prepared by an inframe insertion of the coding region of Nischarin into the pE-GFP-N1 vector (CLONTECH Laboratories, Inc.). Expression plasmids for CD4, human  $\alpha$ 2, and  $\alpha$ v integrin subunits were obtained from Drs. R. Nicholas (University of North Carolina-Chapel Hill, Chapel Hill, NC), L. Parise (University of North Carolina-Chapel Hill), and David Cheresch (The Scripps Research Institute, La Jolla, CA), respectively. Transfection of mammalian cell lines was usually done with Lipofectamine (GIBCO BRL) or Superfect (QIAGEN), according to the manufacturer's specifications.

### Northern Blot Analyses

A mouse multiple tissue Northern blot (CLONTECH Laboratories, Inc.) was probed with a 0.45-kb fragment of the  $\alpha$ 5 integrin-binding region of Nischarin (probe 1) (nucleotides 1,305–1,743), with fragments from the far 5' end (probe 2) (nucleotides –334–+56), or the 3' end (probe 3) (nucleotides 2,936–3,748). RNA was isolated from various cell lines, run on agarose-formaldehyde gels, and hybridized with probes 1–3, using previously described techniques (Alahari et al., 1996).

### Antibodies

The predicted ORF of Nischarin was used to design two peptides represented at the far COOH terminus of the protein. The peptides (EALCGRELPVELTGA-C and LDDGRRVRDLDRVL-C) were obtained from the University of North Carolina-Protein Core Laboratory. Both peptides were conjugated to keyhole limpet hemocyanin (Pierce Chemical Co.) and sent to Aves Laboratories for production of chicken pAbs. Anti- $\alpha$ 5 cytoplasmic domain pAb was a gift from Richard Hynes (Massachusetts Institute of Technology, Cambridge, MA). Anti-myc mAbs and pAbs were purchased from Babco. pAbs to  $\alpha$ v cytoplasmic domain were provided by Guido Tarone (University of Torino, Torino, Italy). Rat anti-mouse  $\alpha$ 5 mAb, and control rat IgG were purchased from Pharmingen and Sigma-Aldrich, respectively. mAbs to vinculin and phosphotyrosine were purchased from Sigma-Aldrich and Upstate Biotechnology. Fluorescent phalloidin was bought from Sigma-Aldrich. A partially purified preparation of the human  $\alpha$ 5 $\beta$ 1 integrin (Chemicon) was sometimes used as a control.

### Binding To GST Fusion Proteins

GST-Nischarin fusion proteins expressed from pGEX vectors (Amersham Pharmacia Biotech) were prepared in a standard manner and bound to glutathione-Sepharose 4B beads for "pull down" experiments. CHO cells (clone B2 $\alpha$ 27), which overexpress the human  $\alpha$ 5 integrin subunit, were used as the source of integrins (Bauer et al., 1993).  $\alpha$ 5-deficient cells (CHO B2) were used as controls. CHO cells were lysed in a buffer containing nonionic detergent and protease inhibitors. The CHO lysate was added to the GST protein-containing beads, incubated for 1 h at 4°C, and washed four times with buffer. Bound CHO proteins were eluted by boiling in 2 $\times$  SDS sample buffer and analyzed by Western blotting using anti- $\alpha$ 5 cytoplasmic domain antibody.

### Coimmunoprecipitation Experiments

CHO B2 $\alpha$ 27 and B2 cells were transiently transfected with myc vector, myc-Nischarin (434–581), or myc-Raf. After 48 h of transfection, cells were lysed in a 0.5% Triton X-100 buffer. These lysates were immunoprecipitated with anti-myc antibody, resolved by 7% SDS-PAGE, electrophoretically transferred to nylon membranes, and Western blotted with anti- $\alpha$ 5 cytoplasmic domain antibody. In further studies with full-length Nischarin, cells were lysed in a buffer containing 0.1% Triton X-100 (Borowsky and Hynes, 1998). In one set of experiments, lysates of Cos7 cells cotransfected with myc-Nischarin and  $\alpha$ 5,  $\alpha$ v, or CD4, were immuno-

precipitated with anti-myc and blotted with anti- $\alpha 5$  extracellular domain antibody (Transduction Laboratories), anti- $\alpha v$ , or anti-CD4 antibodies (Santa Cruz Biotechnology, Inc.). For mouse NB41A3 cells, endogenous  $\alpha 5$  was immunoprecipitated with rat anti- $\alpha 5$  mAb and the immunoprecipitate was blotted for endogenous Nischarin using the chicken anti-Nischarin pAb described above.

### Cell Migration Experiments

Wound-type cell migration experiments were performed as described previously (Bauer et al., 1992, 1993), with minor modifications. In brief, 3T3 cells were cotransfected with 1  $\mu$ g of  $\beta$ -galactosidase plasmid and various amounts of full-length myc-Nischarin construct and plated on gridded tissue culture dishes. After 48 h, the cell layer was scraped along the center of the dish with a sterile razor blade. After overnight incubation at 37°C in serum-containing medium, cells migrating into the scraped area were detected by staining for  $\beta$ -galactosidase. The percent of transfected cells that migrated across the line and into the denuded area was calculated by counting blue cells in several gridded fields from the unscraped area and in several fields from the scraped area. At least 50 migrant cells were counted for each condition. The ratio of migrant transfected cells to total transfected cells  $\times 100$  was taken as the percent migration.

Cell migration studies using Nischarin-transfected or control-transfected 3T3 cells or CHO cells were also performed using a transwell assay, according to a previously described procedure (Keely et al., 1997). The transfected cells were marked by use of a GFP expression plasmid. Fibronectin or other matrix proteins were coated on the underside of the transwell, the cells were plated on the upper surface, and the percent of Nischarin or control transfectants migrating across the 8- $\mu$ m pore size membrane was determined by visual inspection in a fluorescence microscope after overnight incubation in BSA-containing medium. Transwell experiments were performed with wild-type 3T3 cells, 3T3 sublines over-expressing human  $\alpha 5$  or  $\alpha 2$  subunits (Aplin et al., 1999b), and CHO B2 cells lacking  $\alpha 5$ , as well as CHO B2a27, its  $\alpha 5$  transfectant (Bauer et al., 1993).

### Subcellular Fractionation

Cos7 cells transfected with myc-Nischarin and untransfected Neuro 2A cells were subjected to subcellular fractionation, as described previously (Gu and Majerus, 1996), with minor modifications. Cell lysates were centrifuged briefly at 1,500 rpm to remove nuclei and intact cells. The supernatant was further spun at 100,000 g for 30 min at 4°C; this supernatant was considered to be the cytosolic fraction. The pellet was solubilized in a 1% Triton X-100-containing solution and centrifuged at 100,000 g for 30 min; this supernatant was considered to be the membrane fraction. Membrane and cytosolic fractions were resolved by 8% SDS-PAGE, electrophoretically transferred onto a nylon membrane, and blotted with anti-Nischarin antibodies, as described above.

### Fluorescence Microscopy

Immunofluorescence studies with antibodies to integrins or focal contact proteins were conducted according to procedures described previously (Burrige et al., 1992). Rat embryonic fibroblasts (REFs) were cotransfected with 1  $\mu$ g of GFP plasmid and 2  $\mu$ g of myc-Nischarin plasmid, or 1  $\mu$ g of GFP alone, per well on six-well plates. After 48 h, cells were trypsinized and plated onto fibronectin-coated cover slips for 3 h in serum-containing medium. The cells were washed three times with cold PBS, fixed for 10 min in 0.37% formaldehyde, and permeabilized in 1% Triton X-100 for 5 min. Then, cells were washed several times and blocked in 2% BSA for 1 h at room temperature. Primary antibody incubation was done in a moist chamber overnight in a cold room. Anti-tubulin, anti-PY, anti-vinculin, and anti-vimentin antibodies were used at a dilution of 1:100. After rinsing in PBS, coverslips were incubated with an appropriate TRITC-conjugated secondary antibody for 1 h at room temperature. For actin staining, cover slips were incubated with TRITC-phalloidin (1:1,000) for 15 min.

In some cases, the subcellular distribution of Nischarin was evaluated using the full-length Nischarin-GFP chimera described above. This was transfected into 3T3 cells at a level of 2  $\mu$ g per well (it should be noted that levels of expression of Nischarin-GFP chimeric protein were substantially lower than expression of myc-Nischarin protein when equivalent amounts of plasmid were transfected). After 48 h of transfection, cells were plated onto fibronectin coverslips, as described above, and incubated with antibodies to vinculin or integrins, and then with TRITC-conjugated secondary antibody, or with TRITC-phalloidin to visualize actin. In all cases, coverslips were observed on a ZEISS Axioscop fluorescence micro-

scope using a 40 $\times$  oil immersion objective. Images were recorded using a CCD camera and a computer with Metamorph image analysis software.

### Rho GTPase Experiments

For studies on Rho-mediated signaling, NIH 3T3 cells were cotransfected with 1  $\mu$ g of luciferase reporter under the control of the c-fos promoter (c-fos-Luc) (Hill et al., 1995), 3  $\mu$ g of pAX142 vector, pAX142 Rac Q61L (Whitehead et al., 1988), or an activated MEK construct (pFC-MEK1; Stratagene) and various amounts of pcDNA myc-Nischarin or pcDNA vector, using Superfect. The pAX142 vectors were provided by Drs. I. Whitehead and C. Der (University of North Carolina-Chapel Hill). After 4 h of transfection, cells were washed with PBS, maintained in 0.5% serum for 24 h, and lysed in luciferase buffer, as described above. Additional experiments were done with commercial luciferase reporter systems (Stratagene) using either Rac-driven c-Jun transcriptional activation or protein kinase A-driven activation of the cyclic AMP-response element (CRE)-response element. In all transfections, DNA quantities were normalized with the pcDNA vector. Luciferase activity was measured by normalizing for total protein content or by coexpression of *Renilla* luciferase.

To study the effect of Rho-family GTPase on the cytoskeleton, 3T3 cells were transfected with a plasmid expressing an activated (Q61L) form of Rac and with a Nischarin plasmid or with a vector control. A small amount of a GFP-expressing plasmid was used to mark the transfectants. After 48 h, the actin filaments were stained with TRITC-phalloidin and the cells were observed by fluorescence microscopy, as described above.

## Results

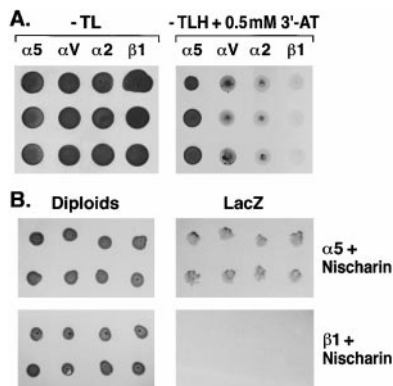
### Detection of a Novel $\alpha 5$ -interacting Protein

We used a yeast two-hybrid screen to identify proteins that interact with the  $\alpha 5$  cytoplasmic domain. A protein composed of the complete cytoplasmic tail of  $\alpha 5$  fused with the DNA-binding domain of Lex A was expressed from the yeast plasmid pBTM $\alpha 5$ . We searched mouse embryonic libraries for proteins that interact with the  $\alpha 5$  cytoplasmic tail. Protein domains from the libraries were fused to the VP16-transactivating domain and expressed from the yeast plasmid pVP16. Cotransformants of pBTM $\alpha 5$  and pVP16 in the yeast L40 were screened for conversion to histidine prototrophy. Out of  $1.7 \times 10^7$  transformants screened, 120 colonies were positive for histidine; of those, 45 were also positive for LacZ activation. To determine specificity, several other "baits" were tested for interaction with the  $\alpha 5$ -binding library protein(s). In particular, we tested for interactions with the cytoplasmic domains of the  $\alpha 2$ ,  $\alpha v$ , or  $\beta 1$  integrin subunits, or with the irrelevant protein lamin. As seen in Fig. 1 and Table I, the  $\alpha 5$  bait strongly interacted with the library protein and activated expression of the histidine and LacZ markers. The

Table I. Summary of Yeast Two-Hybrid Interactions

Baits	Nischarin	
	Histidine prototrophy	$\beta$ -gal positivity
$\alpha 5$ tail	++++	++++
$\alpha 2$ tail	+	--
$\alpha v$ tail	+	--
$\beta 1$ tail	--	--
Lamin	--	--
Vector	--	--

The AMR 70 yeast strain, which is suitable for mating with the L40 strain, was transformed with pBTM  $\alpha 5$ , pBTM  $\alpha 2$ , pBTM  $\alpha v$ , pBTM lamin, or pBTM vector alone. These cells were mated with L40 cells that contained Nischarin-positive pVP16 library plasmids to yield diploids. Positive interactions were detected by  $\beta$ -galactosidase assay and histidine prototrophy, whereas growth of only diploid cells was assured by use of selective media.



**Figure 1.** Two-hybrid analysis for specificity of Nischarin interactions. (A) Activation of His3 by  $\alpha$  subunits. A yeast strain that contains the  $\alpha 5$ -reactive (Nischarin) fragment isolated from the yeast library screen was transformed with  $\alpha 5$ ,  $\alpha V$ ,  $\alpha 2$ , and  $\beta 1$  cytoplasmic tails and lamin and screened for histidine transactivation. The left side confirms the presence of the bait and library plasmids by growth on tryptophan- and leucine-deficient plates (-TL). The transformants were tested for their ability to grow on histidine-deficient plates containing 0.5 mM 3'-amino-1,2,4-triazole. The  $\beta 1$ -transformed cells have growth similar to lamin-transformed or -untransformed yeast cells and are considered to be at background levels. (B) Activation of the lacZ reporter gene. This diploid interaction analysis was performed as described in Materials and Methods. The left two panels show growth of the diploids on selective media that excludes growth of haploid cells. The right two panels show X-gal staining of these diploids and illustrates that use of an integrin  $\alpha 5$  subunit cytoplasmic domain bait plasmid activates  $\beta$ -galactosidase, whereas use of a  $\beta 1$  subunit bait plasmid does not. Results using several bait plasmids are summarized in Table I.

$\alpha V$  and  $\alpha 2$  baits only weakly activated the histidine reporter, and were unable to activate LacZ. These data suggest that the  $\alpha 5$ -positive library protein may interact weakly with several integrin  $\alpha$  subunits, but binds strongly to the  $\alpha 5$  subunit.

Restriction enzyme analysis and DNA sequencing revealed that the insert in all positives tested was comprised of the identical 450 nucleotide sequence. This sequence comprises an ORF, but no start or stop codons. To find the full-length sequence of this cDNA, we screened a mouse brain library in the lambda zap II vector. We identified two overlapping cDNA fragments; and sequence analysis of these fragments revealed the complete ORF for this gene. This consists of 4,062 nucleotides and codes for a novel protein of 1,354 amino acids, with a predicted molecular weight of 148,053. As mentioned above, we have termed this protein Nischarin, a name which describes its effects on cell migration. The predicted amino acid sequence of Nischarin is shown in Fig. 2. The validity of this ORF as a protein-coding region is suggested by the presence of a well conserved Kozak sequence immediately 5' of the ATG, the presence of a poly A tail in the 3' untranslated region, the fact that the observed message size (5.5 kb, see below) is consistent with the cDNA size, and the fact that several mouse expressed sequence tags, which have the correct reading frame, overlap our predicted ORF region.

### Homologies with Other Proteins

BLAST analysis indicates that Nischarin lacks significant homology with any known protein with a well-described

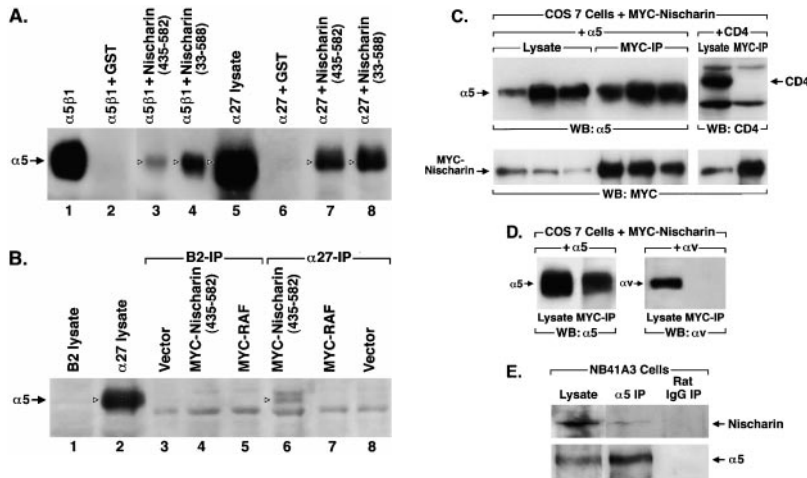
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MSVRFPSATSMKEVLAPEASEFDEWEPEGTATLGGPVTAIIPQWALTTLDLSHNSICEIDES 62
VKLIPKIEYLDLSDHNGLRVVDNLQHLVNLVHLDLSYNKLSLEGVHTKLGNVKTLNLAGNFL 124
ESLSGLHKLYSLVMVDLRDNRIEQLDDEVKSTGSLPCLERLTLNPLSIIIPDYRTKVLSPQFG 186
ERASEICLDDVATTEKELDTVEVLKAIQKAKDVKSKLSNTEKKAGEDFRLLPAPACIRPGGSP 248
PAAPASASLPQPILSNQGMFVQEEALASSLSSTDSLPPEDHRPIARACSDSLESIPAGQVA 310
SDDLDRVPGAVGVSPDHAEEFVQVVPGSGQIIFLFPFCIGYTATNQDFIQRLSTLIRQAIE 372
RQLPAWTEAANQREEAHGEQGESEEEEEEDVAENRYFEMGPPDAEEEGSGQGEDEDEDE 434
DEEAEEERLALWALGADEDFLLEHIRILKVLWCFLIHVQGSIRQFAACLVLDTDFGIAVFEI 496
PHQESRGSSQHILSSLRVFCFPHGDLTEFGFLMPELCLVLKVRHSENTLFIISDAANLHEF 558
HADLRSCFPAPQHMMALMCSFILYGSHTTLQEFRLQLLTPYKVAGGSQERSQCFVVLVYSDK 620
RMVQTPAGDYSGNIWASCTLCSAVRRSCCAPSEAVKSAATPYWLLLTSQLHNVIKADFNPM 682
PNRGTHNCRNRNSFKLSRVPLSTVLLDPTRSCTQPRGAFADGHVLELLVGVRFVTAIFVLP 744
EKPFHLRVYVQLRASLDLKTVVISKNPSAKPRNQPAKSRASAEQRLQETPADAPAAVPP 806
TASAPAPAEALAPDLAPVQAPGEDRGLTSABEAPAAAEAPAAAEAPAAAEAPAAAEAPAAEA 868
PAAAEAPAPAEAPAAAEAPAAAEAPAAAEAPAAAEAPAAAEAPAPNQAPAPARGPAPARGPA 930
PAGGPAPAGGPAPAEALAQAEVPAQYPSERLIQSTSEENQIPSHLFPVCPSLQHIARLRGRAI 992
IDLFNHNSIAEVENEELRHLLWSSVVFYQTPGLEVTACVLLSSKAVYFILHDGLRRYFSEPLQ 1054
DFWHQKNTDYNNSPFHVSQCFLVKLSDLSQVNVGLFDQYFRLTGSSPTQVVTCLTRDSYLTH 1116
CFLQHMLVLSLLERTSPPEVDKDFYSEFGDKNTGKMMENYELIHSSRVKFTYPSSEEVGDL 1178
TYIVAQKMDPAKPNALSILLYIQAFQVVTPLHGRGRGRLPKTLTLLSABEIFFLDDYIHY 1240
PLPEFAKEPPQRDRYRLDDGRRVRLDRVLMGYYPYQALTLVFDDETQGHDLMGSVTLDFHG 1302
EMPGGPGRVGGQREVQVQVFVPSAESREKLISLLARQWEALCGRELPELVTG 1354

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**Figure 2.** The primary sequence of Nischarin (sequence data available from GenBank/EMBL/DDBJ under accession no. AF315344). The amino acid sequence of the Nischarin ORF is shown. Notable features include (a) the proline-rich region (795–955), (b) the cytochrome motif (558–567), and (c) two leucine-zipper motifs (449–470) and (1211–1232). The integrin-binding region identified in the original two-hybrid screen (434–581) is in bold. Potential SH3 domain-binding sites are indicated by dotted underlining. The COOH-terminal peptides used for chicken antibody production are underlined.

function. A close human homologue of Nischarin protein has been reported in GenBank (sequence data available from GenBank/EMBL/DDBJ under accession no. AF082516). It has been suggested, based on limited evidence (Ivanov et al., 1998), that this protein is a neuroreceptor for imidazoline compounds. The human protein is very similar to Nischarin. Thus, there is 82% identity in a 792 NH<sub>2</sub>-terminal region that includes the 150 residue integrin-binding region originally identified by two-hybrid screening. There is also 84% identity in a 400 residue COOH-terminal region. However, there is less homology in the central proline-rich region (see below). The NH<sub>2</sub>-terminal region of Nischarin also displays a strong homology to a *Caenorhabditis elegans* protein (sequence data available from GenBank/EMBL/DDBJ under accession no. Z69383) of unknown function that resembles the regulatory subunits of protein phosphatases. In addition, there are two *Drosophila* homologues of the NH<sub>2</sub>-terminal region (AE003611 and AE003811). When BLAST searching is performed with the filter for low complexity regions inactivated, the central region of Nischarin, which has multiple repeats of proline, alanine, and glutamic acid residues, is found to resemble regions seen in neurofilament proteins (e.g., accession no. Z31012) and dermal proteins (accession nos. P17437 and X51394). Despite the existence of



**Figure 3.** Specific binding of Nischarin to  $\alpha 5\beta 1$  in vitro and in vivo. (A) GST pull-down experiments were performed as described in Materials and Methods. Cell lysates using a nonionic detergent were obtained from  $\alpha 5$  positive-CHO B2a27 cells. Alternatively, a partially purified  $\alpha 5\beta 1$  was used. The GST proteins used were GST-Nisch (435–582), GST-Nisch (33–588), and unmodified GST; these were bound to glutathione-Sepharose beads. The lysate material retained on the GST beads was resolved by SDS-PAGE and Western blotted with an anti- $\alpha 5$  antibody. The  $\alpha 5$  band is marked with an arrowhead. Lane 1, purified  $\alpha 5\beta 1$ ; lanes 2–4, pull-downs of partially purified  $\alpha 5\beta 1$  by GST, GST-Nisch(435–582), GST-Nisch(33–588); lane 5, lysate from CHO B2a27 cells; lanes 6–8, pull-downs of B2a27 lysate by GST, GST-Nisch(435–582), GST-Nisch(33–588). (B)  $\alpha 5$  positive-CHO B2a27 cells and  $\alpha 5$  negative-CHO B2 cells were

transiently transfected with plasmids expressing myc-Nisch (435–582), myc-Raf, or with empty vector. Transfected cell lysates were immunoprecipitated with anti-myc antibody, followed by Western blotting with an anti- $\alpha 5$  antibody. The  $\alpha 5$  band is marked with an arrowhead. Lane 1, lysate from CHO B2 cells; lane 2, lysate from CHO B2a27 cells; lanes 3–5, anti-myc immunoprecipitates from CHO B2 cells; lanes 6–8 anti-myc immunoprecipitates from CHO B2a27 cells. (C) Cos 7 cells were transiently cotransfected with full-length myc-Nischarin and  $\alpha 5$  plasmid or myc-Nischarin and CD4 plasmid. The lysates were immunoprecipitated with anti-myc antibody and blotted with anti- $\alpha 5$  antibody (Transduction labs) or anti-CD4 antibody (Santa Cruz Biotechnology, Inc.). The first three lanes represent lysates from  $\alpha 5$  and myc-Nischarin-transfected cells in three different experiments and the next three lanes represent their respective myc immunoprecipitates. On the right side, CD4- and myc-Nischarin-transfected cell lysate and its myc immunoprecipitate are shown. The bottom row indicates the expression of myc-Nischarin in all conditions. (D) Cos 7 cells were transiently cotransfected with myc-Nischarin and  $\alpha 5$  or  $\alpha v$  plasmids. Myc immunoprecipitates and lysates were Western blotted with  $\alpha 5$  or  $\alpha v$  antibodies. In each western blot, the first lane is lysate and the second lane is its myc-immunoprecipitate, both blotted with anti-integrin subunit antibodies. In panel (E) lysates of mouse NB41A3 cells were exposed either to rat anti-mouse  $\alpha 5$  monoclonal IgG or an equivalent amount of native rat IgG; the lysates were then precipitated using protein G agarose beads. The immunoprecipitates were then Western blotted with either chicken anti-Nischarin antibody (top) or anti- $\alpha 5$  antibody (bottom).

a highly proline-rich region, no consensus-binding sites for SH3 domains (Sparks et al., 1996) were identified in this region (however, see below). The integrin-binding region of Nischarin identified in the two-hybrid screen does not have any close homologues other than in its human direct counterpart. We have used several software programs to look for specific functional motifs that might be present in Nischarin. Aside from the few exceptions noted below, we were unable to find well-known protein functional motifs in Nischarin. There is a cytochrome p450 motif (FHADLRSCFA) 558–567 that may be indicative of an enzymatic function; there are two leucine zipper repeats 449–470 and 1,211–1,232 (LGADEDFLLEHIRILKVLWCFL and LGRGRGPLRPKTLTLLTSAEIFL) that may be predictive of protein–protein interaction sites. There are also potential SH3 domain-binding motifs, two in the NH<sub>2</sub>-terminal region and two in the COOH-terminal region. Thus, the primary sequence of Nischarin offers few clues to the biological function of this protein.

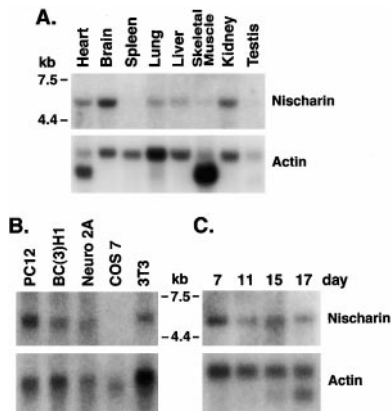
### Nischarin Binds Integrin $\alpha 5$ Subunit In Vitro and In Vivo

To confirm that the interaction between the  $\alpha 5$  cytoplasmic tail and Nischarin detected by two-hybrid analysis also occurs in vitro, two GST-Nischarin constructs were made, GST-Nisch (435–582) and GST-Nisch (33–588). GST-Nisch (435–582) corresponds to the integrin-binding region of Nischarin identified in the two-hybrid system, whereas the second construct contains additional NH<sub>2</sub>-terminal residues. The Nischarin-GST fusion proteins were immobilized on a glutathione-agarose matrix and incubated with purified  $\alpha 5\beta 1$  protein, with a cell lysate of

CHO B2a27 (human  $\alpha 5$ -transfected cells) or CHO B2 ( $\alpha 5$ -deficient cells). The proteins retained on the glutathione matrix were analyzed by SDS-PAGE and immunoblotting for  $\alpha 5$ . Consistent with the yeast data, the GST-Nischarin fusion proteins, but not GST alone, were able to interact with purified  $\alpha 5\beta 1$  or with  $\alpha 5\beta 1$  from a cell lysate (Fig. 3 A). These data indicate Nischarin binds the  $\alpha 5$  integrin subunit in vitro.

Several types of coimmunoprecipitation experiments were performed to confirm that Nischarin interacts selectively with the  $\alpha 5$  integrin subunit in mammalian cells. First, CHO B2a27 cells were transiently transfected with a construct expressing a truncated myc epitope-tagged segment of Nischarin (435–582), with myc-tagged Raf, or with myc vector alone. Cells were lysed in a buffer containing nonionic detergent, immunoprecipitated with anti-myc antibody, and blotted with anti- $\alpha 5$  or anti-myc antibodies. Western blotting with anti- $\alpha 5$  antibody indicated that  $\alpha 5$  coimmunoprecipitated with anti-myc antibody only in cells transfected with myc-Nischarin (435–582), but not in cells transfected with myc-Raf or myc vector alone (Fig. 3 B). Expression of similar amounts of myc-Nischarin and myc-Raf was confirmed by Western blotting (data not shown). In experiments with CHO B2 cells, which lack  $\alpha 5$ , Nischarin did not coimmunoprecipitate a band in the  $\alpha 5$  region. This indicates that myc-tagged Nischarin (435–582), but not an irrelevant myc-tagged protein, can bind  $\alpha 5\beta 1$  integrin.

Further coimmunoprecipitation experiments were done using full-length Nischarin. To show that Nischarin interacts preferentially with  $\alpha 5$  integrins and not with other transmembrane proteins, coimmunoprecipitations were done with CD4, another protein having a single transmembrane domain. Cos 7 cells were transiently cotransfected



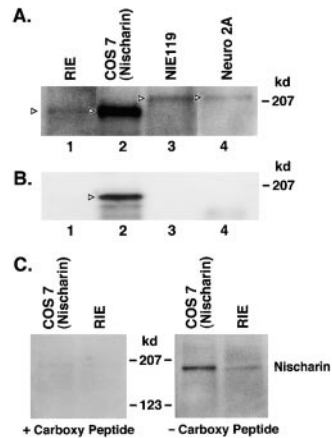
**Figure 4.** Northern blot analysis of Nischarin message. (A) A commercial multiple tissue membrane was hybridized with a 0.45-kb Nischarin probe, stripped, and rehybridized with an actin probe. The position of the 5.5-kb Nischarin message corresponds to the major band. (B) The same procedure was applied to RNA extracted from several cell lines. (C) A commercial membrane containing mouse RNA from several developmental stages was analyzed by the same procedure.

with myc-Nischarin and pcDNA- $\alpha 5$  or myc-Nischarin and pcDNA-CD4. These cells were lysed and myc immunoprecipitates were processed for blotting with anti- $\alpha 5$  or anti-CD4 antibodies. As shown in Fig. 3 C, myc-Nischarin specifically immunoprecipitated  $\alpha 5$ , but not CD4.

To further examine the alpha subunit selectivity of Nischarin, Cos 7 cells were cotransfected with myc-Nischarin and plasmids expressing the human  $\alpha 5$  or  $\alpha v$  subunits. These cells were lysed and myc immunoprecipitates were processed for blotting with anti- $\alpha 5$  or anti- $\alpha v$  antibodies. As seen in Fig. 3 D, Nischarin coimmunoprecipitated substantial amounts of  $\alpha 5$ , but barely detectable  $\alpha v$  subunits. Thus, the strong preference of Nischarin for the  $\alpha 5$  subunit, which was detected in the two-hybrid analysis, seems to be borne out in the cellular setting.

Coimmunoprecipitation experiments were also done for endogenous Nischarin and endogenous  $\alpha 5$  subunit. NB41A3 cells, a mouse neuronal-derived line, were lysed and immunoprecipitates formed using rat anti-mouse  $\alpha 5$  mAb, or rat IgG as a control. The immunoprecipitates were Western blotted using chicken anti-Nischarin pAb. As seen in Fig. 3 E, a band for Nischarin was detected in the  $\alpha 5$  immunoprecipitate, but not in the control. It was not possible to examine other integrin  $\alpha$ -subunit immunoprecipitates, since only low levels of expression were found in NB41A3 cells for other integrins for which anti-mouse mAb are available.

The experiments shown in Fig. 3 A–E indicate: (a) full-length Nischarin or truncated Nischarin (435–582) can bind to the  $\alpha 5$  integrin subunit, (b) other proteins (e.g., myc-Raf) do not bind  $\alpha 5$  under the conditions used for immunoprecipitation, and (c) full-length Nischarin does not bind to coexpressed irrelevant proteins. Furthermore, Nischarin seems to prefer the  $\alpha 5$  subunit compared with other tested  $\alpha$  subunits. These findings demonstrate a selective interaction between Nischarin and the  $\alpha 5$  integrin subunit in mammalian cells. This interaction is able to occur under physiological conditions, as indicated by the coimmunoprecipitation of endogenous  $\alpha 5$  and Nischarin.



**Figure 5.** Expression of Nischarin in cell lines. Various rodent cell lines, as well as Cos7 cells transfected with full-length myc-tagged Nischarin, were lysed in a non-ionic detergent-containing buffer and the lysates were resolved by SDS-PAGE. The samples were Western blotted using either anti-myc antibody or a chicken antibody to a peptide sequence from the COOH-terminal region of Nischarin. (A) A comparison of the expression of Nischarin in several rodent cell lines and in transfected Cos cells, using the chicken anti-Nischarin antibody (the Nischarin bands are marked with arrowheads). (B) The lysates are the same as in A, except the membrane was blotted with anti-myc antibody, demonstrating that the anti-myc and anti-Nischarin antibodies react with the same protein in transfected Cos cells. (C) The peptide used for immunization can block the binding of chicken anti-Nischarin antibody both to endogenous Nischarin in RIE cells and to expressed Nischarin in Cos7 cells.

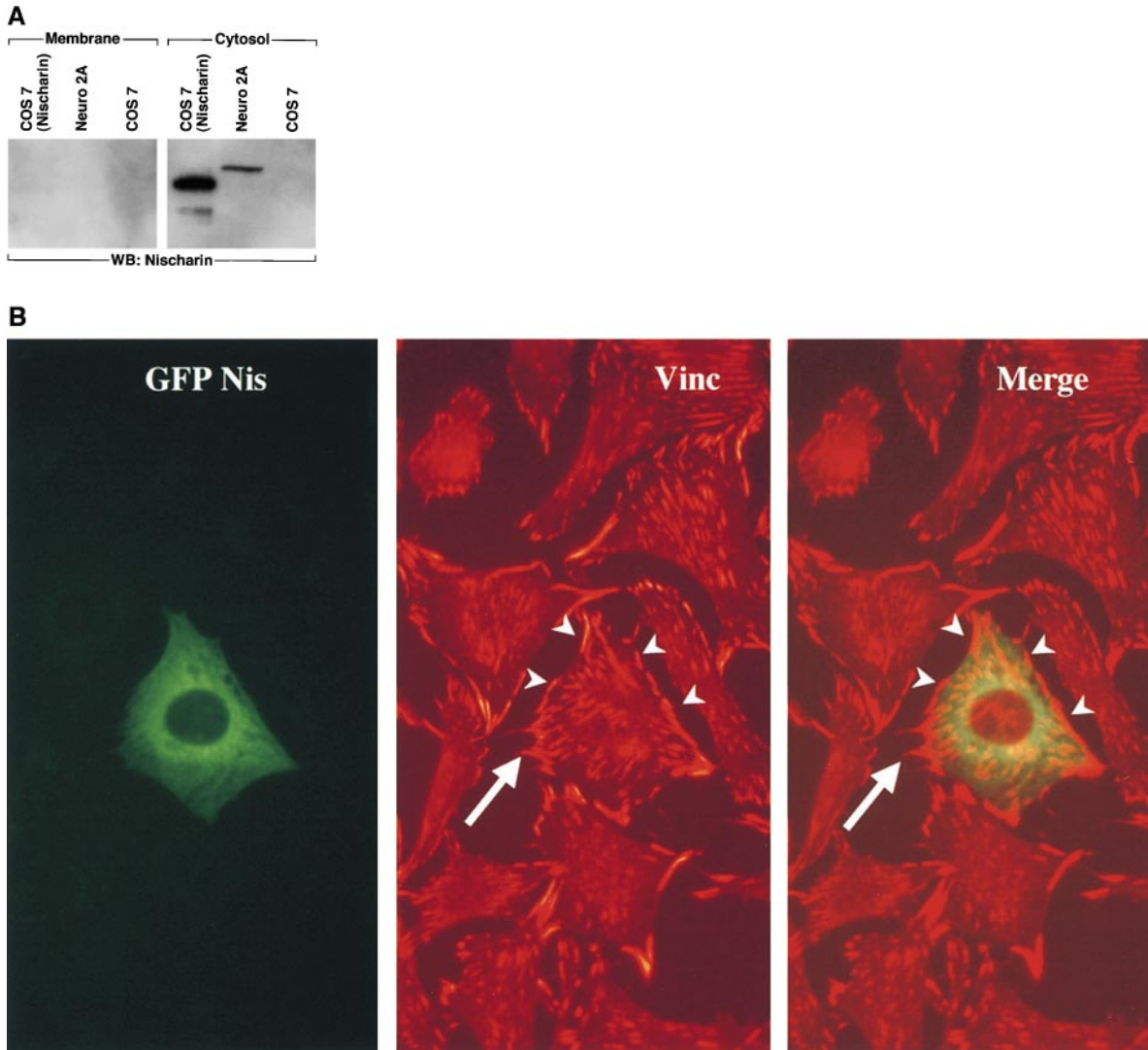
### Tissue Distribution of Nischarin

To determine the tissue distribution of Nischarin mRNA, a mouse multiple tissue Northern blot was hybridized with a PCR probe from the Nischarin integrin-binding region. A single mRNA of  $\sim 5.5$  kb was detected. In further analysis (not shown), three probes from different regions of the cDNA (extreme 5' end, integrin-binding region, and extreme 3' end of the ORF) were used; all three probes detected the same message. Nischarin mRNA expression was highest in brain and kidney, expression levels were lower in heart, liver, lung, and skeletal muscle, whereas no expression was seen in spleen and testis (Fig. 4 A). To address the expression of Nischarin in various cell types, we performed Northern blot analysis on several cell lines. Nischarin message was present in various rodent epithelial, fibroblast, and neuronal cell lines, though higher levels tended to be present in neuronal cells (Fig. 4 B and data not shown). Analysis of a mouse embryo RNA blot indicated the presence of Nischarin message as early as 7 d of development (Fig. 4 C).

### Expression of Nischarin in Cells and Its Subcellular Localization

The expression of transfected myc-tagged full-length Nischarin and expression of endogenous Nischarin were evaluated by Western blotting of cell lysates with anti-myc antibodies, as well as chicken pAbs directed against polypeptides from the COOH-terminal region of the predicted Nischarin ORF. In Cos7 cells transfected with myc-tagged full-length Nischarin, both antibodies recognized the same band of  $\sim 190$  kD (Fig. 5, A and B); this is somewhat larger than the predicted molecular weight of expressed Nischarin, but the reason for this is unclear. The expression of endogenous Nischarin was evaluated in detergent lysates of several cell lines by Western blotting with chicken anti-Nischarin antibodies (Fig. 5 A). In rat intestinal epithelial (RIE) cells (Oldham et al., 1996), a pro-



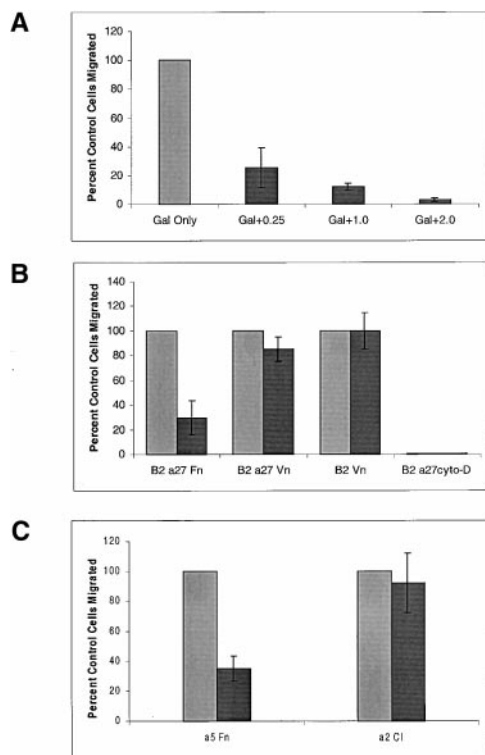


**Figure 6.** Subcellular localization of Nischarin. (A) Biochemical fractionation data is shown. Unmodified Neuro2A cells and Cos7 cells transfected with myc-tagged full-length Nischarin were used. Cell homogenates were separated into a 100,000 *g* cytosolic fraction and a membrane fraction. Each fraction was resolved by SDS-PAGE and then Western blotted with an anti-myc antibody, or chicken anti-Nischarin antibody. On the left side (anti-Nischarin Western blot), lanes represent the membrane fractions from Nischarin-transfected Cos7 cells, untransfected Neuro 2A cells, and untransfected Cos 7 cells. On the right side, lanes represent the respective cytosolic fractions. (B) Immunofluorescence data. NIH 3T3 cells stably expressing human  $\alpha 5$  were transiently transfected with plasmids expressing GFP-tagged full-length Nischarin. Cells were grown on fibronectin-coated coverslips, fixed, stained with anti-vinculin antibody (Sigma-Aldrich) and rhodamine-labeled secondary antibody, and observed by two-color fluorescence microscopy. Subcellular distribution of Nischarin-GFP was visualized by examination of green fluorescence. Cells transfected with Nischarin-GFP are marked by large white arrows. Representative vinculin-rich focal contacts are marked by small white arrowheads.

tein band of  $\sim 190$  kD was detected; this band comigrated with the immunoreactive band in Nischarin-transfected Cos7 cells. In NIH 3T3 cells, the chicken anti-Nischarin antibody detected a very weak band of 190 kD (data not shown). In several mouse neuronal cell lines (NIE 119, NB41A3, BC(3)H1, and Neuro 2A), an immunoreactive band of somewhat higher apparent molecular weight was detected (Fig. 5 A, and data not shown). The immunoreactivities of the bands detected in Nischarin-transfected Cos7 cells, or in nontransfected RIE cells, were competed out upon addition of the Nischarin COOH-region peptide used for chicken immunization (Fig. 5 C). Thus, the bands detected by the chicken antibody in the various rodent cell lines or in the transfected Cos7 cells are very likely to rep-

resent Nischarin. The detection of immunoreactive bands of differing apparent molecular weights in the various cell types examined suggests differences in splicing or post-translational modifications. The fact that only a single Nischarin message is seen by Northern analysis seems to militate against the possibility of splicing differences. However, alternate splicing of a short region of the mRNA might not be readily detected. The data of Fig. 5, A–C, indicate that Nischarin is widely expressed in various rodent cell lines, with higher levels found in cells of neuronal origin.

Both biochemical and fluorescence microscopy techniques were used to evaluate the subcellular localization of Nischarin. Overall distribution of Nischarin was assayed



**Figure 7.** Effects of Nischarin overexpression on cell migration. Mouse 3T3 fibroblasts or CHO cells were transfected with various amounts of a plasmid expressing myc-tagged full-length Nischarin, and cotransfected with a marker plasmid (A,  $\beta$ -galactosidase for wound-type migration assays; B and C, GFP for transwell assays). The transfected cell populations were then analyzed for cell migration, either by wound-type assays or transwell assays, as described in Materials and Methods. (A) An illustration of the wound-type migration behavior of Nischarin transfectants versus 3T3 cells transfected with  $\beta$ -galactosidase alone. The amount of transfected Nischarin plasmid is shown on the abscissa; migration results are reported as percent of the  $\beta$ -galactosidase only control. (B) An illustration of the transwell migration behavior of  $\alpha$ 5-deficient CHO B2 cells or  $\alpha$ 5-expressing CHO B2 $\alpha$ 27 cells on membranes coated with 10  $\mu$ g/ml fibronectin (Fn) or vitronectin (Vn). Light bars represent cells transfected only with GFP plasmid, whereas dark bars represent cells transfected with GFP and 2  $\mu$ g of Nischarin plasmid. Results are presented as percent of the GFP control for each cell type and coating. Treatment of B2 $\alpha$ 27 cells with 2  $\mu$ g/ml of cytochalasin D is used as a negative control to illustrate total inhibition of migration. It should be noted that CHO B2 cells do not adhere to surfaces coated with fibronectin and thus cannot migrate on these surfaces. (C) An illustration of the transwell migration of 3T3 cells stably transfected with human  $\alpha$ 5 or  $\alpha$ 2 and plated on membranes coated with 10  $\mu$ g/ml fibronectin (Fn) or collagen (Cl), respectively. Light bars represent cells transfected only with GFP plasmid, whereas dark bars represent cells transfected with GFP and 2  $\mu$ g of Nischarin plasmid. Results in A–C represent the means and standard errors of three determinations.

by subcellular fractionation. Cos7 cells were transfected with plasmids expressing myc-tagged full-length Nischarin. These cells, as well as untransfected Neuro 2A cells, were fractionated as described in Materials and Methods. Fractions containing membranes or 100,000 g supernatant were prepared, resolved by SDS-PAGE, and subjected to Western blotting with chicken anti-Nischarin antibodies (Fig. 6

A). Both transfected and endogenously expressed Nischarin were primarily found in the 100,000 g supernatant fraction, indicating that Nischarin is a soluble rather than a transmembrane protein.

We have used the Nischarin–GFP chimera described in Materials and Methods and fluorescence microscopy to obtain further information on the subcellular localization of Nischarin (Fig. 6 B). NIH 3T3 cells expressing the Nischarin–GFP chimera were counterstained with antibodies to the focal contact protein vinculin (Fig. 6 B). Nischarin–GFP did not enter the nucleus and was primarily found diffusely distributed in the cytosol. However, a greater concentration of Nischarin was seen in the perinuclear region partially associated with punctate structures that may be endomembrane vesicles. There was no evidence of Nischarin–GFP accumulation at vinculin-rich focal adhesion sites. Staining of focal contacts and fibrillar structures with anti- $\alpha$ 5 antibody also failed to reveal any obvious colocalization with Nischarin (not shown). Similar studies using an antiphosphotyrosine antibody to detect focal contact sites yielded the same result (data not shown). Thus, Nischarin is found primarily in the cytosol, with some concentration in the perinuclear area, but does not concentrate at “classical” focal adhesion sites.

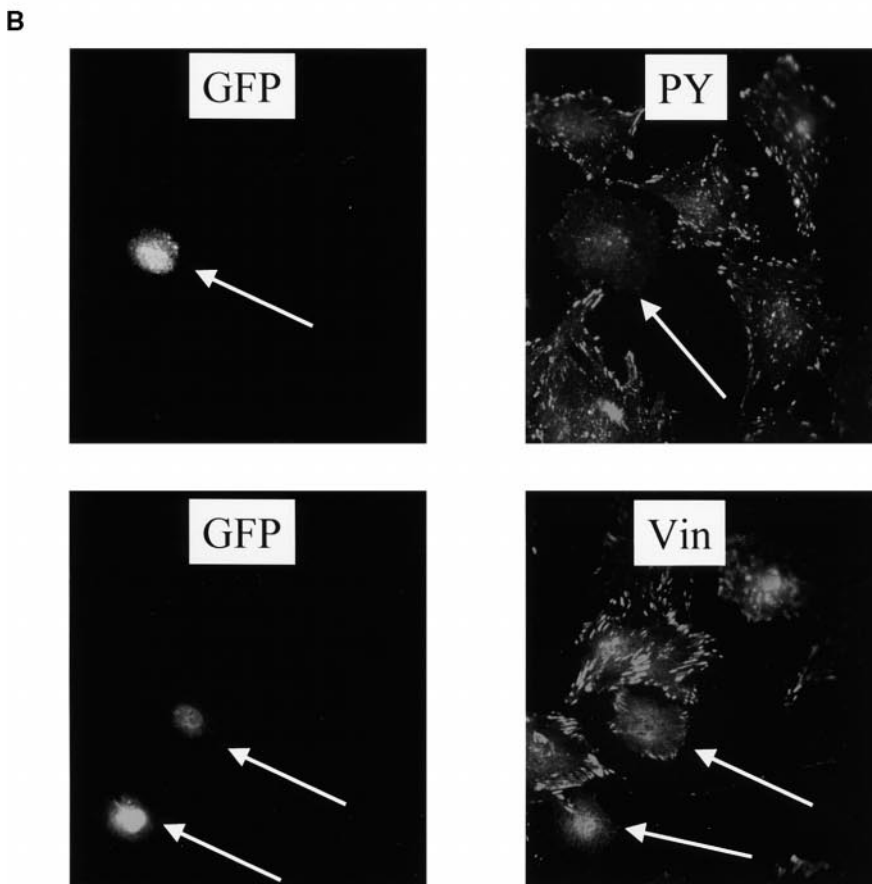
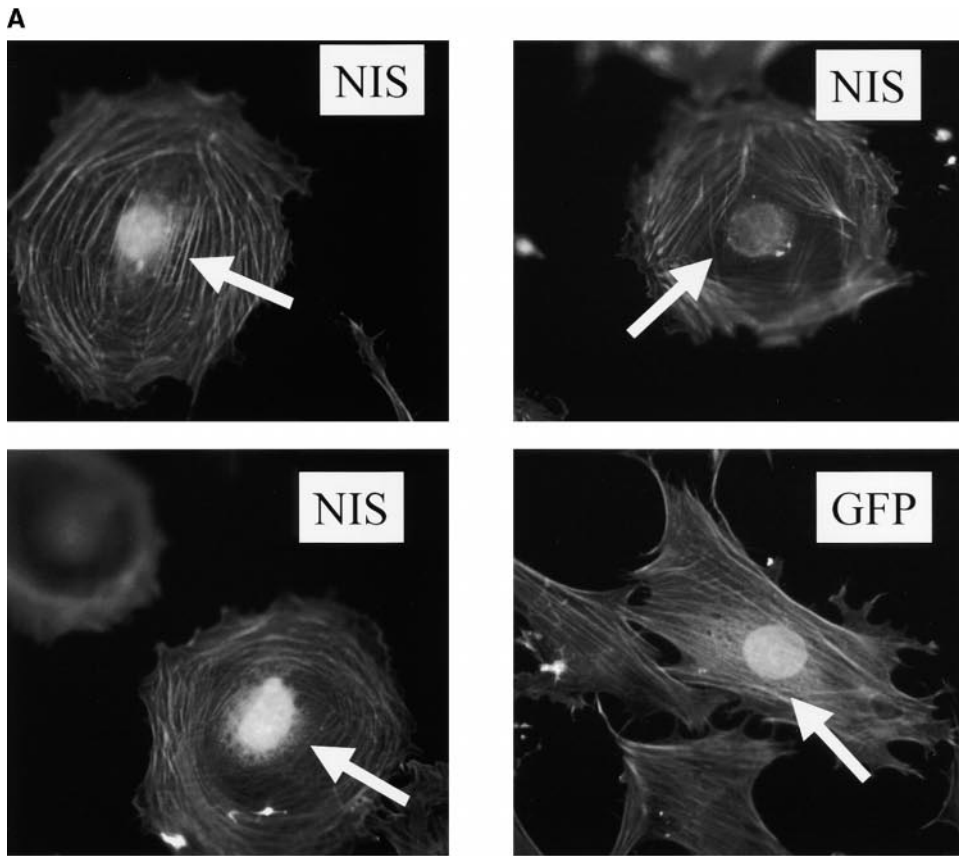
The observations in Fig. 6 indicate that Nischarin is largely a cytosolic protein, and its overall distribution does not coincide with focal adhesion structures. Since Nischarin can clearly associate with the  $\alpha$ 5 subunit, as demonstrated by coimmunoprecipitation, this may indicate that only a small fraction of the total cellular pool of Nischarin is associated with the  $\alpha$ 5 $\beta$ 1 integrin at any given time.

### Nischarin Inhibits Cell Migration

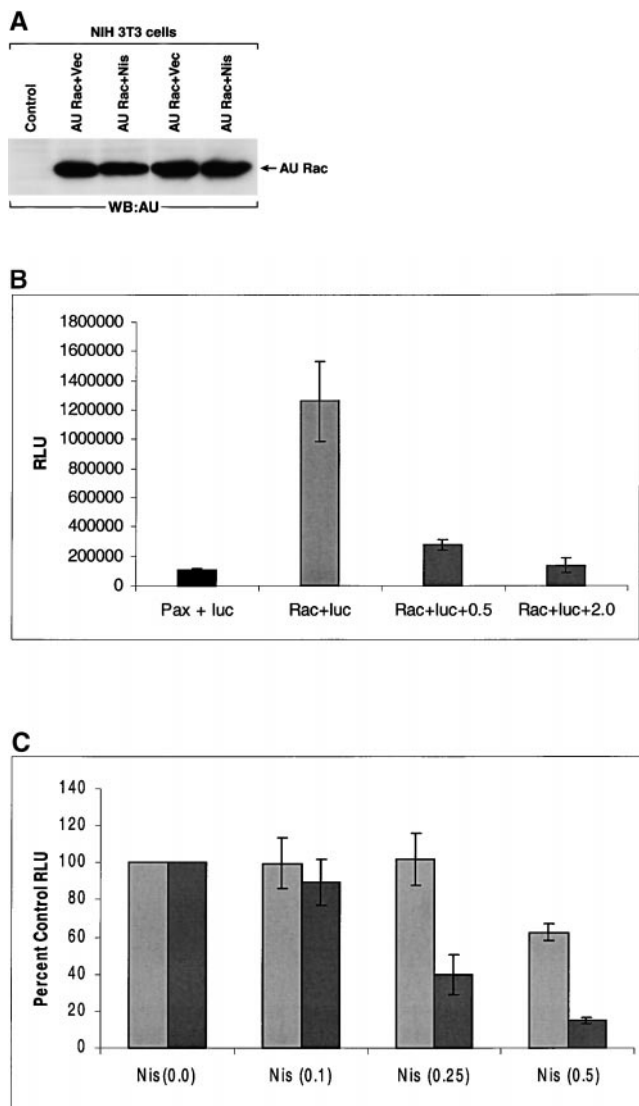
To investigate the biological role(s) of Nischarin, we focused on the finding that Nischarin seems to interact most strongly with the  $\alpha$ 5 subunit and on the knowledge that  $\alpha$ 5 $\beta$ 1 plays an important role in cell motility. The effect of Nischarin on cell migration was initially evaluated using a monolayer “wounding” assay (Bauer et al., 1993). NIH 3T3 cells were cotransfected with various amounts of a plasmid expressing full-length Nischarin and with a  $\beta$ -galactosidase marker plasmid. After transfection and recovery, the transfected cell monolayers were scraped with a razor blade. The migration of transfected ( $\beta$ -galactosidase positive) cells across the wound boundary was quantitated, as described in Materials and Methods. As seen in Fig. 7 A, cells overexpressing Nischarin showed significant inhibition of migration compared with cells transfected with  $\beta$ -galactosidase plasmid alone. Increasing the dose of Nischarin plasmid resulted in a progressive decrease in migration. This is unlikely to be due to toxicity since increasing the amount of Nischarin transfected did not reduce the survival of the transfectants, as judged by the fraction of  $\beta$ -galactosidase-positive cells in the total cell population.

Nischarin effects on cell movement were also evaluated using a transwell assay (Keely et al., 1997) where the cells migrate across a membrane containing 8  $\mu$ m pores. These studies also undertook to examine whether the effect of Nischarin on migration displayed any specificity in terms of the integrin involved in migration. One set of experiments used CHO sublines differing in  $\alpha$ 5 $\beta$ 1 expression (Bauer et al., 1993). CHO B2 cells lack  $\alpha$ 5 $\beta$ 1 and do not





*Figure 8.* Effects of Nischarin on cytoskeletal organization. REF cells were transiently transfected with a plasmid expressing full-length Nischarin and cotransfected with a marker plasmid expressing GFP. After 48 h, the cells were replated on fibronectin-coated cover slips for 3 h and processed for immunofluorescence, as described in Materials and Methods. (A) TRITC-phalloidin staining of the actin cytoskeleton in Nischarin transfectants (NIS) and in control cells transfected only with the GFP plasmid (GFP). The transfected cells expressing GFP are marked with arrows. (B) Cells stained for the focal contact markers vinculin (VIN) or phosphotyrosine (PY) using a TRITC-labeled secondary antibody and visualized using a filter for red fluorophores. Transfected cells expressing GFP are visualized using a filter for green fluorophores; the GFP-expressing cells are marked with arrows.



**Figure 9.** Effects of Nischarin on signaling by Rac GTPase. Mouse 3T3 cells were transiently cotransfected with a plasmid containing luciferase driven by the *c-fos* promoter, with plasmids expressing activated forms of the Rac GTPase, or with a plasmid expressing activated MEK, and with various amounts of a plasmid expressing full-length myc-tagged Nischarin. All transfections were normalized for total DNA using vector plasmid. Nischarin expression was confirmed by Western blotting (not shown). Activation of the luciferase reporter was determined by luminometry and normalized based on total cell protein. (A) An anti-AU epitope Western blot for expression of transfected AU-epitope-labeled Rac, with or without cotransfection with 2  $\mu$ g Nischarin plasmid. The control is the blot for untransfected 3T3 cells. (B) An illustration of the effect of transfection with the plasmid pAX142 RacQ61L(Rac) in activating the *c-fos*-luciferase reporter (Luc) and the effects of cotransfection with various amounts (shown in  $\mu$ g) of Nischarin plasmid. Transfection with the pAX142 “empty” vector (Pax) serves as a negative control. (C) An illustration of the effects of transfection with the plasmid pAX142 RacQ61L(Rac) (dark bars) or the plasmid pFC-MEK1 (light bars) in activating the *c-fos* luciferase reporter (both normalized to 100%) and the effects of cotransfection with 0.1–0.5  $\mu$ g of Nischarin plasmid (Nis). Results in A–C are the means and standard errors of three determinations.

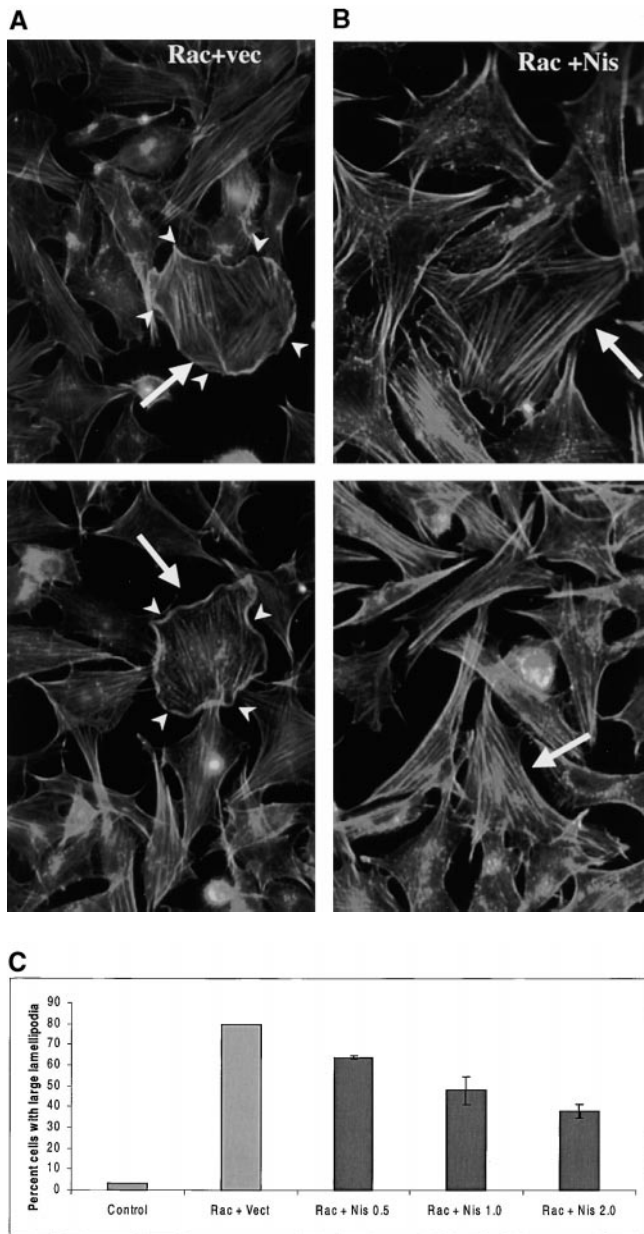
adhere to fibronectin, but express other integrins that allow adhesion and migration on vitronectin or other matrix proteins. CHO B2a27 cells derive from B2, but have been stably transfected with human  $\alpha 5$ ; these cells migrate on fibronectin in a completely  $\alpha 5\beta 1$ -dependent manner. The transwell membranes for these assays were coated with either fibronectin or vitronectin. As seen in Fig. 7 B, transfection of B2a27 cells with Nischarin led to a major reduction of their migration on fibronectin-coated membranes, but only a modest reduction on vitronectin-coated membranes. Furthermore, migration of the B2 cells on vitronectin-coated membranes was not at all affected by expression of Nischarin. Treatment of cells with cytochalasin D completely abolished migration of either cell type.

In another set of experiments, transwell migration assays were performed with 3T3 cells stably transfected with human  $\alpha 5$  or  $\alpha 2$  subunits (Aplin et al., 1999b). The adhesion, and presumably migration, of these cells is strongly influenced by the transfected integrin subunit. As seen in Fig. 7 C, transfection with Nischarin markedly inhibited migration of the  $\alpha 5$ -overexpressing cells on fibronectin, but had little effect on the migration of the  $\alpha 2$ -overexpressing cells on collagen.

Thus, Nischarin overexpression can profoundly inhibit cell migration. This effect displays substantial integrin subunit specificity and is much more dramatic for migration on fibronectin mediated by  $\alpha 5\beta 1$  than for migration on other matrix proteins mediated by other integrins.

#### *Effects of Nischarin on the Cytoskeleton*

Since overexpression of Nischarin resulted in substantial alterations in cell migration, we wished to determine if this was accompanied by changes in the organization of the cytoskeleton. REF cells were transfected with plasmids expressing full-length Nischarin and GFP. Cells were probed with the actin-binding reagent phalloidin or were immunostained for phosphotyrosine, vinculin, tubulin, or vimentin. Phalloidin staining (Fig. 8 A) indicated that many of the transfected REF cells had a unique phenotype, with a more or less circular shape and having actin filaments arranged in “basket” structures around the periphery, rather than as the linear stress fibers commonly seen in adherent fibroblasts. Although this phenotype was not universal,  $\sim 60\%$  of the REF cells cotransfected with Nischarin and GFP showed the basket-like actin structures. In contrast, only a few percent of the control GFP transfectants had this phenotype. We next looked for the effects of Nischarin on focal adhesions by staining for vinculin and phosphotyrosine. As seen in Fig. 8 B, vinculin-containing focal contacts and phosphotyrosine in focal contacts were somewhat reduced in REF cells transfected with Nischarin compared with control cells. Staining, with anti-tubulin or anti-vimentin antibodies, suggested that Nischarin expression had little effect on the organization of microtubules or intermediate filaments in REF cells (not shown). Similar effects were observed in WI-38 cells, another well spread cell line (not shown). However, the “basket” phenotype was not apparent in Nischarin-transfected NIH 3T3 cells or in Cos7 cells, both of which are less well spread. The highly organized actin filaments of REFs may allow easier visualization of the relatively subtle effects of Nischarin on cytoskeletal architecture.



**Figure 10.** Inhibition of Rac-induced lamellipodia formation by Nischarin. NIH 3T3 cells were transiently cotransfected with RacQ61L and vector alone (A), or RacQ61L and Nischarin (B). To monitor transfected cells, a GFP plasmid was also included. After 48 h of transfection, cells were serum starved for 4 h, trypsinized, and plated onto fibronectin-coated coverslips for 4 h. Actin polymerization was visualized by staining with rhodamine-phalloidin, as described above. Transfected cells positive for GFP are marked with large white arrows. Areas of lamellipodia formation are marked with white arrowheads. (C) The effect of increasing amounts of cotransfected Nischarin plasmid on the number of Rac-transfected cells displaying large lamellipodia.

### Effects of Nischarin on Rac GTPase-mediated Signaling

Since the organization of the actin cytoskeleton, as well as cell motility, are strongly influenced by the activity of the Rac GTPase (Mackay and Hall, 1998), we decided to evaluate whether Nischarin might affect Rac-mediated func-

tions. As an initial test of the effects of Nischarin on signaling by Rac, NIH 3T3 cells were transfected with a reporter plasmid that uses the c-fos promoter to drive luciferase expression. Cells were then cotransfected with plasmids expressing activated versions of Rac, as well as with various amounts of a plasmid expressing full-length Nischarin. As seen in Fig. 9 A, transfection with Nischarin plasmid did not affect levels of expression of cotransfected Rac. Activated Rac strongly stimulated luciferase expression (Fig. 9 B), most likely through well-known effects on transcription factors that recognize the serum response element (SRE) in the c-fos promoter (Hill et al., 1995). However, coexpression of Nischarin blocked Rac-mediated stimulation of the c-fos-Luc reporter in a dose-dependent manner (similar effects were observed with activated CDC42, whereas much more modest effects of Nischarin were observed with Rho, not shown). This inhibition was not due to non-specific effects on transcription or translation, since stimulation of c-fos-Luc by a plasmid expressing constitutively active MEK was only weakly affected by coexpression of Nischarin (Fig. 9 C). Thus, Nischarin strongly inhibits signaling mediated by Rac, but is less effective in blocking signaling mediated by an effector in the Erk/MAPK cascade. The amount of Nischarin plasmid required to strongly block Rac-induced activation of the c-fos reporter correlates well with the amount that produces a dramatic reduction in cell migration (compare Figs. 7 and 9). A similar strong inhibition of reporter gene expression by Nischarin was also observed using a commercial system that detects Rac-driven activation of c-Jun. Furthermore, Nischarin only modestly inhibited expression of a reporter gene that is responsive to protein kinase A-mediated activation of a CRE element (Reddig, P., and R.L. Juliano, unpublished observations). Thus, overexpression of Nischarin seems to preferentially affect signaling through Rac-driven pathways rather than other well-known signaling pathways.

To further evaluate the possible interplay between Nischarin and the Rac GTPase, we examined the effect on Nischarin on a characteristic cytoskeletal function of Rac, namely the enhanced formation of lamellipodia (Mackay and Hall, 1998). NIH 3T3 cells were transfected with a plasmid expressing an activated form of Rac (Q61L), and, in some cases, were cotransfected with full-length Nischarin. A small amount of a GFP plasmid was used to mark the transfected cells. As seen in Fig. 10 A, expression of activated Rac produced the expected enhancement of lamellipodia formation, with >80% of the transfectants showing large and distinct areas of membrane ruffling. When cells were cotransfected with active Rac and Nischarin there was a strong inhibition of the Rac effect, with only ~35–40% of the cells displaying large lamellipodia when higher doses of Nischarin were transfected (Fig. 10 C). Some of the Rac plus Nischarin-transfected cells resembled untransfected 3T3 cells (Fig. 10 B). However, various cells at each dose of Nischarin displayed intermediate phenotypes with partial ruffling and incomplete lamellipodia (not shown). Thus, overexpression of Nischarin can inhibit lamellipodia formation, one of the most characteristic effects of Rac on the cytoskeleton and associated with cell movement. These findings suggest that Nischarin inhibits cell migration, at least in part, through its actions on pathways regulated by the Rac GTPase.

## Discussion

Recent studies suggest that individual integrin  $\alpha/\beta$  heterodimers can play unique roles in the regulation of cell migration, growth, survival, and differentiation (Pozzi et al., 1998; Farrelly et al., 1999; Liu et al., 1999; Lochter et al., 1999; Sastry et al., 1999; Lee and Juliano, 2000). This may come about via specific interactions between the cytoplasmic domains of individual integrins and intracellular proteins involved in signal transduction or other aspects of cell regulation. The  $\alpha5\beta1$  integrin is particularly interesting in this regard, since it has been implicated in the control of both cell growth and programmed cell death (Varner et al., 1995; Zhang et al., 1995; O'Brien et al., 1996; Sastry et al., 1999; Lee and Juliano, 2000). Here, we have reported the identification and characterization of mouse Nischarin, a soluble intracellular protein that is capable of interacting with the cytoplasmic domain of the integrin  $\alpha5$  subunit. Using both two-hybrid analysis and coimmunoprecipitation of expressed proteins in cells, Nischarin was found to interact with the  $\alpha5$  subunit much more strongly than with the other two  $\alpha$  subunits tested. Furthermore, immunoprecipitation of endogenous  $\alpha5\beta1$  from a mouse neuronal cell line resulted in coimmunoprecipitation of endogenous Nischarin. Thus, current evidence suggests that Nischarin interacts preferentially with the  $\alpha5$  subunit, and this interaction can occur under physiological conditions. However, we cannot rule out the possibility that Nischarin may interact with other examples of the many known  $\alpha$  subunits.

Nischarin bears limited resemblance to identified proteins with a well-known functions. Only two close homologues of Nischarin have been reported in the DNA data bases. The human homologue of Nischarin has been described as a putative imidazole receptor (Ivanov et al., 1998) (sequence data available from GenBank/EMBL/DDBJ under accession no. AF082516). Imidazolines are thought to be neurotransmitters and, thus, their receptors would presumably be transmembrane proteins. However, our results clearly show that endogenous Nischarin is primarily a soluble protein and most likely rules out a role as a transmembrane neurotransmitter receptor. A second close homologue of the NH<sub>2</sub>-terminal region of Nischarin has been reported from the *C. elegans* genome project (accession no. Z69383) and two similar proteins are found in *Drosophila*. However, the function of these proteins is completely unknown. The human protein contains a segment that is highly homologous to the integrin-binding region of mouse Nischarin that we detected by two-hybrid screening. There are also substantial homologies between the central proline-rich region of Nischarin and portions of several neurofilament proteins, but the functional significance of this is unclear. Examination of the primary sequence of Nischarin, using programs that search for common protein structural or functional motifs, yielded few clues as to the biological role of this molecule.

Nischarin is expressed in many cell types and is found both in the adult mouse and in the developing embryo. Higher amounts of Nischarin are found in neuronal-derived cell lines than in epithelial cells or fibroblasts, but some Nischarin is expressed in all of these cell types. Western blotting of various cell lines for endogenous Nischarin

revealed proteins of two distinct sizes; in some cells, an  $\sim 190$  kD form is found that comigrates with expressed Nischarin. However, in neuronal cells, a larger form of the protein is seen. The basis for this difference is currently unknown, but may reflect cell-type specific alternative splicing, use of alternate start codons, or posttranslational modification.

Immunofluorescence and biochemical fractionation studies indicate that Nischarin is largely a soluble cytosolic protein. It is clear from fluorescence microscopy studies that Nischarin is not concentrated in vinculin-rich focal contacts. Although, one might expect a protein that interacts with  $\alpha5\beta1$  integrin to be localized to focal contacts, this is not always the case. For example, members of the TM4 family of proteins clearly interact specifically with certain integrins, but TM4 proteins are not found in "classic" focal contacts (Porter and Hogg, 1998; Berditchevski and Odintsova, 1999). This is also true of calveolin, which has been found to interact with certain integrins (Wary et al., 1996). At present it is unknown whether there is any physiological regulation of the association between the  $\alpha5\beta1$  integrin and Nischarin that might affect its subcellular distribution.

It seems clear that Nischarin can selectively bind to the cytoplasmic domain of the  $\alpha5$  integrin subunit, based both upon two-hybrid analysis and coimmunoprecipitation of full-length Nischarin with native  $\alpha5\beta1$  in mammalian cells. However, at any given time, only a small fraction of the total Nischarin in a cell is likely to be bound to the integrin, since most Nischarin is found in the cytosolic fraction. This type of situation is often seen in signaling pathways, where only a minority of a cytosolic effector molecule associates with its membrane-bound partner molecule. The well-known association between Ras and Raf-1 is a good example, where Raf is primarily found in the cytosol, despite its clear ability to interact with membrane-bound Ras (Wartmann et al., 1997; Campbell et al., 1998).

Overexpression of full-length Nischarin results in major changes in cell behavior and also affects cytoskeletal organization. The most dramatic aspect is the profound inhibition of cell migration caused by Nischarin. At this point, it is unclear whether the reduced cell migration observed in the "wounding" and transwell assays used here is due to a reduction in innate motility or to an impairment of directional movement (Gu et al., 1999). This will be an important issue for further investigation. The effects of Nischarin on cell migration are quite  $\alpha$ -subunit selective. Thus,  $\alpha5\beta1$ -dependent migration on fibronectin is inhibited far more strongly than migration on other substrata mediated by other integrins.

The overexpression of Nischarin in certain fibroblasts leads to substantial changes in focal contact and actin filament organization. Thus, Nischarin-transfected REF cells display fewer linear stress fibers and a reduction in mature, vinculin-positive focal contacts. Instead, the actin filaments form unusual "basket" structures around the cell periphery. These effects are clearly seen in well spread fibroblasts such as REF and WI-38 cells, but are much less apparent in cell lines such as 3T3 and Cos. The dramatic effects of Nischarin on cell migration and actin filament organization described here may be due, at least in part, to the fact that the transfected molecule is expressed at sub-

stantially higher levels than the normal amount of endogenous Nischarin. However, even the somewhat skewed effects triggered by overexpression may provide important clues in eventually ascertaining the physiological role of Nischarin.

The observed effects of Nischarin on cell motility and cytoskeletal organization suggested that Nischarin might impact the pathways used by some Rho-family GTPases to regulate individual pools of actin filaments (Mackay and Hall, 1998). Thus, it was satisfying to find that overexpression of Nischarin strongly blocked the ability of active forms of Rac to drive reporter gene expression from the serum-response element of the c-fos promoter. Failure to strongly block MEK-induced activation of this same promoter indicates that Nischarin acts preferentially on Rac-mediated events rather than other signaling cascades. The theme that Nischarin can block Rac functions clearly associated with cytoskeletal organization and cell motility was extended by the observation that Nischarin can inhibit or reverse the well-known action of Rac in promoting lamellipodia formation. Thus, it seems likely that Nischarin can have a substantial impact on the signaling and cytoskeletal functions of Rac. There is little in the primary sequence of Nischarin to suggest a mechanism for its influence on Rac GTPase pathways, that is, no obvious homologies to exchange factor, GAP, or GDI domains (Sasaki and Takai, 1998) are apparent.

Lately, the mechanistic basis underlying integrin-mediated cell movement has received a good deal of attention. It is clear that FAK is a key regulator of cell migration in most cells (Ilic et al., 1995; Cary et al., 1998; Sieg et al., 2000). Overexpression of the PTEN tumor suppressor, a dual specificity phosphatase, results in the dephosphorylation of FAK and a reduction in directional cell motility (Tamura et al., 1998; Gu et al., 1999). The focal contact protein p130Cas is tyrosine phosphorylated by FAK; subsequently, the adaptor protein Crk can bind phosphotyrosyl sites on Cas. The Cas-Crk complex has been implicated in the control of cell migration, whereas the Rac GTPase seems to be a downstream mediator of the Cas/Crk pathway (Cary et al., 1998; Klemke et al., 1998). In epithelial cells, an important connection has been made between cell motility and a signaling pathway involving phosphatidylinositol-3-kinase and the Rac and CDC42 GTPases (Keely et al., 1997; Shaw et al., 1997). Interestingly, integrin-mediated cell adhesion has been shown to directly activate Rac and CDC42 (Price et al., 1998), whereas Ras, CDC42, Rac, and Rho have all been implicated in cooperative regulation of cell movement (Clark and Brugge, 1995; Nobes and Hall, 1999). Thus, though many of the molecular details remain to be determined, it seems clear that a pathway (perhaps branched) involving integrins, FAK, Cas/Crk, phosphatidylinositol-3-kinase, and Rho-family GTPases positively regulates cell motility. However, other than the role of PTEN in FAK dephosphorylation, there has been little evidence, to date, of physiological inhibitors of cell motility. In this context, Nischarin may play an important role by negatively impacting cell motility pathways controlled by Rac.

In summary, we have identified and characterized a novel protein that we have named Nischarin. This protein can bind selectively to the cytoplasmic tail of the integrin

$\alpha 5$  subunit. Overexpression of Nischarin has potent effects in terms of retarding cell migration, and it acts preferentially on migration mediated by the  $\alpha 5 \beta 1$  integrin. Nischarin overexpression also influences actin filament organization in some cell types. These effects may be mediated through Nischarin's selective action on pathways regulated by the Rac GTPase. Thus, one important aspect of Nischarin's biological role may be to counterbalance the effects of Rac in promoting directed cell movement.

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