A membrane proximal region of the integrin α5 subunit is important for its interaction with nischarin

Suresh K. ALAHARI[1] and Hani NASRALLAH

Department of Pharmacology, University of North Carolina School of Medicine, Chapel Hill, NC 27599-7365, U.S.A.

In a previous study [Alahari, Lee and Juliano (2000) J. Cell Biol. **151**, 1141–1154], we have identified a novel protein, nischarin, that specifically interacts with the cytoplasmic tail of the α5 integrin subunit. Overexpression of this protein profoundly affects cell migration. To examine the nischarin–α5 interaction in detail, and to find the minimal region required for the interaction, several mutants of nischarin and α5 were created. The results obtained for the yeast two-hybrid system indicate that a 99-aminoacid region of nischarin (from residues 464 to 562) is indispensable for the interaction. Also, we demonstrate that the membrane proximal region (from residues 1017 to 1030) of the α5 cytoplasmic tail is essential for the interaction. To characterize more directly the properties of the interaction between nischarin and α5, we performed surface-plasmon resonance studies in which peptides were immobilized on the surface of a sensor chip, and the recombinant nischarin protein fragments were injected. Consistent with the two-hybrid results, recombinant nischarin binds well to immobilized α5 peptides. In addition, mutational analysis revealed that residues Tyr^{1018} and Lys^{1022} are crucial for α5–nischarin interactions. These results provide evidence that nischarin is capable of directly and selectively binding to a portion of the α5 cytoplasmic domain. Further studies demonstrated that the minimal α5 binding region of nischarin does not affect cell migration.

Key words: biacore, integrin, migration, nischarin, yeast two-hybrid.

INTRODUCTION

Integrins consist of three domains: extracellular, transmembrane and intracellular regions. Integrin cytoplasmic domains articulate directly or indirectly with a number of cytoskeletal proteins, including actin, vinculin, talin, paxillin, as well as signalling molecules, including Src, FAK (focal adhesion kinase), Ras, Raf, MAPK (mitogen-activated protein kinase), MEK (MAPK/ERK kinase) and c-Jun N-terminal kinase [1–3]. This suggests that integrins play an important role in converging signals from the membrane to the inside of cells (outside in signalling). The β cytoplasmic domains have been shown to play a major role in integrin-mediated signal-transduction events [4]. In addition, β cytoplasmic domains are required for cell spreading, particularly that mediated by R-Ras, phosphoinositide 3-kinase and Rac1 [5], and for the regulation of fibronectin binding by $\alpha v\beta 3$ [6]. The role of α subunits in integrin function is not very well known. However, several reports indicate that the α cytoplasmic tails are important in signalling through interaction with calreticulin [7,8], and essential for stress fibre formation and cell migration in CHO (Chinese-hamster ovary) cells [9]. In contrast, the cytoplasmic tails of α4 and α9 inhibit cell spreading and enhance cell migration [10]. All α integrin subunits are characterized by highly conserved GFFKR regions. Whereas many proteins have been shown to bind to β integrin cytoplasmic domains [11–13], only a few proteins have been shown to bind to α subunits. These include calcium- and integrin-binding protein binding to the αIIb cytoplasmic tail [14], talin binding to the cytoplasmic tails of αIIb and β3 [15], paxillin binding to the α4 cytoplasmic tail [16] and nischarin binding to the α5 cytoplasmic domain [17].

We previously reported that the α5 cytoplasmic region selectively interacts with nischarin. By yeast two-hybrid analysis and co-immunoprecipitation experiments, nischarin has been shown to interact with the α5 integrin subunit more strongly than any other integrin subunit tested [17]. Although nischarin is ubiquitously expressed, it is most highly expressed in neuronal cells. Nischarin is a protein of 190 kDa (measured molecular mass), and its overexpression inhibits cell migration and affects the actin cytoskeleton. This inhibitory effect was more dramatic when cells migrated on fibronectin matrix, mediated by α5, compared with cells migrating on matrices mediated by other integrins. Our recent results indicate that nischarin selectively inhibits Rac-driven cell migration through p21-activated kinase [18].

The exact interacting regions on α5 as well as on nischarin have not been mapped. To understand further the interaction, we used a chimaeric yeast two-hybrid system to map the binding domain of nischarin. In the present study, we describe a minimal domain containing 99 amino acids as the essential region for the interaction with the α5 integrin subunit. The interactions detected by two-hybrid analysis were further validated using surface-plasmon resonance studies. To explore the importance of specific sequences in α5 for binding to nischarin, we analysed the binding of nischarin truncations to full-length α5 cytoplasmic tail as well as binding of a series of α5 peptides to nischarin. Our results show that the IYILYKLGFFKRSL sequence is required for the formation of nischarin–α5 complex as monitored by BIAcore analysis, whereas Tyr^{1018} and Lys^{1022} seem to be crucial for the interaction. In addition, functional studies showed that the MIBD (minimal integrin-binding domain) of nischarin did not have any effect on α5-mediated cell motility indicating that the IBD (integrin-binding domain) is insufficient for the inhibitory function of nischarin.

MATERIALS AND METHODS

Reagents

Streptavidin chips for plasmon resonance studies were purchased from BIAcore (Piscataway, NJ, U.S.A.). Oligonucleotides were

Abbreviations used: CHO, Chinese-hamster ovary; DSC, differential-scanning calorimetry; GST, glutathione S-transferase; IBD, integrin-binding domain; MIBD, minimal IBD; NIBD, non-IBD.

[1] To whom correspondence should be addressed (e-mail alahari@med.unc.edu).

Table 1 Primers used to make truncations of IBD of Nischarin

IBD construct	Primer	Site
Trunc 1	5′-CAGCGGCCGCTCAAGCTGGAGTGGGCCCTGGGC-3′, 5′-CAGCGGCCGCGCTGCCGTAGAGGATGGG-3′	*Not*I
Trunc 2	5′-CAGCGGCCGCTCAAGGATGAGGACTTCCTGCTG-3′, 5′-CAGCGGCCGCGCTGCCGTAGAGGATGGG-3′	*Not*I
Trunc 3	5′-CAGCGGCCGCTCAAGATCCTCAAGGTGCTCTGG-3′, 5′-CAGCGGCCGCGCTGCCGTAGAGGATGGG-3′	*Not*I
Trunc 3.1	5′-CAGCGGCCGCTCAAGAAGGTGCTCTGGTGCTTC-3′, 5′-CAGCGGCCGCGCTGCCGTAGAGGATGGG-3′	*Not*I
Trunc 3.2	5′-CAGCGGCCGCTCAAGTGGTGCTTCCTGATCCAC-3′, 5′-CAGCGGCCGCGCTGCCGTAGAGGATGGG-3′	*Not*I
Trunc 3.3	5′-CAGCGGCCGCTCAAGCTGATCCACGTGCAAGGC-3′, 5′-CAGCGGCCGCGCTGCCGTAGAGGATGGG-3′	*Not*I
Trunc 4	5′-CAGCGGCCGCTCAAGCACGTGCAAGGCAGCATC-3′, 5′-CAGCGGCCGCGCTGCCGTAGAGGATGGG-3′	*Not*I
Trunc 11	5′-CAGCGGCCGCGGCCATGTGCTGCGGTGC-3′, 5′-CAGCGGCCGCTCAAGGACGAGGAGGCGGAGGAG-3′	*Not*I
Trunc 12	5′-CAGCGGCCGCGCGTAGATCAGCATGGAA-3′, 5′-CAGCGGCCGCTCAAGGACGAGGAGGCGGAGGAG-3′	*Not*I
Trunc 13	5′-CAGCGGCCGCGGCAGCATCCGAGATGATGAA-3′, 5′-CAGCGGCCGCTCAAGGACGAGGAGGCGGAGGAG-3′	*Not*I
Trunc 14	5′-CAGCGGCCGCCTCACTGTGCCGCACCTT-3′, 5′-CAGCGGCCGCTCAAGGACGAGGAGGCGGAGGAG-3′	*Not*I

Table 2 Primers used to make truncations of α5 cytoplasmic domain

α5 construct	Primer	Site
Trunc 1	5′-GGGAATTCAAGCTTGGATTCTTCAAACGCTCCCTCCCATATGGCACCGCCTG-3′	*Eco*RI
	5′-GGCTGCAGGGCATCAGAGGTGGCTGGAGGCTTGAGCTGAGCTTTTTCCATGGCGGTGCCATATGG-3′	*Pst*I
Trunc 2	5′-GGGAATTCGGATTCTTCAAACGCTCCCTCCCATATGGCACCGCCATG-3′	*Eco*RI
	5′-GGCTGCAGGGCATCAGAGGTGGCTGGAGGCTTGAGCTGAGCTTTTTCCATGGCGGTGCCATATGG-3′	*Pst*I
Trunc 3	5′-GGGAATTCTCCCTCCCATAT-3′	*Eco*RI
	5′-GAGGATCCGGCATCAGAGGT-3′	*Bam*HI
Trunc 11	5′-GACTGCAGTGGAGGCTTGAGCTGAGCTTTTTCCATGGCGGTGCCATATGGGAGGGAGCGTTTGAA-3′	*Eco*RI
	5′-GAGAATTCATCTACATCCTCTACAAGCTTGGATTCTTCAAACGCTCCCTC-3′	*Pst*I
Trunc 12	5′-GACTGCAGCTGAGCTTTTTCCATGGCGGTGCCATATGGGAGGGAGCGTTTGAA-3′	*Eco*RI
	5′-GAGAATTCATCTACATCCTCTACAAGCTTGGATTCTTCAAACGCTCCCTC-3′	*Pst*I
Trunc 13	5′-GAGAATTCATCTACATCCTCTACAAGCTTGGATTCTTC-3′	*Eco*RI
	5′-GACTGCAGTCAGAGGGAGCGTTTGAAGAATCCAAGCTT-3′	*Pst*I

synthesized by the University of North Carolina (UNC) DNA core facility. Peptides conjugated with biotin at the N-terminus were synthesized by the UNC peptide synthesis core facility.

Construction of plasmids

To map the minimal interaction region on the α5 cytoplasmic tail as well as on the IBD of nischarin, we prepared several constructs as described below.

IBD constructs

The constructs were based on the IBD of nischarin previously reported by us [17]. All IBD constructs were synthesized by using full-length nischarin as the template and amplified by PCR using the appropriate primers (see Table 1). The resulting fragments were digested with *Not*I and fused to the VP16 *trans*-activating domain of pVP16 vector (Table 1). The N-terminal truncations were 1–4, 3.1, 3.2 and 3.3, and the C-terminal truncations were 11–14. All the constructs were confirmed by sequencing.

α5 constructs

All α5 truncations were constructed as lex A DNA-binding fusions in the vector pBTM116. DNA fragments of truncated versions of the integrin α5 cytoplasmic domain were amplified by PCR using the appropriate primers (see Table 2). These fragments were generated using full-length α5 cDNA as template, or simply by fill-in extensions using approx. 60-mer oligonucleotide primers (see Table 2). The fragments were subcloned into pBTM 116 utilizing either *Bam*HI/*Eco*RI or *Pst*I/*Eco*RI sites. N-terminal truncations are trunc 1–3 and C-terminal fragments are trunc 11–13. All constructs were confirmed by DNA sequencing.

Table 3 Primers used to make GST-IBD, GST-MIBD and GST-NIBD

GST construct	Primer	Site
IBD	5′-CTGAATTCGATGAGGACTTCCTGCTGGAG-3′	*Eco*RI
	5′-GCCTCGAGGGCCATGTGCTGCGGTGCAAA-3′	*Xho*I
MIBD	5′-CTGAATTCAAGGTGCTCTGGTGCTTCCTG-3′	*Eco*RI
	5′-GCCTCGAGGCGTAGATCAGCATGGAACTC-3′	*Xho*I
NIBD	5′-CTGAATTCTTCCTGATCCACGTGCAAGGC-3′	*Eco*RI
	5′-GCCTCGAGACACAGCTCGGGCATGAGGAA-3′	*Xho*I

Glutathione S-transferase (GST) constructs

Three GST-fusion constructs were prepared as shown in Table 3. Fragments were amplified by PCR with the appropriate primers (see Table 3) and subcloned into GST vector (pGEX5X.1; Amersham Biosciences, Piscataway, NJ) in the *Xho*I/*Eco*RI region as GST fusions and confirmed by sequencing. We made three GST-fusion plasmids: (i) GST-IBD, GST fused to the originally described IBD (residues 435–581); (ii) GST-MIBD, GST fused to MIBD (residues 464–562) as determined here by yeast two-hybrid approach; and (iii) GST-NIBD (where NIBD stands for non-IBD), GST fused to a region of the MIBD that cannot bind to α5 as identified by two-hybrid analysis (residues 469–534) (Table 3).

Myc constructs

To generate Myc-tagged IBD, MIBD and NIBD, the respective sequences were isolated by PCR as described for GST constructs

and inserted into pcDNA3.1 vector (Invitrogen, Carlsbad, CA, U.S.A). Primers for these constructs were exactly the same as for GST constructs except that we included a Kozak sequence (GCCACCATGG).

Yeast two-hybrid screening

Interactions of α5 integrin cytoplasmic domain constructs and the IBD region of nischarin were examined using a yeast two-hybrid system in L40 yeast strain [17]. These plasmids were introduced into cells by the standard lithium acetate method, and the interaction was examined by screening for histidine prototrophy and Lac Z positivity. All these experiments were performed as described previously [17].

Protein purification

GST-fusion constructs were transformed into BL21 cells. GST–nischarin fusion proteins were purified as described previously [17] with minor modifications. Briefly, the proteins were induced with IPTG, and the cells were lysed in NETN buffer (20 mM Tris/100 mM NaCl/1 mM EDTA/0.5 % Nonidet P40) [17] with aprotinin, and EDTA-free protease inhibitor cocktail tablet (Roche Biochemicals). The lysates were sonicated, spun down, and both the pellet and supernatant fractions were saved. Since these proteins were not soluble, the pellet fraction was solubilized in a buffer containing 10 mM Tris (7.8), 150 mM NaCl, 1 mM EDTA and 0.5 % *N*-dodecanoylsarcosine along with protease inhibitors. The proteins were visualized by SDS/PAGE followed by staining with Coomassie Blue. Proteins were immobilized on to glutathione beads and the proteins that bound to glutathione–Sepharose beads were eluted with 50 mM glutathione and dialysed against PBS with Mg^{2+} and Ca^{2+} buffer. The concentration and purity of the proteins were estimated by comparing with BSA standards on a Coomassie Blue-stained SDS/polyacrylamide gel. Before using in BIAcore assays, GST-fusion proteins were further verified for purity and apparent molecular mass by size-exclusion chromatography using HiPrep 16/60 Sephacryl S-100 column, running on an AKTA-FPLC (Amersham Biosciences).

Surface-plasmon resonance with BIAcore

Binding analyses were performed by surface-plasmon resonance using a BIAcore 2000; these experiments were performed at the University of North Carolina Macromolecular Interactions facility. Sensor chips were activated by using the NaCl/NaOH buffer according to the manufacturer's specifications.

Surface-plasmon resonance experiments were performed as described previously [19,20]. 5′-Biotinylated 14-residue α5 peptides (Table 4) were immobilized on the streptavidin-coated surface of the sensor chip (sensor chip SA, BIAcore). All peptides were diluted to 100 μM in the BIAcore buffer and they were injected at a flow rate of 10 μl/min. The injections were stopped once the immobilization of the peptide reached 300 resonance units (RU) on the chip. Fusion proteins were diluted to 40 nM in the running buffer and injected at a flow rate of 20 μl/min for 3 min. All proteins were injected using the kinject command. Sometimes, the injections with GST-fusion proteins were repeated to ensure that the surface-immobilized peptides on the sensor chip were saturated with GST-fusion proteins. Proteins bound to the sensor chip were allowed to disassociate for 180 s before the surface was regenerated with 0.05 % SDS. The resulting sensorgrams were edited using the BIAevaluation software (version 3.0). For re-usage, the chip was washed using the quickinject command with 0.05 % SDS at a flow rate of 20 μl/min, and the injected volume was 5 μl. After this quick injection, the chip was washed with 100 μl of BIAcore buffer at a flow rate of 20 μl/min.

Table 4 Peptides used in BIAcore experiments

No.	Biotinylated peptide
1	IYILYKLGFFKRSL
2	IYIL**A**KLGFFKRSL
3	I**A**ILYKLGFFKRSL
4	IYILY**A**LGFFKRSL
5	IYILYKLGFFARSL
6	IYILYKLGFFK**A**SL
7	IYILYKLGFFKR**A**L
8	FGFLKYKLIRYSIL (SC)

Differential-scanning calorimetry (DSC)

DSC experiments were performed using a Microcal VP differential-scanning calorimeter (Microcal Incorporated, Northampton, MA, U.S.A.) at the UNC Macromolecular Interactions Facility. This technique is one of the best methods to evaluate protein-unfolding transitions. GST and GST-MIBD proteins were purified as described above. The protein was dialysed overnight against 2 litres of PBS that was used to establish the baseline. The sample solutions and buffer were filtered and degassed under vacuum. The calorimetric samples were pressurized and scanned from 20 to 95 °C at a rate of 1°/min and protein concentrations of 0.5 mM GST-MIBD and 2 mM GST. Baselines were collected for PBS and subtracted from the scans of GST-MIBD samples. Data were analysed using the Microcal Origin Software (version 4.0). Each sample was heated and cooled for five cycles to evaluate folding reversibility. Unfolding of the protein was irreversible and accompanied by precipitation.

Transwell migration assays

CHO B2 cells lacking α5 as well as CHO B2a27, its α5 transfectant [21], were transiently transfected with full-length nischarin (pcDNA-mycNis) and its truncations (pcDNA myc-IBD, pcDNA myc-MIBD, pcDNA myc-NIBD) or vector control using LIPOFECTAMINE™ (Invitrogen). Cell migration studies were performed using a transwell assay, according to a previously described procedure [17]. The transfected cells were marked using a β-galactosidase expression plasmid. The underside of each transwell was coated with 10 μg of fibronectin or vitronectin; the cells were plated on the upper surface and the percentage of full-length nischarin, truncated forms of nischarin or control transfectants migrating across the 8 μm pore-size membrane was determined by staining for β galactosidase after overnight incubation in BSA-containing medium. The ratio of migrant transfected cells to total transfected cells $\times$100 was taken as the percentage of migration.

RESULTS

Construction of IBD and α5 truncations

To map the minimal interaction region on the α5 cytoplasmic tail as well as on the IBD of nischarin, we prepared 17 constructs (see the Materials and methods section). Of these, six were α5 mutants and 11 were nischarin IBD mutants. As described in the Materials and methods section, seven different N-terminal IBD-truncated constructs and four C-terminal truncations were

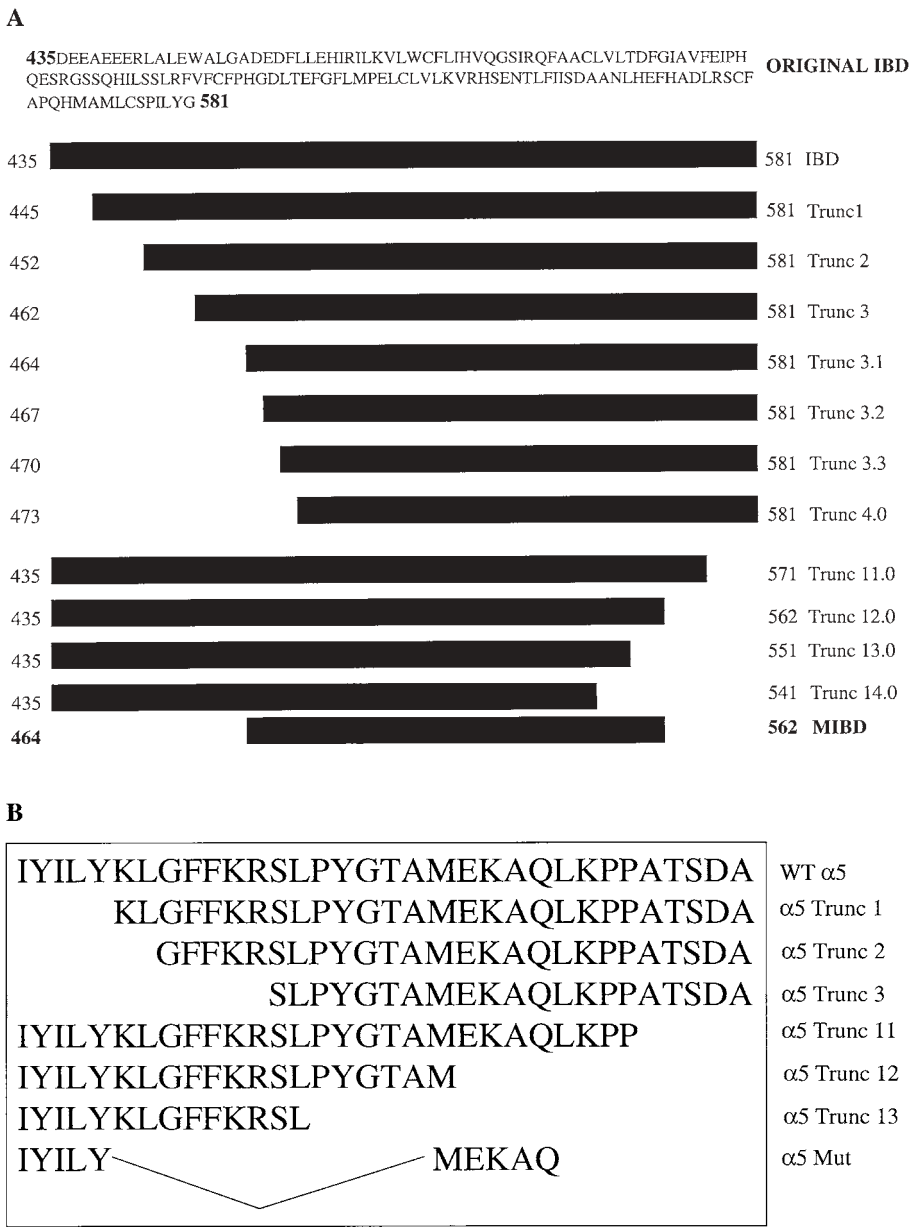


Figure 1 Schematic representation of the IBD of nischarin and of α5 cytoplasmic domain truncations

(**A**) Amino acid sequence and schematic representation of IBD-truncated constructs. The wild-type amino acid sequence of the previously described IBD is shown, the truncated analogues are depicted and the starting and ending amino acid numbers are shown. These truncations were created by PCR approach using the primers described in Table 1. (**B**) Amino acid sequence of α5-truncated constructs. These constructs were prepared using the primers described in Table 2. The names of the constructs are shown to the right in (**A**) and (**B**).

made by PCR approaches (Figure 1A). Furthermore, three α5 N-terminal and three C-terminal truncations were made (Figure 1B).

Yeast-two-hybrid analyses

Characterization of α5 binding sites in the IBD of nischarin

Several IBD truncations described above were tested against the α5 cytoplasmic tail using a two-hybrid analysis. As reported previously, the originally described [17] IBD of nischarin interacts strongly with the α5 cytoplasmic region, but not the vector control or other α subunits (Figure 2, position #12, [17]). Fragments of nischarin with N-terminal truncations (trunc1: 445–581, trunc2: 452–581, trunc3: 462–581; Figures 2B and 2C, positions #1–3) also interacted strongly with the α5 cytoplasmic region, whereas trunc 4 (473–581) did not interact (Figures 2B and 2C, position #7). To identify further the exact region of interaction, three other constructs including trunc 3.1 (464–581), trunc 3.2 (467–581) and trunc 3.3 (470–581) were made. Trunc 3.2 and trunc 3.3 (positions #5 and 6) were unable to interact, whereas trunc 3.1 (position #4) was able to interact (Figures 2B and 2C), suggesting that truncations beyond 467 amino acids disrupted the interaction.

To demarcate the C-terminal boundary of the region, C-terminal truncations were created. C-terminal fragments, truncs 11 (435–571) and 12 (435–562), interacted well (Figure 2, positions #8 and 9), whereas truncs 13 (435–551) and 14 (435–541) did not

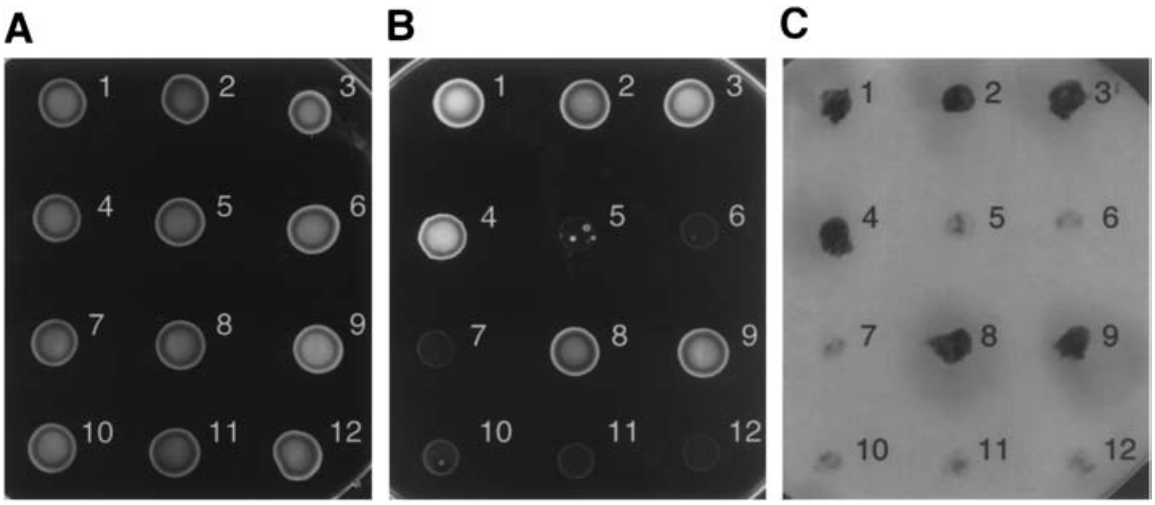


Figure 2 Two-hybrid analysis to identify minimal region of nischarin necessary for interaction with α5 integrin cytoplasmic tail

Yeast cells were transformed with pBTM-α5 and selected for tryptophan prototrophy. Tryptophan-positive cells were again transformed with truncs 1 (position #1), 2 (position #2), 3 (position #3), 3.1 (position #4), 3.2 (position #5), 3.3 (position #6), 4.0 (position #7), 11 (position #8), 12 (position #9), 13 (position #10), 14 (position #11) or vector control constructs (position #12) (described in Table 1 and Figure 1A), and screened for tryptophan and leucine prototrophy by growing them on plates that lack leucine and tryptophan (-TL) (**A**). The cells that grew on -TL were replica-plated on to plates that lack tryptophan, leucine and histidine residues (**B**). The cells in (**A**) were also transferred to a filter, and X-gal staining was performed to confirm the interactions further. Only cells with strong interaction turn blue under these conditions (**C**).

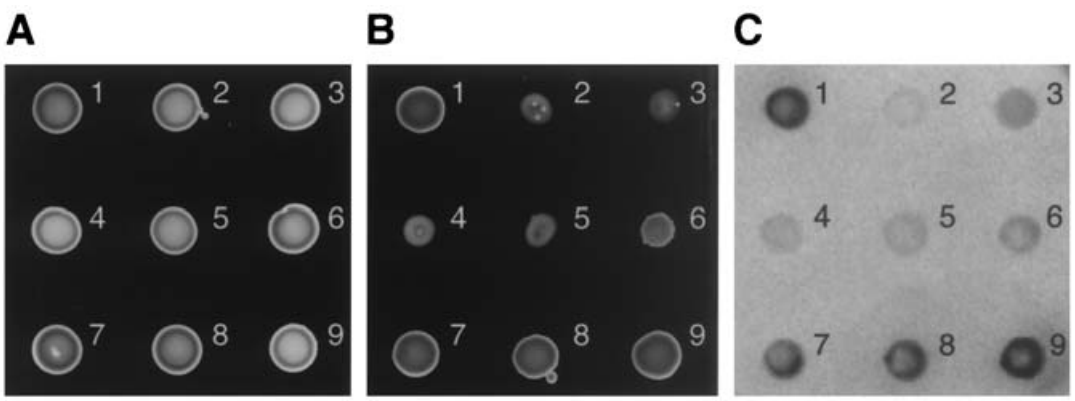


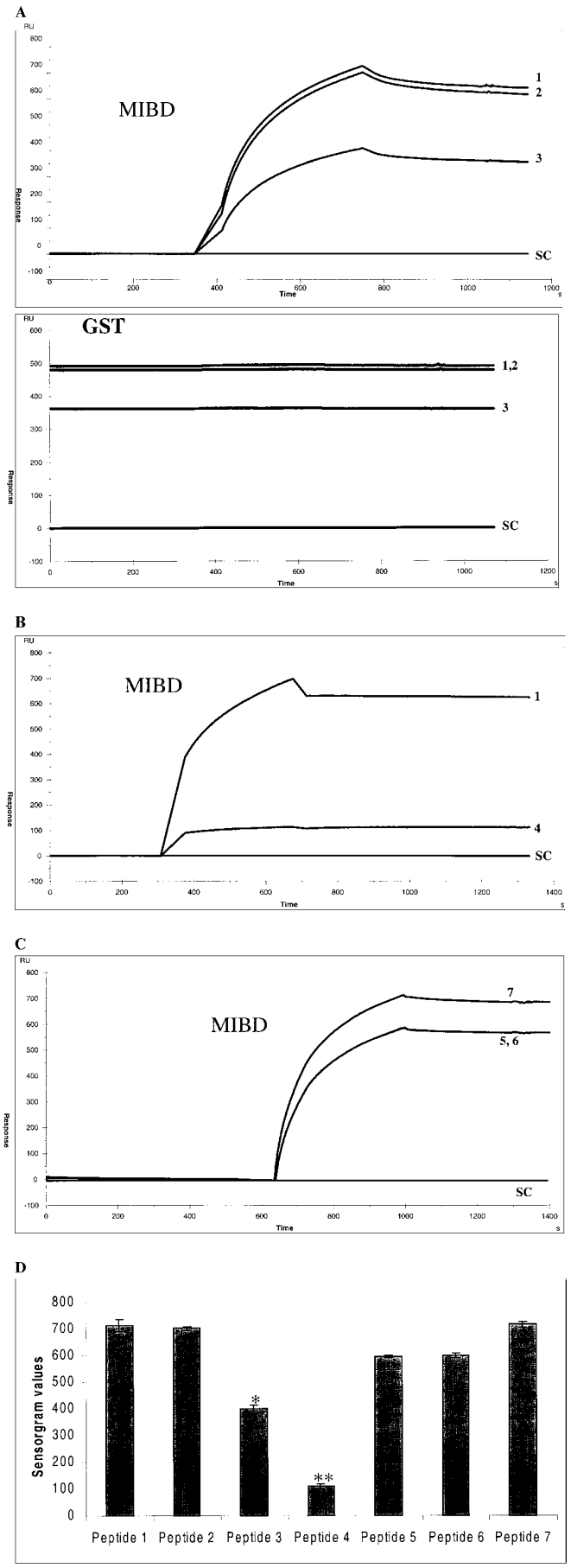
Figure 3 Two-hybrid analysis to identify minimal region of α5 necessary for interaction with nischarin

L40 yeast strain was transformed with pVP-IBD plasmid and selected for leucine prototrophy. The yeast cells that contain the pVP-IBD plasmid were transformed with wild-type α5 (position #1), α2 (position #2), trunc 1 (position #3), trunc 2 (position #4), trunc 3 (position #5), mutant (position #6), trunc 11 (position #7), trunc 12 (position #8) or trunc 13 (position #9) constructs (described in Table 2 and Figure 1B), and selected for leucine and tryptophan prototrophy. All transformed cells grow well under these conditions (**A**). The same cells in (**A**) were replica-plated on to plates that lack histidine in addition to leucine and tryptophan (**B**). The same cells in (**A**) were transferred to a filter paper and stained for β-galactosidase (**C**).

(Figure 2, positions# 10 and 11). C-terminal truncations of the region up to amino acid 462 retained α5 binding, since both truncs 11 (435–571) and 12 (435–562) bound to α5. Deletion of amino acids from the C-terminus beyond residue 561 disrupted α5 interaction. These results suggest that deletions beyond amino acids 464–562 were not dispensable for interaction. Thus the region (amino acids 464–562) was designated as MIBD.

Characterization of nischarin-binding sites in the cytoplasmic domain of α5

The α5 tail fragments were subcloned into pBTM bait vector, and co-transfected with pVP-nischarin IBD constructs (Figures 1B and 3). As reported previously, IBD interacted with the full-length α5 cytoplasmic tail (Figure 3, position #1), whereas the α2 cytoplasmic tail did not interact (Figure 3, position #2). The α5 N-terminal truncations, namely trunc 1 (lacks IYILY), trunc 2 (lacks IYILYKL) and trunc 3 (lacks IYILYKLGFFKR), failed to interact with the IBD of nischarin (Figure 3, positions #3–5). In the C-terminal mutants, deletion of residues beyond GFFKRSL seems to be dispensable for the interaction, as these mutants strongly interacted with the IBD (truncs 11–13) (Figure 3, positions #7–9). The GFFKR region is one of the most highly conserved regions of the peptide among different integrin α subunits. Thus we created a mutant that lacks only KLGFFKRSL (Figure 1B). This mutant was unable to interact with the IBD of nischarin (α5 mutant) (Figure 3, position #6). These results indicate that neither IYILYKL nor GFFKRSL alone can interact with nischarin and the entire region of IYILYKLGFFKRSL (residues 1017–1030 of α5 integrin) is required for nischarin binding.



The BIAcore assay for interaction between nischarin and α5

Recombinant nischarin proteins (GST-IBD, GST-MIBD and GST-NIBD) and GST alone were purified as described in the Materials and methods section. The interaction of these proteins with α5 was further examined using a BIAcore assay which has been widely used to detect protein–protein interactions [19,20]. Our experiments used α5 peptides conjugated to biotin at their N-terminus (Table 4). A total of eight peptides of α5 were analysed for their binding capacity to nischarin-fusion proteins. As a positive control, we used a wild-type α5 sequence (peptide 1), whereas a scrambled peptide served as a negative control. The biotinylated peptides were immobilized via a biotin group bound to the streptavidin biosensor surface. The recombinant GST-IBD (results not shown) or GST-MIBD nischarin proteins were flowed over the complex. As a control, the analyte buffer containing GST alone or GST-NIBD (a region that cannot bind to nischarin) was injected.

As expected, GST-MIBD (residues 464–562) bound strongly to peptide 1 (IYILYKLGFFKRSL) (Figure 4A), whereas GST alone did not interact. Both GST-NIBD (results not shown) and GST alone displayed negligible binding to peptide-immobilized streptavidin chip (Figure 4A, bottom panel). Analysis of the sensorgrams demonstrated that peptide 1 (IYILYKLGFFKR), but not scrambled peptide, was able to bind to GST-MIBD (Figure 4A). Thus nischarin interacts directly with α5 peptides. To identify which residues are important for binding, a series of mutated peptides were examined by a surface-plasmon resonance approach. First, we attempted to determine if either of the tyrosine residues in the ^{1017}IYILYKL^{1023} region is important for the binding; thus both tyrosine residues were mutated to alanine residues. The membrane-distal tyrosine (residue 1021; peptide 2) did not have any effect on binding. However, membrane proximal substitution of tyrosine (residue 1018; peptide 3) affected binding significantly (Table 4, Figure 4A), indicating that this region might be important for binding to nischarin. Lysine is highly conserved among all α and β subunits and predicted to play a role in exposing the cytoplasmic tail for interactions with cytoplasmic proteins [22]. Thus we mutated this residue; substitution of alanine for lysine (residue 1022; peptide 4) resulted in almost complete loss of binding (Figure 4B), indicating that this residue is critical for interaction. Both GST-NIBD and GST alone failed to bind peptides on the chip (results not shown).

Several conserved features constitute the cytoplasmic tail of integrin, including the highly conserved GFFKR region. To our surprise, single mutations in the GFFKRSL region did not have any effect on binding to nischarin. As shown in Figure 4(C), substitution of alanine for lysine (residue 1027; peptide 5), and

Figure 4 GST-IBD of nischarin binding to α5 peptides as assessed by surface-plasmon resonance

(**A**) Top panel: Membrane-proximal Tyr^{1018} of α5 is important for binding to nischarin. Peptides IYILYKLGFFKRSL (1), IYILAKLGFFKRSL (2), IAILYKLGFFKRSL (3) or FGFLKYKLIRYSI (scrambled peptide, SC) were immobilized to streptavidin-coated biosensor surfaces and 60 μl of 50 nM GST-MIBD was injected (time, 360 s; flow rate, 10 μl/min). Background for binding of nischarin to the scrambled peptide has been subtracted. RU, resonance units; time in S. Bottom panel: the same biosensor surfaces in (**A**), top panel, were stripped, and GST alone was injected. (**B**) Lys^{1022} is important for interaction. Peptides IYILYKLGFFKRSL (1), IYILYALGFFKRSL (4), or FGFLKYKLIRYSIL (SC) were immobilized to strepatavidin-coated surface and 50 nM of GST-MIBD was injected as in (**B**). (**C**) Single mutations in GFFKR region did not strongly affect binding. IYILYKLGFFARSL (5), IYILYKLGFFKASL (6), or IYILYKLGFFKRAL (7) were immobilized as above, and GST-MIBD was injected. (**D**) Quantification of BIAcore data: SPR data of seven peptides were converted into Microsoft Excel, and values of peptide binding peaks were plotted. Binding of peptides 3 and 4 are significantly low compared with peptide 1. For peptide 3, $^{*}P < 0.005$ ($P = 0.003$); for peptide 4, $^{**}P < 0.0005$ ($P = 0.0004$). Results are the means $\pm$ S.E.M. from three determinations.

alanine for arginine (residue 1028; peptide 6) and alanine for serine (residue 1029; peptide 7) had no significant decrease in binding to IBD of nischarin. These results further support that the membrane-proximal 14-amino-acid residue of the α5 cytoplasmic domain contains the binding site for nischarin, and reveals that residues Tyr^{1018} and Lys^{1022} are important for nischarin binding. Our results show that nischarin MIBD binds strongly to α5 peptides. We were not able to calculate affinity constants. However, size-exclusion chromatography revealed that GST-MIBD was a dimer (see below) rather than a monomer. Thus, we could not obtain meaningful dissociation constants. However, in semi-quantitative terms, it is clear that peptides 3 (mutation of residue 1018) and 4 (mutation of residue 1022) have significantly lower binding than the other α5 peptides (Figure 4D).

DSC experiments

Since the GST-fusion proteins were initially insoluble, we performed several biophysical experiments to examine the nature of these proteins. Gel-filtration chromatography experiments revealed that the GST-MIBD exists as a monodisperse dimer (results not shown), similar to the GST protein that is known to exist as a dimer [23–25]. Also, we have examined whether GST-MIBD is a folded domain by DSC experiments. The proteins were heated at a scanning rate of 60 °C/h up to 95 °C, cooled and rescanned under the same conditions. The peak of a DSC curve yields the melting temperature (T_m) for denaturation. GST thermally denatured as a single peak in DSC at approx 58 °C (Figure 5). The unfolding of GST-MIBD showed two peaks, one with a midpoint at approx 58 °C and the other at approx 82 °C, probably representing GST and MIBD domains respectively. The DSC profile for MIBD showed a broad unfolding transition, which is somewhat similar to the curve shown by Garcia-Mira et al. [26], indicating that unfolding probably started at even lower temperatures. The observed transitions were irreversible as no transitions were noticed in subsequent scans. Heating of the sample above 80 °C leads to complete and irreversible denaturation of GST-MIBD.

In DSC, most proteins were not able to re-fold after heat denaturation, especially for proteins larger than 20 kDa [27]. Irreversible thermal denaturation has also been observed in several MHC proteins [28]. Thus, our results revealed the structural stability of the fusion proteins and suggest that MIBD is a folded domain.

Migration effects

Although it is clear that nischarin plays an important role in the regulation of cell migration, the role of the IBD is not known. To elucidate the role of the MIBD in cell migration, we performed cell migration assays using the IBD, MIBD or NIBD of nischarin. Surprisingly, none of these moieties had any effect on haptotactic migration on fibronectin and on vitronectin, whereas full-length nischarin was capable of inhibiting cell migration on fibronectin, as reported in [17] (Figure 6). In addition, migration of B2 cells (that lack α5) on vitronectin was not affected by any of these constructs. These results indicate that the IBD is not sufficient for the migration inhibitory effects of nischarin, and thus the other regions of this protein may be necessary for this function.

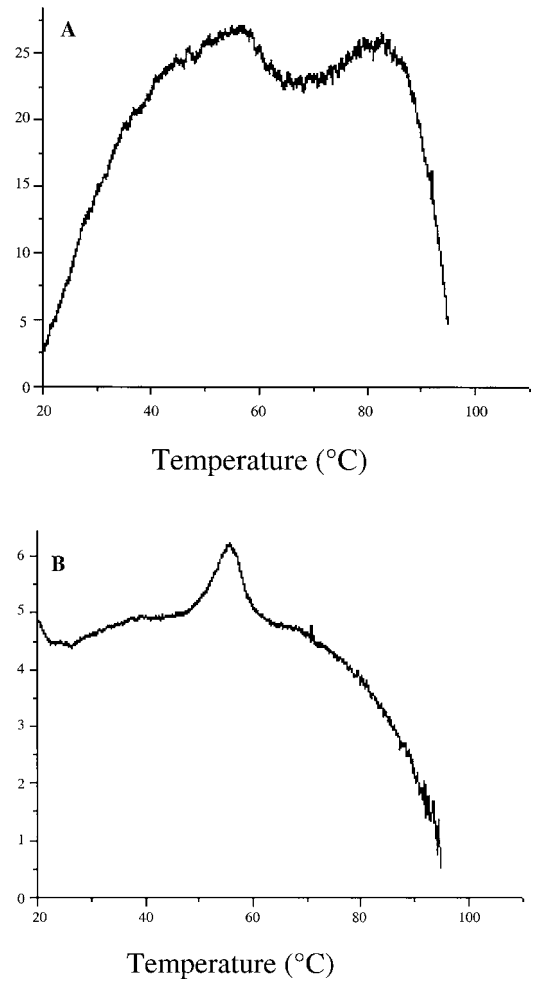


Figure 5 DSC thermograms

DSC was performed in PBS at 1 °C/min, and from 20 to 95 °C. For clarity, starting values of C_p (cal\°C) on the *y*-axis were normalized to zero. (**A**) Thermal denaturation profile of (**A**) GST-MIBD (protein concentration was 0.5 mM) and (**B**) GST (protein concentration was 2 mM).

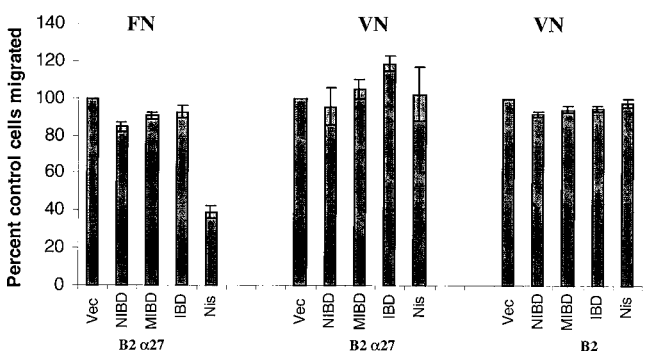


Figure 6 Migration is not affected by the nischarin IBD

Transwell migration assays were performed using α5-deficient CHO B2 cells or α5 overexpressing CHO B2α27 cells transfected with full-length nischarin (Nis) (pcDNA-Myc nischarin), pcDNA-Myc IBD, pcDNA-Myc MIBD or pcDNA-Myc NIBD. Cells migrating either on fibronectin (FN) or vitronectin (VN) were counted and plotted. Results represent means ± S.E.M. from three determinations. Vec, vector (control).

DISCUSSION

We identified nischarin as a protein that binds selectively to the α5 integrin subunit and inhibits cell migration. Since nischarin selectively affects cell migration on fibronectin, and binds $\alpha5\beta1$ integrin, it is of interest to identify the minimal α5 binding region on nischarin, and the nischarin-binding region on the cytoplasmic domain of the α5-integrin subunit. Nischarin is the only

protein known to date, which interacts selectively with the integrin-α5 subunit. Previously, we reported [17] that a 147 amino acid residue nischarin IBD directly binds to the cytoplasmic tail of α5. In the present study, we have demonstrated that another 51 amino acids are dispensable for this interaction; thus we have identified 99 residues as the minimal binding domain of nischarin for α5. Also, we mapped the nischarin-binding site on the α5 cytoplasmic domain to a 14-residue stretch that includes membrane-proximal as well as putative intra-membrane portions of the α5-integrin subunit. BIAcore surface-plasmon resonance experiments allowed monitoring of interactions between a recombinant MIBD and various truncated α5 cytoplasmic tail peptides. We have identified residues Tyr^{1018} and Lys^{1022} (I**Y**ILY**K**L-GFFKRSL) to be important in nischarin–α5 interaction. This is somewhat surprising since the IYILYKGFF sequence is thought to be partially within the membrane [22]; however, some soluble proteins penetrate membranes [29], and this may allow interactions with intra-membrane sequences. Several possibilities for the interaction of this region with cytosolic proteins have been proposed by Armulik et al. [22]. It was suggested that the region is pushed out of the membrane when its hydrophobicity is decreased; Lys^{1022} is highly conserved in all integrin subunits, and it is believed that this residue may facilitate the entry of the adjacent residues into the cytosol. Another possibility is that the region is buried in the membrane when integrins are in inactive conformation and on activation these will be accessible to intracellular proteins. Thus it is possible that nischarin interacts with α5 at the membrane-proximal sequence through one of the scenarios described [22]. Interestingly, the interacting region contains a membrane-proximal IYILY region that is exclusively present in the α5 subunit; this explains the reason for selective binding to α5 cytoplasmic tail as demonstrated by us previously [17].

This is consistent with other reports demonstrating the importance of integrin-membrane-proximal regions in association with other membrane or cytoplasmic proteins [30]. Talin is a cytoskeletal protein that connects integrins to the actin cytoskeleton. Talin has been shown to interact with peptides from the membrane-proximal region of β3 integrin [31]. The membrane-proximal NPXY motif of the β3 cytoplasmic tail is essential for talin binding [32]. Interaction of the membrane-proximal region of β3 with integrin leads to integrin activation [33]. More precisely, a FERM (four point one, ezrin, radixin and moesin) domain of the head domain of talin has been shown to bind to the β3 cytoplasmic tail, and is important in integrin linkage to the interior of the cell [34]. F3, a subdomain of FERM, induces activation of integrin αIIbβ3 and in turn may control cell migration [32]. These results suggest that association of integrins with cytoplasmic proteins plays a major role in integrin activation and regulation of cell migration.

Most of the integrin subunit cytoplasmic domains are relatively small (20–70 residues), with the exception of the β4 integrin cytoplasmic domain. A deletion of seven membrane-proximal amino acids resulted in the activation of integrin αIIb, which further suggests that membrane-proximal residues are important for integrin function [35,36]. Cytohesin 1 has been shown to interact with the nine membrane-proximal amino acids of the β2 cytoplasmic tail [37]. In contrast, ICAP1α (integrin cytoplasmic domain-associated protein-1α) has been shown to bind to the C-terminus of the integrin β1 subunit [38]. Recently, a binding site for the calcium- and integrin-binding protein has been mapped to a 15-amino-acid membrane-proximal region of αIIb (some residues in the region are thought to be embedded in the membrane) [30]. The KVGFFKR sequence of αIIb has been shown to be necessary but not sufficient for the α-β heterodimerization [39–41]. More recently, it was shown that transmembrane helix-cytoplasmic domain of β3 regulates changes in the αIIbβ3 activation state [42]. At present, we do not know whether nischarin has a regulatory effect on α5β1 ligand-binding function and/or in post-receptor occupancy events; these possibilities need further exploration.

It has been shown that several integrin cytoplasmic domain-binding proteins regulate several aspects of signal transduction and cell migration. For instance, ICAP1 has been shown to regulate cell adhesion and cell migration [43,44]. Also paxillin binding to the α4 integrin cytoplasmic tail selectively inhibits cell migration [16]. Previously, we reported that full-length nischarin inhibits α5-dependent cell migration [17]. We tested whether the minimal-binding region (99 amino acids) of nischarin is sufficient for this inhibition. As shown in Figure 6, neither the 147-residue fragment nor the 99-residue fragment was able to inhibit migration of CHO cells overexpressing α5 integrin, indicating that other regions of nischarin contribute to its motility inhibitory ability.

In summary, in the present study, we have mapped the α5 integrin-binding region in nischarin and the nischarin binding region in α5 integrin. We found that (1) a region of residues between Lys^{464} and Arg^{562} is sufficient for binding to the α5 cytoplasmic tail; (2) the IYILYKLGFFKRSL sequence is sufficient for binding to the IBD of nischarin and Tyr^{1018} and Lys^{1022} are crucial for this interaction; and (3) the 99-residue fragment that binds to the α5 cytoplasmic domain is not sufficient for inhibition of migration. Thus, it will be important to examine how the IBD of nischarin works with other regions of this protein to provide the full biological activity of the molecule. Also this knowledge will be helpful for future structural and functional studies of nischarin.

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