Regulation of p21-activated Kinase-independent Rac1 Signal Transduction by Nischarin*

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Nischarin regulates Rac1-dependent cell motility by interaction with and inhibition of the p21-activated kinase (PAK1). In addition to regulating the activation of PAK1, Rac1 controls multiple downstream pathways to regulate cell growth and differentiation, as well as cell motility. Signaling by a constitutively activated Rac1 mutant deficient in PAK binding (Rac1Q61L-40C) was examined to determine whether Nischarin impinges on these other Rac1 effector pathways. Nischarin formed immunoprecipitable complexes with Rac1Q61L and Rac1Q61L-40C when the proteins were co-expressed. In NIH3T3 cells, Rac1Q61L and Rac1Q61L-40C stimulation of a minimal NF-κB response element or the cyclin D1 promoter, a downstream target of NF-κB, was inhibited by co-expression of Nischarin. Additionally, suppression of endogenous Nischarin protein with small interfering RNA in PC12 cells enhanced Rac1Q61L and Rac1Q61L-40C activation of NF-κB. In further support of Nischarin suppressing PAK independent Rac signaling, foci formation in monolayers of NIH3T3 cells by Rac1Q61L-40C in cooperation with c-Raf/CAAX was inhibited by the presence of Nischarin. Nischarin alters the cellular localization of Rac1Q61L and Rac1Q61L-40C to vesicles and this positively correlates with the repression of the Rac1 signal. Thus, Nischarin, in addition to regulating the PAK strand of Rac1 signaling, can also regulate other links in the web of Rac1 signaling pathways.

The Rho GTPases are a family of 20 proteins that act as molecular switches that assist in the transduction of signals through the interior of the cells. Activation of a variety of cell surface receptors shifts the Rho GTPases from the inactive GDP bound state to the active GTP bound state through the activity of numerous guanine nucleotide exchange factors. The active GTPase can then interact with a myriad of effectors and modulate their function. GTPase activating proteins suppress active GTPase and can act independently of PAK to regulate processes such as cell proliferation and transformation (2–7). To mediate these effects on cellular phenotype, Rac1 interacts with a variety of effector proteins. For example, Rac1 regulation of cell motility is mediated, in part, by interaction with the family of p21-activated kinases (PAK). Activated PAK stimulates the formation of cortical actin networks, which are important for Rac-induced lamellipodia, by suppressing the actin-depolymerizing activity of coflin via the activation of LIM kinase. PAK also readies the cell for movement by suppressing stress fiber formation and increasing focal adhesion turnover, in part, by disrupting the actin myosin network through inactivation of myosin light chain kinase (8). Independently from PAK, Rac1 interacts with the dormant, multiprotein WAVE complex through PIR121 and Nap125, to stimulate the dissociation of WAVE and allowing it to activate the actin polymerizing Arp2/3 complex. Rac has also been found to complex with WAVE through IRSp53 to modulate WAVE function (9, 10). Similarly, Rac1 can regulate proliferative signals through various means such as activation of PAK and its subsequent phosphorylation of Rafmitogen-activated protein kinase components or via other mechanisms, such as stimulation of NF-κB through the NF-κB inducing kinase (11–16).

Nischarin was cloned from a mouse embryonic cDNA library using the cytoplasmic domain of the integrin α5 subunit as “bait” (17). Nischarin interacts directly with α5 through membrane proximal residues of the α5 cytoplasmic domain (18). Elevated levels of intracellular Nischarin impede α5-dependent cell motility in fibroblast and epithelial cells (17). Overexpression of Nischarin inhibits Rac1-stimulated cell motility and this directly correlates with its ability to inhibit signaling via the Rac1 effector PAK1 (18, 19). Nischarin preferentially binds activated PAK1 and forms a complex with PAK1 via its N terminus and the kinase domain of PAK. The association of Nischarin with PAK1 inhibits PAK1 activation and, thus, PAK1-stimulated migration (20).

As described above, in addition to PAK, Rac1 has multiple downstream effectors and can act independently of PAK to regulate processes such as cell proliferation and transformation (2, 3, 6, 16, 21). Using a PAK binding-deficient mutant of Rac, Rac1Q61L-40C, we have found that Nischarin can interact with activated Rac independently of its interaction with PAK. This interaction is functionally important because Nischarin inhibits PAK-independent Rac1 signal transduction and cell

1 The abbreviations used are: PAK, p21-activated kinase; ERK1, extracellular signal-regulated kinase 1; GFP, green fluorescent protein; HA, hemagglutinin; IRAS, imidazoline receptor antisera-selected cDNA; NF-κB, nuclear factor-κB; EGF, epidermal growth factor; DMEM, Dulbecco’s modified Eagle’s medium; aa, amino acids; PBS, phosphate-buffered saline; siRNA, small interfering RNA; FITC, fluorescein isothiocyanate; CAAX, a prenylation motif where A is an aliphatic amino acid (italicized letters represent unknowns); IκB, NF-κB inhibitor.
transformation. Thus, in addition to regulating the PAK1 arm of Rac signal transduction, Nischarin may regulate Rac1 signaling via other Rac effector pathways.

MATERIALS AND METHODS

Cell Culture—COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 4,500 mg/liter d-glucose and sodium pyruvate supplemented with 1-glutamine and 10% fetal bovine serum (Sigma). NIH3T3 cells were cultured in DMEM supplemented with 2-glutamine and 10% calf serum (Colorado Serum Company, Denver, CO). PC12 cells were cultured in DMEM with 10% horse serum (GIBCO®, Invitrogen Cell Culture, Carlsbad, CA) and 5% fetal bovine serum.

Vectors—The cDNA for full-length mouse nischarin, the N terminus (aa 1–502), and the C terminus (aa 970–1354) were inserted at the EcoRI/NsiI sites in the multiple cloning site of pcDNA3.1 B Myc/His (pcDNA; Invitrogen). Full-length or truncated PAK1 cDNA (provided by J. Chernoff) and β-galactosidase were expressed from pcDNA3.1 B-Myc/His. Myc-GFP was expressed from the pCMV/myc/cytGFP vector from Invitrogen. HA-tagged Rac1Q61L, HA-Rac1Q61L-40C, and cRaf/CAAX (provided by C. Der) were expressed from the pCGN vector (21). The AU5-Rac1 wild type expression vector was provided by K. Burridge. The AU5-Rac1 C terminus, or Myc-N terminus, Myc-C terminus, or Myc-β-galactosidase and 1 µg of HA-Rac1Q61L, HA-Rac1Q61L-40C, or pcDNA plasmids and 500 ng of 3×NF-xB-LUC. Twenty-four hours after transfection the cells were washed with PBS, starved in serum-free DMEM for 12–15 h, harvested, and assayed for luciferase activity.

For suppression of endogenous Nischarin protein levels by siRNA, a previously described 21-base pair siRNA oligonucleotide for rat Nischarin, rCRUcrGurGrUrArCrUUrGArCrCUrGTT (20), or a nonspecific control duplex siRNA (#D-001206-07-20) were synthesized by Dharmacco, Lafayette, CO. The siRNA oligomers at a concentration of 150 nM were co-transfected with 500 ng of HA-Rac1Q61L, HA-Rac1Q61L-40C, or pcGNN plasmids and 500 ng of 3×NF-xB-LUC into PC12 cells with Lipofectamine™ 2000 as described above. Forty-eight hours after transfection the Nischarin antibody (BD Biosciences). α-Tubulin was detected with a monoclonal anti-α-tubulin antibody (Sigma). All of the luciferase assays were performed in triplicate, and the luciferase activity was normalized to protein levels.

Foci Forming Assays—NIH3T3 cells were plated in 60-mm dishes at 4 × 105 cells per plate. The cells were transfected with 1 µg of Rac1Q61L-40C, 100 ng of c-Raf/CAAX, both, or the pcGNN vector control and pcDNA using SuperFect™. The plates were kept at confluence for 21–24 days with media changes every 3–4 days. The colonies were visualized and counted after staining with 0.1% crystal violet. The experiments were performed with triplicate or quadruplicate plates for each treatment.

Confocal Microscopy—NIH3T3 cells were plated in 6-well plates at 2 × 105 cells per well and grown overnight. The cells were transiently transfected with 3 µg of Myc-Nischarin, Myc-N terminus, Myc-C terminus, or pcDNA vectors and 1 µg of HA-Rac1Q61L, HA-Rac1Q61L-40C, or pcGNN with Lipofectamine™ 2000 (Invitrogen). After 24 h of transfection, the cells were trypsinized, and half of the cells were replated on fibronectin-coated coverslips (100 g of plasmid for Myc-Nischarin, Myc-N terminus, Myc-C terminus, pcDNA vectors and 1 µg of HA-Rac1Q61L, HA-Rac1Q61L-40C, or pcGNN with Lipofectamine™ 2000). For Nischarin/Rac1 binding, COS-7 cells were transfected with 3 µg of Myc-Nischarin or Myc-N terminus, Myc-C terminus, or Myc-β-galactosidase and 1 µg of HA-Rac1Q61L or HA-Rac1Q61L-40C, or pcGNN plasmids and 1 µg of Myc-Nischarin antibody (BD Biosciences). α-Tubulin was detected with a monoclonal anti-α-tubulin antibody (Sigma). All of the luciferase assays were performed in triplicate, and the luciferase activity was normalized to protein levels.

RESULTS

Nischarin Interacts with Rac1 Independently of PAK—Nischarin directly interacts with activated PAK1 and suppresses PAK1 kinase activity. Additionally, co-expression of activated Rac1 with PAK1 and Nischarin stimulates association of PAK1 with Nischarin without preventing association of Rac1 with PAK1. All three proteins can be found in Nischarin immunoprecipitates from cells overexpressing Nischarin, PAK1, and activated Rac1 (20).

The presence of Rac1 in the Nischarin-PAK1 intracellular complex may solely depend on the association of Rac1 with PAK1. Alternatively, Rac1 could associate with Nischarin through PAK1-independent interactions. Overexpression of HA-tagged, constitutively activated Rac1 (HA-Rac1Q61L) with Myc-tagged Nischarin (Myc-Nischarin) in the absence of exogenous PAK1 in COS-7 cells allowed co-precipitation of Myc-Nischarin with the immunoprecipitated HA-Rac1Q61L (Fig. 1A). Conversely, immunoprecipitation of Myc-Nischarin also co-precipitates HA-Rac1Q61L (Fig. 1B). Nischarin does not exhibit detectable interaction with the unrelated protein, HA-ERK1, under these experimental conditions nor does HA-Rac1Q61L interact with Myc-GFP. This indicates that the Nischarin/Rac1 interaction is specific. The retention of the Rac1/Nischarin interaction in the absence of exogenously expressed PAK1 suggests that the binding of Rac1 to Nischarin may have a PAK-independent component because the levels of endogenous PAK are unlikely to be sufficient to mediate this interaction.

To directly test if the interaction of Nischarin with Rac1...
required PAK1, a PAK binding-deficient mutant of Rac1, HA-Rac1Q61L-40C, was used. Several laboratories have used this Rac1 effector domain mutant to investigate PAK-independent functions of Rac1 (16, 21). Myc-Nischarin and Myc-PAK1 were co-expressed in COS-7 cells with HA-Rac1Q61L or HA-Rac1Q61L-40C. Immunoprecipitation of Nischarin or PAK1 by their Myc epitopes consistently co-precipitated HA-Rac1Q61L. In agreement with previous studies (16, 21), HA-Rac1Q61L-40C did not co-precipitate with immunoprecipitated PAK1. However, HA-Rac1Q61L-40C was repeatedly co-immunoprecipitated with Nischarin (Fig. 1C). Thus, the complex formation between Nischarin and active Rac1 does not require the interaction of Rac1 with PAK. Previously described Rac1 effector domain mutants (E31V, F37L, and N43D) were also examined in the co-immunoprecipitation assay and they did not abrogate the Rac1/Nischarin interaction (data not shown) (16, 21).

To determine whether Nischarin and Rac1 can interact at the normal, endogenous levels of the proteins, PC12 cells were used because Nischarin is expressed well in PC12 cells (17, 20). Lysates were made from exponentially growing cells, and Rac1 was immunoprecipitated with a Rac1-specific monoclonal antibody. The endogenous Nischarin was co-immunoprecipitated in the co-immunoprecipitation assay and they did not abrogate the interaction of Rac1 with PAK and the 40C substitution does not alter the interaction of Rac1 with either domain. Additionally, the interaction of Rac1 with the C terminus of Nischarin supports the PAK-independent nature of this interaction because PAK1 exhibited no interaction with the C terminus of Nischarin (20).

**Growth Factor-stimulated Interaction of Rac and Nischarin—** Rac switches to its activated state in response to signals from various cell surface receptors (2, 3). To test if a natural cell stimulus could stimulate the Rac1/Nischarin interaction, COS-7 cells co-expressing wild type AU5-Rac1 and Nischarin were treated with EGF. The cells were harvested after 10 min of treatment with EGF. Myc-Nischarin was immunoprecipitated and the precipitates were examined for the presence of AU5-Rac1. Rac1 was detected in the Myc-Nischarin immunoprecipitate after EGF stimulation (Fig. 3). This mimicked the stimulated binding of PAK1 to Rac1 in parallel immunoprecipitations. This EGF-stimulated interaction between Rac1 and Nischarin points to an important physiological role for this interaction.

**Nischarin Regulation of PAK-independent Rac Signaling—** The observation of a PAK-independent interaction between Nischarin and Rac1 suggested that Nischarin may be able to regulate Rac1 functions that are not dependent on PAK for their execution. NIH3T3 cells have been used in conjunction with other cell types for many studies, and NIH3T3 cells are known to lack PAK activity (20). Thus, NIH3T3 cells were used because they lack PAK activity to study the effect of Nischarin on Rac1 activity in the absence of PAK.

**FIG. 1. Nischarin co-precipitates with activated Rac1.** A, COS-7 cells were co-transfected with Myc-tagged Nischarin, PAK1, or GFP and HA-Rac1Q61L or HA-ERK1 expression vectors. Forty-eight hours after transfection cells were lysed in Rac lysis buffer and immunoprecipitated (IP) with a 1:50 dilution of the monoclonal HA antibody. The blot was probed with anti-Myc or anti-HA antibodies as indicated. B, activated Rac1 co-precipitates with Nischarin. COS-7 cells were co-transfected with Myc-Nischarin, Myc-PAK1, or Myc-GFP and HA-Rac1Q61L or HA-ERK1 vectors. Forty-eight hours after transfection cells were lysed in Rac lysis buffer and immunoprecipitated with a 1:100 dilution of the anti-Myc antibody. The blot was probed with anti-HA or anti-Myc antibodies as indicated. C, Nischarin co-precipitates with PAK binding-deficient HA-Rac1Q61L-40C. Myc-Nischarin or Myc-PAK1 and HA-Rac1Q61L or HA-Rac1Q61L-40C vectors were co-transfected into COS-7 cells. The cells were treated and harvested as in A, and Nischarin or PAK1 were immunoprecipitated in parallel with a 1:100 dilution of the anti-Myc antibody. The blots were probed for the Myc and HA epitopes as indicated. D, endogenous interaction of Nischarin and Rac1. Exponentially growing PC12 cells were harvested in Rac lysis buffer + 0.1% Triton X-100. Rac1 was immunoprecipitated with a monoclonal Rac1 antibody from the cleared lysates. An equivalent amount of lysate was incubated with an equal concentration of normal mouse IgG in parallel. Nischarin was detected by immunoblotting with a polyclonal anti-Nischarin antibody.

The C terminus also co-immunoprecipitated with HA-Rac1Q61L, but with reduced efficiency (Fig. 2A). Immunoprecipitation of the Nischarin fragments via the Myc epitope produced a similar binding pattern (data not shown).

The HA-Rac1Q61L-40C mutant exhibited a similar co-immunoprecipitation pattern with Nischarin and its fragments as HA-Rac1Q61L (Fig. 2B). Thus, the formation of Nischarin intracellular complexes with activated Rac1 by either domain of Nischarin does not require the interaction of Rac1 with PAK and the 40C substitution does not alter the interaction of Rac1 with either domain. Additionally, the interaction of Rac1 with the C terminus of Nischarin supports the PAK-independent nature of this interaction because PAK1 exhibited no interaction with the C terminus of Nischarin (20).
with the Rac1Q61L-40C effector domain mutant to identify PAK-independent pathways downstream of Rac1 (16, 21, 22).

One Rac1 pathway that appears to be independent of PAK in NIH3T3 cells is the NF-κB pathway (12, 23, 24). We examined the effect of Nischarin on Rac1 stimulation of NF-κB using a 3×NF-κB response element linked to a luciferase reporter construct, 3×NF-κB-LUC. Expression of activated Rac1Q61L or Rac1Q61L-40C in NIH3T3 cells stimulated the NF-κB response element 5- and 6-fold, respectively (Fig. 4A). An activated mutant of PAK1, PAK1-T423E, which mimics the kinase domain activation loop phosphorylation, was unable to stimulate 3×NF-κB-LUC. This underscores the PAK independence of the NF-κB signaling in NIH3T3 cells. Co-expression of Nischarin with Rac1Q61L or Rac1Q61L-40C suppressed the Rac1-stimulated NF-κB response by 59 and 73%, respectively. Expression of the N terminus also significantly suppressed Rac1Q61L activation of NF-κB by 72% and Rac1Q61L-40C stimulation by 80%. However, expression of the C terminus did not suppress the activation of NF-κB by either form of activated Rac (Fig. 4A). Thus, the N terminus of Nischarin inhibits Rac1-mediated stimulation of the NF-κB pathway independently of PAK function.

Activated Rac1 stimulates cyclin D1 gene expression. This stimulation can occur independently of PAK activation and is mediated in part by NF-κB activation (21, 23, 25). Co-transfection of 963-CD1LUC with Rac1Q61L or Rac1Q61L-40C, respectively, stimulated the cyclin D1 promoter by 10- and 7-fold (Fig. 4B). Expression of PAK1-T423E had little effect on the level of cyclin D1 promoter activity. Co-expression of Nischarin with Rac1Q61L suppressed cyclin D1 promoter activation by 46%. Rac1Q61L-40C-stimulated cyclin D1 promoter activation was inhibited 58% by co-transfected Nischarin. The N terminus of Nischarin suppressed Rac1Q61L stimulation of the cyclin D1 promoter by 73% and it inhibited the Rac1Q61L-40C stimulation by 78%. Co-expression of the C terminus of Nischarin with either activated Rac1 did not suppress the activation of the cyclin D1 promoter (Fig. 4B). Thus, the ability of Nischarin to inhibit the Rac1-stimulated cyclin D1 maps to the N terminus and does not require Rac1 to PAK signaling.

To determine whether the physiological role of Nischarin in Rac signal transduction is to act as a suppressor of Rac1 signaling, the effect of siRNA suppression of endogenous Nischarin on NF-κB activation by Rac1 was examined in PC12 cells. Similar to the observations in NIH3T3 cells, the overexpression of Nischarin in PC12 cells with Rac1Q61L or Rac1Q61L-40C suppressed Rac1 stimulation of the NF-κB pathway by 50 and
Co-transfection of siRNA for Nischarin with either Rac1Q61L or Rac1Q61L-40C in PC12 cells elevated the level of Rac1 stimulation of the NF-κB pathway by 2.2-fold for Rac1Q61L and 1.6-fold for Rac1Q61L-40C compared with the level of NF-κB activation in cells transfected with control siRNA (Fig. 4D). This elevation of the Rac-stimu-
for the vector controls, Myc
co-transfected with Rac1Q61L and c-Raf-C
10:1 and Nischarin or

expression vectors. The focus forming activity of the various combinations were as-
sayed 22–24 days after transfection. The combination of
Rac1Q61L–40C and c-Raf-C
AAX
increased the level of focus
formation by 3.6-fold over background. The presence of Nischa-
rin with Rac1Q61L–40C and c-Raf-C
AAX
stimulated focus formation by Nis-
charin. NIH3T3 cells were transfected
with 1 μg of HA-Rac1Q61L–40C vector, 100 ng of c-Raf-C
AAX
vector, both, or vec-
tor controls (pCGN) and 2 μg of Myc-β-
galactosidase or Myc-Nischarin vector.
The cells were kept at confluence for
22–24 days with media changes every 3–4
days. The transformed foci were visual-
ized after staining with 0.1% crystal vio-
et. The data are from four experiments
performed with triplicate or quadrupli-
cate plates. The data are presented as
mean ± S.E. The level of foci formation
for the vector controls, Myc-β-galactosid-
ase/pCGN, was set equal to 1 (*, p < 0.05,
for Nischarin/HA-Rac1Q61L–40C/c-Raf-
CAAX compared with β-galactosidase/
HA-Rac1Q61L–40C/c-Raf-CAAX; unpaired
Student’s t test).

vesicular structures and in the cytoplasm. The co-localization
did not normally extend to the plasma membrane. The Nischa-
rin staining extended from the perinuclear region and stopped
prior to membrane protrusions, whereas the Rac1Q61L stain-
ing could be found at the plasma membrane. Nischarin did not
localize to the nucleus with Rac1Q61L, which displayed vari-
able levels of nuclear staining (Fig. 6B). A subset of cells did not
exhibit the vesicular structures, but still displayed the strong
cytoplasmic co-localization. Cells expressing both Nischarin
and Rac1Q61L displayed variable phenotypes, but they were
generally intermediate between the Nischarin and Rac1Q61L
alone phenotypes. The expression of Rac1Q61L–40C in NIH3T3
cells induced morphological changes similar to that of
Rac1Q61L with the addition of more filopodial protrusions (Fig.
6A). Nischarin exhibited a similar pattern of co-localization
with Rac1Q61L–40C as that seen with Rac1Q61L. The proteins
were found to co-localize in vesicles and in the cytoplasm with
the co-localization extending from the perinuclear region to just
short of the plasma membrane. As with Rac1Q61L, a portion of
the cells did not exhibit the vesicular structures but still dis-
played the strong cytoplasmic co-localization. The morphologi-
cal changes induced by Rac1Q61L–40C were muted, but not
eliminated, by the co-expression of Nischarin (Fig. 6C).

The localization pattern of the N and C terminus in NIH3T3
cells was examined to determine whether this varied from
Nischarin itself. When expressed alone, the N terminus exhib-
it a staining pattern similar to that of Nischarin. All of the
cells exhibited strong cytoplasmic and weak nuclear staining
with a subset of the cells exhibiting the vesicular structures
similar to full-length Nischarin (Fig. 6A). The C terminus
showed a widespread, diffuse staining pattern with the nucleus
and cytoplasm containing protein. The C terminus never ex-
hibited the vesicular structures seen with Nischarin or its N
terminus (Fig. 6A).

expression of Rac1Q61L or Rac1Q61L–40C with the N
terminus led to a pattern of co-localization similar to that of
full-length Nischarin. Activated Rac1Q61L–40C and the N
terminus displayed cytoplasmic and vesicular co-localization.
The cytoplasmic co-localization did not extend to the plasma
membrane; Rac1 was detected at the membrane in ruffles, but the N
terminus staining stopped prior to reaching the membrane.
The N terminus also did not co-localize with nuclear Rac1 (Fig.
6D, data not shown). Thus, Nischarin and its N terminus,
which suppress Rac1 signaling, exhibit the same pattern of
vesicular co-localization with Rac1.

Expression of the C terminus and Rac1Q61L–40C together
produced a broad pattern of staining. Rac1Q61L–40C and the C
terminus were seen in the nucleus and the cytoplasm, more

Nischarin Regulation of Rac1 Signaling

FIG. 5. Inhibition of Rac1Q61L–40C-
stimulated focus formation by Nis-
charin. NIH3T3 cells were transfected
with 1 μg of HA-Rac1Q61L–40C vector, 100 ng of c-Raf-C
AAX
vector, both, or vec-
tor controls (pCGN) and 2 μg of Myc-β-
galactosidase or Myc-Nischarin vector.
The cells were kept at confluence for
22–24 days with media changes every 3–4
days. The transformed foci were visual-
ized after staining with 0.1% crystal vio-
et. The data are from four experiments
performed with triplicate or quadrupli-
cate plates. The data are presented as
mean ± S.E. The level of foci formation
for the vector controls, Myc-β-galactosid-
ase/pCGN, was set equal to 1 (*, p < 0.05,
for Nischarin/HA-Rac1Q61L–40C/c-Raf-
CAAX compared with β-galactosidase/
HA-Rac1Q61L–40C/c-Raf-CAAX; unpaired
Student’s t test).

Co-localization of Rac1 and Nischarin in NIH3T3 Cells—
The proper cellular localization of Rac1 is important for efficient
transmission of its signal (26–29). Integrin-directed transloca-
tion of activated Rac to lipid-rich rafts in the plasma membrane
appears to be critical for Rac1 signaling (26, 27, 29). Addition-
ally, Rac1 translocation to the endosomal compartment and the
nucleus may be important in the regulation of Rac1 signaling
(29–31).

NIH3T3 cells were transfected with Myc-Nischarin and HA-
Rac1Q61L or HA-Rac1Q61L–40C. Following a time course sim-
ilar to the NF-κB and cyclin D1 regulation experiments, the
localization of Nischarin and Rac1Q61L were examined 2 days
post-transfection. Cells expressing Nischarin alone exhibited
two distinct patterns of staining. In the majority of the cells
Nischarin displayed a diffuse cytoplasmic appearance with mi-
nor nuclear staining. A subset of cells Nischarin displayed a
vesicular staining pattern in addition to the general diffuse
cytoplasmic staining. The cells expressing Nischarin generally
retained their fibroblast phenotype (Fig. 6A). Expression of
Rac1Q61L in NIH3T3 stimulated the classic Rac phenotype of
well spread cells with numerous membrane ruffles. Rac1Q61L
in NIH3T3 cells was found throughout the cytoplasm, the nu-
cleus, some vesicles, and in membrane ruffles (Fig. 6A). Ex-
pression of both Nischarin and Rac1Q61L led to strong co-
localization of the two proteins. They co-localized to numerous

A
A

B
B

C
C

D
D
strongly in the perinuclear region. Additionally, the presence of the C terminus was detected in membrane ruffles with Rac1Q61L-40C. Unlike Nischarin or the N terminus, the non-inhibitory C terminus exhibited no detectable vesicular co-staining with Rac1Q61L-40C in cells co-expressing these proteins. Rac1Q61L exhibited a similar pattern of staining with the C terminus when they were expressed together in NIH3T3 cells (Fig. 6E, data not shown).

**DISCUSSION**

Nischarin appears to be an important modulator of Rac1-regulated signal transduction pathways. Nischarin suppresses Rac1-stimulated migration by directly interacting with the kinase domain of PAK1 and inhibiting the kinase activity of PAK1 (20). In this work, we demonstrate that Nischarin also regulates Rac1 signal transduction pathways that are PAK independent.

Nischarin interacts with Rac1 in its activated state. Both the N and C terminus of Nischarin exhibit the ability to form a complex with active Rac1. The intracellular interaction between Nischarin and Rac1 does not require the interaction of PAK with Rac1 because the PAK binding-deficient mutant, Rac1Q61L-40C, also binds to Nischarin through interactions with both the N and C termini. Activated PAK interacts with Nischarin via multiple contacts in the N terminus, but PAK makes no detectable contacts with the C terminus (20). Thus, the formation of intracellular Rac1-Nischarin complexes, although not absolutely requiring PAK, would likely be facilitated by both PAK-dependent and PAK-independent interactions with Nischarin. Furthermore, the interactions between these three proteins are not mutually exclusive because Nischarin, PAK1, and Rac1 can be co-immunoprecipitated from cells in the presence of activated Rac1, but the interaction of Nischarin with PAK1 or Rac1 does not require the binding of the other protein to Nischarin (20).

In NIH3T3 cells Rac1 has the ability to signal to multiple effector pathways that are not dependent on activation of PAK (16, 21). The activation of NF-κB function and the cyclin D1 promoter positively correlate with cellular transformation by Rac1 (23, 32). Importantly, the activation of these pathways also appears to be important in human cancer (33). Rac1 activation of these pathways in NIH3T3 cells does not require PAK activation (21, 23, 24). The interaction of Nischarin with Rac1Q61L-40C suppresses the ability of Rac1 to stimulate the function of NF-κB, the cyclin D1 promoter, and the formation of foci. Thus, the ability of Nischarin to suppress these pathways does not require Nischarin repression of PAK function. The enhanced Rac1 to NF-κB signal after repression of endogenous Nischarin levels in...
Integrins signal to NF-κB through phosphatidylinositol 3-kinase and Rac1 (34). Rac1, as well as Cdc42 and RhoA, can stimulate the phosphorylation of IkB, an inhibitor of NF-κB function, leading to its degradation and NF-κB activation (35, 36). The phosphorylation of IkB downstream of active Rac1 is mediated by the activation of the IkB kinase β through Rac1 activation of the NF-κB inducing kinase, NIK. In NIH3T3 cells, the Rac-NIK-IkB kinase β pathway appears to be the primary pathway for regulating NF-κB function (12). In other cell types, Rac1 can stimulate NF-κB in a mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase 1-dependent manner or by increasing the level of intracellular reactive oxygen species (37–41). Reactive oxygen species can stimulate IkB kinase β via Src activation and subsequent stimulation of protein kinase D activity via protein kinase Cδ and the Abl tyrosine kinase (42, 43). Activated PAK1 can stimulate NF-κB function, but it alone does not stimulate the activity of IkB kinase α or β (12, 44, 45). However, activated Rac1 with a mutation of tyrosine at residue 40 was able to stimulate NF-κB in NIH3T3 cells (23). In NIH3T3 cells, we observed no stimulation of NF-κB by activated PAK1, whereas Rac1Q61L-40C efficiently stimulated NF-κB function. Thus, disruption of the Rac1 to NF-κB signal by Nischarin does not require PAK, but may involve the pathways described above.

Rac1 plays a central role in mediating adhesion-dependent signaling to cyclin D1 (25). In NIH3T3 cells, activated Rac1 stimulates cyclin D1 transcription via activation of NF-κB through cooperative interactions at a NF-κB and an ATF-2 binding site in the cyclin D1 promoter. An activated Rac1 with a mutated tyrosine at residue 40 was able to stimulate NF-κB in NIH3T3 cells (23). In NIH3T3 cells, we observed no stimulation of NF-κB by activated PAK1, whereas Rac1Q61L-40C efficiently stimulated NF-κB function. Thus, disruption of the Rac1 to NF-κB signal by Nischarin does not require PAK, but may involve the pathways described above.

The inhibition of Rac1 function maps to the N terminus of Nischarin. However, both Rac1Q61L and Rac1Q61L-40C form complexes with both the N and C terminus of Nischarin. The difference between these interactions with Rac1 may be their differential cellular localization. Nischarin and the N terminus exhibited similar patterns of co-localization with Rac1. Both were found with Rac1 in the cytoplasm and vesicles. They exhibited no co-localization with Rac1 in the nucleus. This localization was not dependent on PAK because both Rac1Q61L and Rac1Q61L-40C displayed a similar co-localization. The C terminus exhibited a diffuse cytoplasmic and nuclear localization when present with both forms of activated Rac1. There was not any strong vesicular co-localization with the C terminus.

The cellular localization of Rac is critical for its function. The translocation of active Rac1 to the plasma membrane facilitates the transduction of Rac1 signals to its effectors. Rho-GDI bound to Rac1 in the cytoplasm inhibits its signaling. Activated integrins direct translocation of Rac1 to cholesterol-rich, lipid raft signaling complexes at the plasma membrane where Rac1 can signal. Internalization of these Rac1 containing lipid raft signaling complexes dampen the Rac1 signal (26, 27). Disruption of macropinocytotic internalization of Rac1 with a dominant negative dynamin-2 leads to aberrant localization of active Rac1 and inhibition of lamellipodia formation (29). Additionally, movement of Rac1 to the endocytic compartment in an Arf6-dependent manner can repress the induction of membrane ruffles of Rac1 (30). Interestingly, recent investigations indicate that active Rac1 stimulates the translocation of the IkB NF-κB complex to the membrane. This membrane localization of IkB/NF-κB facilitates activation of NF-κB through IkB degradation (46). The human Nischarin homolog, IRAS, has been recently demonstrated to co-localize to early/spotting and recycling endosomes and was postulated to be a sorting nexus. This localization was dependent on a Phox domain and a coiled-coil domain in the N terminus of IRAS. Elevated levels of IRAS shifted α5 integrin from the membrane to the endosomal compartment (47). Nischarin lacks the Phox domain, but retains a coiled-coil domain at aa 380–405 within the N terminus (48, 49). In NIH3T3 cells, this coiled-coil domain of Nischarin, and the N terminus, may be sufficient for the formation of vesicles. The co-localization of active Rac1 to these vesicles did not require Rac1 interaction with PAK because the Rac1Q61L-40C was also found to co-localize here. The vesicular co-localization may contribute to the suppression of the Rac1 signal in the presence of Nischarin or its N terminus by altering Rac1 cellular localization as described above. The absence of vesicular co-localization of the C terminus and Rac1 and the lack of an effect of the C terminus on Rac1 signaling further suggests that co-localization in these vesicular structures are important for the negative regulation of Rac1 signaling by Nischarin.

Nischarin represses Rac1 signal transduction pathways by PAK-dependent and -independent mechanisms. The ability to repress the PAK arm of the Rac1 signal transduction cascade results from the direct association of Nischarin with PK. The repression of the PAK-independent signals may result from Nischarin altering the cellular localization of activated Rac1. Additionally, suppression by Nischarin of other Rac1 effector pathways may be mediated by the disruption of Rac1 interactions with other effector molecules or the interaction of Nischarin with and modulation of the function of other Rac1 effectors.

Acknowledgments—We thank Suresh K. Alahari for reagents and advice, Yuko Miyamoto for assistance with confocal microscopy, and Michael Fisher for technical support.

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