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An Experimentally Derived Database of Candidate Ras-Interacting Proteins

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Abstract

We used a TAP-tag approach to identify candidate binding proteins for the related Ras family GTPases: H-Ras, R-Ras, and Rap1A. Protein complexes were isolated from mouse fibroblasts, and component proteins were identified by a combination of nanoflow HPLC and tandem mass spectrometry. H-Ras was found to associate with numerous cytoskeletal proteins including talin-1. R-Ras and Rap1A each associated with various signaling molecules, many of which are membrane-associated. Thus, we have established the first database of potential Ras interactors in mammalian cells.

Keywords

proteomics; mass spectrometry; GTPases; Ras

Introduction

Ras small GTPases comprise a large family of related signaling molecules that mediate a diverse array of cellular functions. Like all GTPases, the activity of Ras proteins is regulated by binding to guanine nucleotides. Ras GTPases are activated by Ras guanine nucleotide

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Supporting Information Available: Supplementary Figure 1. This material is available via the Internet at <http://pubs.acs.org>. The complete compilation of candidate Ras interactors can be found at http://www.cellmigration.org/resource/discovery/proteomics/ginsberg_ras_data.shtml. Each protein in the table was identified from MS/MS spectra of tryptic peptides by both a database search with SEQUEST and manual interpretation of the individual spectra. Each identified protein is shown as SEQUEST protein match hyperlinked to the NCBI record and to Swiss-Prot and Interpro records, followed by the number of experiments in which that protein was identified, and corresponding expectation values. The Microsoft Excel spreadsheet lists all validated proteins arranged by Ras isotype used as bait, and includes cellular component, molecular function and biological process designations derived from the Swiss-Prot pages. For proteins in H-Ras pull-downs, spot numbers correspond to spots cut from two-dimensional gel electrophoresis of TAP-purified H-Ras pull-down samples (shown) from independent experiments, identified by MS sequencing of the spots.

exchange factors (RasGEFs), which mediate GTP loading of Ras. GTP-bound Ras proteins then undergo a conformational rearrangement that exposes effector-binding regions referred to as switch 1 and 2. Conversely, these proteins are inactivated by Ras GTPase Activating Proteins (RasGAPs), which speed the conversion of Ras to the GDP-bound, inactive state. Activating mutations in Ras which induce constitutive GTP binding are found in 30% of human cancers, underscoring the importance of Ras regulation in maintenance of normal cellular growth.¹

Evolutionary divergence among the members of this super-family has created a wide array of functional capabilities among closely related paralogues. For example, R-Ras is 55% identical to H-Ras,² with near-complete identity to the conserved effector binding region of H-, K-, and N-Ras and Rap1. Ras isotypes have different effects on tumorigenesis, activation of specific transcription factors, and cell adhesion.^{3–6} Activated H-, N-, and K-Ras activate Raf-1 and, therefore, the ERK/MAP kinase pathway. In contrast, neither R-Ras nor Rap1 activates Raf-1. This may explain why Rap1 and R-Ras are less active at inducing cellular transformation than their close paralogues.^{5,7–10} Furthermore, closely related paralogues can differ in effects on cell adhesion. In particular, an activated variant of H-Ras (H-Ras(G12V)) suppresses activation of some integrins through its effector, Raf-1,¹¹ and downstream activation of ERK1/2,¹² whereas activated R-Ras (R-Ras(G38V)) reverses H-Ras-induced suppression^{13,14} through a pathway that involves unknown R-Ras effectors.^{4,15} Rap1 seems to be a general activator of integrins; however, Rap1(G12V) does not reverse H-Ras-mediated suppression (unpublished data).¹⁶ In addition, R-Ras activates integrins in some cells via Rap1, indicating that these GTPases can cross-talk.^{5,17} Thus, Ras isotypes signal through distinct pathways, suggesting that unique effectors exist for each isotype.

Few Ras isotype-specific effectors have been identified, possibly because these Ras paralogues exhibit remarkable sequence identity in their effector-binding switch 1 and 2 regions.² Genetic and yeast two-hybrid approaches have had limited success and typically have yielded a small number of known effectors shared among the Ras proteins, such as Raf-1, RalGDS, RapL/NORE1, and PI3-kinase.¹⁸ Post-translational processing and a hypervariable region in the C-terminal residues in Ras GTPases direct their sub-cellular localization in mammalian cells, and this process plays an essential role in their functions.⁶ However, these specific localization signals are missing in the yeast two-hybrid system in which the protein–protein interaction takes place in the nucleus. Furthermore, yeast two-hybrid systems detect only direct interactions. Proteins that associate indirectly with Ras GTPases may have an important role in Ras signaling. It has therefore been difficult to identify isoform-specific effectors through methods that detect only binary interactions and that do so out of the normal cellular microenvironment.

We have developed a rapid, efficient method for isolating and identifying potential Ras family effectors in mammalian cells. This technique uses the tandem affinity purification tag (TAP tag) approach, originally pioneered by Séraphin and colleagues in yeast.^{19,20} We have adapted the yeast protocol and optimized it for mammalian cells. To do this, we generated TAP fusions at the N-termini of Ras cDNAs in the pEF4 vector. The N-terminal TAP fusion allows for appropriate C-terminal isoprenylation of Ras when expressed in fibroblasts, and N-terminal substitution on Ras proteins generally does not perturb sub-cellular localization

or function.^{21,22} We expressed TAP fusions of constitutively active and dominant negative variants of H-Ras, R-Ras, and Rap1A in mouse fibroblasts and isolated Ras protein complexes. Protein components of each complex were identified by nanoflow HPLC coupled to tandem mass spectrometry. These experiments identified numerous shared or isoform-specific signaling proteins with each Ras variant. These results comprise the first database of candidate Ras interactors in mammalian cells.

Materials and Methods

Cell Lines, Complementary DNAs, Transfections, Reagents, and Recombinant Proteins

NIH 3T3 cells were from ATCC (Rockville, MD). CHO cells stably expressing constitutively active chimeric integrins ($\alpha\beta$ -py) were as described.¹¹ The N-terminal TAP plasmid was a generous gift of Dr. Bertrand Séraphin (EMBL, Heidelberg).¹⁹ TAP-Ras fusion constructs were generated by subcloning the TAP tag into pEF4 plasmids (Invitrogen, Carlsbad, CA) containing human Ras variants⁴ with BamHI/XbaI (New England Biolabs, Beverly, MA). A plasmid encoding FLAG-tagged human Abi1 was the generous gift of Ann Pendergast (Duke University, Durham, NC). pEGFP-C1 was from Clontech (Mountain View, CA). The PEA-15 plasmid was as described.²³ Cells were transfected using Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. GST-talin-F23 was generated as described in Calderwood, et al.²⁴ Polyclonal antibodies to RalGDS and p110 subunits of PI3-kinase, and monoclonal antibodies to Raf-1, were from Santa Cruz Biotechnology (Santa Cruz, CA).

Tandem Affinity Purification of TAP-Ras Fusions

NIH 3T3 cells (5×10^8) were transfected with TAP-Ras fusion constructs. After 48 h, cells were harvested by scraping into TEVCB (20 mM Tris-Cl, pH 7.4, 50 mM NaCl, 2 mM $MgCl_2$, 0.5% NP-40, protease inhibitor cocktail (Roche, Indianapolis, IN), 10 μ M GTP) plus 1 mM EGTA. Cell lysates were cleared of insoluble material by centrifugation and incubated with Ni^{++} -coupled sepharose beads (Roche) for 1 h at 4 °C. Beads were pelleted by centrifugation and washed 3 $\times$ in lysis buffer containing 40 mM imidazole. Protein complexes were eluted by mixing for 15 min with CBB (lysis buffer without EGTA and containing 400 mM imidazole and 2 mM $CaCl_2$) at 4 °C. TAP complexes were recaptured from Ni^{++} eluates onto calmodulin-coated sepharose beads (Stratagene, La Jolla, CA) by mixing for 1 h at 4 °C. Beads were pelleted by centrifugation and washed 3 with CBB. Purified protein complexes were eluted with 200 μ L of lysis buffer containing 10 mM EGTA but without detergent by mixing for 1 h at 4 °C.

Mass Spectrometry, Two-Dimensional Gel Electrophoresis, and Database Search

TAP-purified samples were treated with 20 μ L of 200 mM $CaCl_2$ and 20 μ L of 100 mM ammonium bicarbonate, followed by addition of 1 μ L of DTT (20 mM final) for 1 h at 52 °C and then 1 μ L of iodoacetamide (40 mM final) for 1 h at room temperature in the dark. Samples were proteolyzed by addition of 1 μ L of modified trypsin (0.5 μ g; Promega, Madison, WI) and incubation at 37 °C for 9 h. The digest was quenched with 2 μ L of glacial acetic acid, and digested samples were loaded onto a C18 column. Peptides were analyzed by nanoflow reverse phase high performance liquid chromatography micro-electrospray

tandem mass spectrometry (RP-HPLC/ μ ESI/MS/MS) interfaced with Finnigan LTQ or LCQ mass spectrometers (Thermo Electron Corp., Waltham, MA). Peptides were gradient-eluted using a linear gradient of 0–60% B in 120 min (A = 0.1 M acetic acid in NANOpure water, B = 70% acetonitrile in 0.1 M acetic acid). The LTQ MS was operated in a data-dependent top 10 MS/MS mode. The data were then searched against the current mouse GenBank protein database compiled by NCBI (<http://www.ncbi.nlm.nih.gov/Sitemap/ResourceGuide.html>), using the SEQUEST search algorithm (version 27).²⁵ Peptide sequence assignments were verified by manual interpretation of MS/MS spectra. Two-dimensional gel electrophoresis was carried out according to the methods of Görg et al.,²⁶ followed by silver staining, in-gel trypsinization, and peptide sequencing by LC–MS/MS.^{27,28}

Integrin Activation

CHO cells expressing constitutively active chimeric integrins¹¹ were transiently transfected with pEGFP and other plasmids as indicated. Integrin activation was assessed by two-color flow cytometry, in which PAC1 antibody binding was measured in a subset of cells gated for GFP expression.²⁹

Talin/H-Ras Affinity Chromatography

CHO cells were transfected with TAP-Ras constructs, lysed in MLB (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% NP-40, 0.25% NaDOC, 10% glycerol, 10 mM MgCl₂, 1 mM EDTA, protease inhibitors) and cleared by centrifugation. Cell lysate (500 μ g) was incubated with GSH-sepharose beads coated with 20 μ g of recombinant talin proteins^{24,30} or GST for 2 h at 4 °C. Beads were washed 6 $\times$ with lysis buffer and solubilized in SDS buffer, followed by Western blotting with antibodies recognizing the TAP tag (α -MLCK_{sk}) and GST (Santa Cruz Biotechnologies).

Results and Discussion

We expressed N-terminal TAP-tagged Ras constructs in NIH 3T3 cells by cationic liposome-mediated transfection, and optimized a purification scheme. The modified TAP tag consists of a poly-histidine tag and a protein A repeat region at the N-terminus, followed by a hexapeptide TEV protease recognition sequence, and a calmodulin-binding domain (CBD) (Figure 1A). Fusion of the TAP tag to the N-terminus had no effect on the ability of H-Ras to suppress integrin activation in CHO cells or on the ability of R-Ras to rescue suppression by H-Ras in cells (Figure 1B). These data indicate that the addition of the TAP tag does not compromise Ras functions *in vivo*. The published TAP protocols begin with initial isolation of protein complexes from lysates with IgG-coupled sepharose, followed by cleavage of the bead-bound complexes with TEV protease, releasing the protein complexes into the soluble fraction. The complexes are further purified by a recapture step onto calmodulin-coated beads, followed by specific elution by Ca⁺⁺ chelation with EGTA.^{19,20} In preliminary experiments with mouse NIH 3T3 fibroblasts, we expressed TAP-tagged Ras variants and performed initial purification from cell lysates under nondenaturing conditions using IgG-sepharose. This step produced a high yield of the tagged bait protein (not shown). However, subsequent treatment with recombinant TEV pro-tease resulted in a secondary

cleavage within the TAP-Ras protein, either by offsite recognition by the TEV protease itself or by activation of other endogenous proteases present in the cell lysate (not shown). To obviate this problem, we modified the TAP system by taking advantage of the poly-histidine tag at the N-terminus. The modified purification scheme entails initial capture on Ni⁺⁺-coated beads from a cell lysate, followed by specific elution with imidazole. This procedure generates a similar array of proteins as TEV cleavage when viewed by silver stain, suggesting that elution with imidazole provides roughly the same degree of purification as elution with the TEV protease (not shown). The enriched protein eluate fractions were then recaptured on calmodulin-coated beads and processed as described above, yielding a highly purified fraction (Figure 2A). The entire purification procedure from lysis to EGTA elution can be performed in less than 4 h. Approximately 50–70% of the bait protein from the cell lysate was typically recovered in the final fraction, resulting in roughly 150-fold enrichment (Figure 2B). We have used this purification scheme to isolate complexes of endogenous proteins potentially interacting *in vivo* with Ras family members bearing mutations which render them constitutively active (GTP-bound) or inactive (nucleotide-free). As an additional control, we expressed and isolated the TAP tag protein alone. The protein components of each complex were then identified by nanoflow reverse-phase high-performance liquid chromatography micro-electrospray tandem mass spectrometry followed by analysis of the resulting MS/MS spectra by manual interpretation and with a database search with SEQUEST.^{25,31} The TAP screens identified many proteins, in complex with each Ras isotype, which did not bind to the TAP tag alone or to inactive Ras. However, manual interpretation of the MS spectra indicated that only approximately 35% of SEQUEST identifications were correct in each case. We therefore generated a table that contains only those proteins identified by manual interpretation of MS/MS spectra recorded on tryptic peptides. The result is a compilation of putative specific and shared effectors of activated H-Ras, R-Ras, and Rap1A. This data is accessible at http://www.cellmigration.org/resource/discovery/proteomics/ginsberg_ras_data.shtml. All entries in this database were not detected in TAP isolations of the TAP tag protein or of TAP fusions of dominant negative variants of the respective Ras isotypes. Highlights of this database are shown in Table 1.

TAP-tagged Ras GTPases pulled down mixtures of proteins having a wide range of structural and functional properties. There were 21, 28 and 222 proteins identified in pull-downs with TAP-tagged R-Ras(G38V), Rap1A(G12V), and H-Ras(G12V), respectively (Table 1). The large number of proteins observed in the TAP-tagged H-Ras sample is probably the result of using a Finnigan LTQ instrument for this analysis. The LTQ operates with a 50-fold increase in dynamic range and is a factor of 10× more sensitive than the LCQ instrument employed for the analysis of the other samples. Many of the proteins in the H-Ras(G12V) sample were also detected by two-dimensional gel electrophoresis. In-gel digestion and sequence analysis of the resulting tryptic peptides were employed to confirm the identities of proteins in numerous gel spots (Table 1 and http://www.cellmigration.org/resource/discovery/proteomics/ginsberg_ras_data.shtml). Pull-downs with TAP-tagged R-Ras and H-Ras contained three proteins that were common to both samples. Samples from TAP-tagged H-Ras and Rap1A contained twenty proteins in common. In contrast, there was no overlap of the proteins observed in pull-downs with TAP-tagged R-Ras and Rap 1A.

When Rap1A was employed as bait, proteins detected in the pull-down included multiple membrane-targeted signaling molecules, including ENA, VASP and Gab-2, kinases, phosphatases, and several ATPases. VASP binds to the Rap1A effector RIAM,³² suggesting a tri-molecular complex, although RIAM was not detected in this screen.

Among the proteins observed in pull-downs only with R-Ras as bait were several potential direct interactors such as Gapvd1, and Abi1. Abi1 may interact via SH3 domains with the unique proline-rich sequence at the N-terminus of R-Ras. We confirmed an Abi1 interaction with R-Ras by coprecipitation. FLAG-tagged Abi1 formed a complex in cells with R-Ras(G38V) but did not interact with R-Ras(T43N) or with activated H-Ras or Rap1A (Figure 3). R-Ras induces cell spreading by promoting actin polymerization,^{5,33,34} and Abi1 participates in actin remodeling through its association with WAVE.³⁵ Our identification of Abi1 as an interactor of activated R-Ras suggests a functional connection between Abi1 and R-Ras' effects on the cytoskeleton. We also detected RalA-binding protein 1 (RalBP1/RLIP76) as an R-Ras-specific interactor. We have recently shown that RLIP76 binds directly to activated R-Ras to mediate cell spreading and migration, but RLIP76 does not interact with H-Ras or Rap1A. We further demonstrated that RLIP76 potentiates these functions of R-Ras by regulating a GTPase cascade of Arf6 activation leading to activation of Rac1.³⁶ Thus, we have characterized a signaling pathway connecting a newly identified effector, RLIP76, to the cellular functions of R-Ras.

With H-Ras as bait, we detected proteins involved in protein processing and scaffolding, and a large group of proteins associated with the cytoskeleton (Table 1). Talin-1 is a member of this latter group. To validate this surprising result, we used affinity chromatography to confirm that activated H-Ras but not inactive H-Ras binds talin through the F23 region of the talin head domain (Figure 4). The head domain is the region of talin that binds to and activates integrins,³⁰ suggesting a possible connection with the ability of H-Ras to modulate integrin activation.¹¹ Thus, we have validated several interactions and demonstrated Ras isotype specificity as indicated by the TAP pull-downs and mass spectrometry.

Surprisingly, although a known direct interactor of active H-Ras, galectin-1, was detected in the H-Ras pull-down,³⁷ several other known direct binding partners of Ras such as Raf-1, RalGDS, and PI3-kinase were not detected in the pull-downs. This may be due to masking of tryptic peptides derived from these proteins by coeluting peptides. Indeed, Raf-1, RalGDS, and PI3-kinase (p110 subunits) were observed in TAP-purified pull-downs of activated but not nucleotide-free H-Ras, R-Ras and Rap1A by immunoblotting (Supplementary Figure 1, see Supporting Information). Hence, in this screen, as is usual, absence of evidence is not evidence of absence. However, the TAP method successfully identified novel effectors which may contribute to the pleiotropic effects of this gene family. In fact, we have characterized a signaling pathway connecting an R-Ras effector identified in this screen, RLIP76, to R-Ras regulation of cell spreading and migration.³⁶ Thus, this proteomic screen establishes a database of candidate Ras-interacting proteins in mammalian cells, which should be useful in understanding the biology of this important class of signaling molecules.

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Figure 1.

(A) Domain structure of TAP-Ras constructs (R-Ras is shown). CBD, calmodulin-binding domain; PP, proline-rich sequence; SW1/2, switch1/2; TEV, TEV protease cleavage site; *, palmitoylation site;³⁸ ¥, methylation and geranylgeranylation site;³⁹ canonical CAAX sequence shown in red. (B) Regulation of integrin activation by TAP-Ras fusion proteins. Integrin activation was measured in CHO cells expressing constitutively active chimeric integrins. TAP-tagged H-Ras(G12V) suppressed integrin activation in GFP-positive cells (relative to the activation state in control GFP-transfected cells, set to 0). Activated R-Ras with or without a TAP tag fusion or PEA-15 (control) rescued H-Ras-mediated suppression. Data are shown +SEM for two independent experiments.

Figure 2.

(A) Ras purification strategy. TAP-Ras-transfected cells were lysed in NP-40 buffer. TAP-Ras protein complexes were enriched on Ni⁺⁺-sepharose beads, followed by elution with imidazole, recapture on calmodulin-sepharose beads, and final elution with EGTA. Purified protein complexes were digested with trypsin and subjected to LC-MS/MS analysis and protein identification using a combination of manual interpretation of MS/MS spectra and the search algorithm, SEQUEST. (B) Silver stain (top) and immunoblot with antibodies to the TAP tag (bottom). The purified fractions contained the TAP-R-Ras protein, and endogenous proteins which did not co-purify with the isolated TAP tag. These proteins were assigned as putative R-Ras interactors. M, molecular weight marker; S, sample.

Figure 3.

Interaction of Abi1 and activated R-Ras. FLAG-tagged Abi1 was coexpressed with TAP-tagged R-Ras(G38V), R-Ras-(T43N), H-Ras(G12V) or Rap1A(G12V). TAP-Ras proteins were precipitated from cell lysates with IgG-coupled sepharose beads, and bead-bound proteins were visualized by Western blotting with anti-FLAG and anti-TAP antibodies.

Figure 4.

Interaction of activated H-Ras and talin-F23. GST-talin-F23 domain fragment protein (GST-F23) or GST was coupled to GSH-sepharose and incubated with lysates of cells expressing TAP-tagged H-Ras, R-Ras, or Rap1A bearing the indicated mutations. Bead-bound Ras proteins were visualized by immuno-blotting with TAP tag antibodies (TAP); GST-F23 was visualized with GST antibodies.

Table 1

Summary of Candidate Ras-Interacting Proteins

Rap1a	R-Ras	H-Ras
ATP synthase α , β , γ , δ , O subunits	14-3-3 ζ	ATP synthase α subunit
Actin, alpha cardiac	Abi1 (Abl-interactor 1) ^{b,d}	Actin, alpha cardiac
Elongation factor 2	Formin 1 isoforms I/II/III	Elongation factor 2
GRB2-associated binding protein 2 ^c	GTPase activating protein and VPS9 domains 1 ^b	GRB2-associated binding protein 2 ^c
Myosin light polypeptide 6	Oxysterol-binding protein-like protein 3	Myosin light polypeptide 6
Tubulin alpha-3/alpha-7 chain	Oxysterol-binding protein-like protein 7	Tubulin alpha-3/alpha-7 chain
Tubulin, beta 5	RalA-binding protein/RLIP76 ^{b,d}	Tubulin, beta 5
Enabled protein homolog	RalBP1 associated Eps domain-containing protein ^c	Zyxin
Serine/threonine protein phosphatase PP1- β	Tubulin alpha-6 chain	Galectin-1 ^b
Vasodilator-stimulated phosphoprotein ^c	Peroxisomal protein 4	Peroxisomal protein 4
		Talin 1 ^d
		14-3-3 protein γ
		Actinin α 4
		Mitogen activated protein kinase 1 ^c
		ARP2
		Chaperonin subunit 2 (β)
		Chaperonin subunit 3 (γ)
		Afadin DIL domain-interacting protein ^c
		LIM and SH3 protein 1

^a All listed proteins were identified only in association with the indicated GTPase.

^b Putative direct Ras-interacting proteins.

^c Putative Ras effector-interacting proteins.

^d All mass spectroscopic identifications were manually confirmed. The indicated interactions were also confirmed in independent co-precipitation and immuno-blotting experiments.