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Protein binding and signaling properties of RIN1 suggest a unique effector function

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ABSTRACT **Human RIN1 was first characterized as a RAS binding protein based on the properties of its carboxyl-terminal domain. We now show that full-length RIN1 interacts with activated RAS in mammalian cells and defines a minimum region of 434 aa required for efficient RAS binding. RIN1 interacts with the "effector domain" of RAS and employs some RAS determinants that are common to, and others that are distinct from, those required for the binding of RAF1, a known RAS effector. The same domain of RIN1 that binds RAS also interacts with 14-3-3 proteins, extending the similarity between RIN1 and other RAS effectors. When expressed in mammalian cells, the RAS binding domain of RIN1 can act as a dominant negative signal transduction blocker. The amino-terminal domain of RIN1 contains a proline-rich sequence similar to consensus Src homology 3 (SH3) binding regions. This RIN1 sequence shows preferential binding to the ABL–SH3 domain *in vitro*. Moreover, the amino-terminal domain of RIN1 directly associates with, and is tyrosine phosphorylated by, c-ABL. In addition, RIN1 encodes a functional SH2 domain that has the potential to activate downstream signals. These data suggest that RIN1 is able to mediate multiple signals. A differential pattern of expression and alternate splicing indicate several levels of RIN1 regulation.**

RAS is a membrane-associated small G protein that is indirectly coupled to both receptor and nonreceptor tyrosine kinases. RAS activation is regulated at the level of guanine nucleotide binding: the GTP- and GDP-bound states of RAS are distinct in their structures (1, 2) and in their capacity to dispatch downstream signals through effector proteins. The interactions between RAS and its effectors require both the GTP-dependent structural confirmation of RAS and the presence of particular RAS residues including a short sequence referred to as the "effector domain." Some effector pathways can lead to cell transformation and tumorigenesis when constitutively activated by a mutant RAS that is unregulated and predominantly in the active form.

The first identified and best characterized effector of mammalian RAS is RAF1. Interaction with RAS leads to events that stimulate the protein kinase activity of RAF1 (3–5). Other potential RAS effectors have now emerged (reviewed in ref. 6). These include PI3 kinase (7), RAL-GDS (8–10), and RIN1 (11). Although these proteins do not share obvious sequence commonality, they each bind RAS in a manner that is conditional upon RAS activation (GTP binding) and an intact effector domain. In the case of RAF1, expression of the RAS-binding domain (RBD) alone has been shown to block normal RAS signaling (12, 13), presumably due to a nonproductive interaction with RAS that blocks access to wild-type RAF1 and other effector molecules.

For RAF1, binding to activated RAS may also involve 14-3-3 proteins. These small acidic proteins have been found to associate with RAF (14–16), PI3 kinase (17), BCR/ABL (18), and other signaling molecules with functions directly or indirectly dependent on RAS. Although 14-3-3 proteins do not appear to directly stimulate enzymatic activity, they may facilitate protein interactions that lead to activation. 14-3-3 proteins also exist as dimers that may act as bridges between signaling molecules (19) or may induce activation through oligomerization (5).

Also fundamental in eukaryotic signal transduction are SRC homology (SH) sequences. SH2 and SH3 domains are structural units instrumental in the assembly of signal transduction protein complexes and in the regulation of nonreceptor tyrosine kinases such as SRC and c-ABL (reviewed in ref. 20). In some cases, as with SRC, SH2- and SH3-based interactions with "adaptor" proteins can serve as a link to the RAS activation machinery. In other cases, such as c-ABL, these domains are essential for function, but downstream effectors have not been identified.

The carboxyl-terminal domain of RIN1 can suppress an activated RAS allele in yeast (21) and was later shown to bind RAS protein *in vitro* (11). We now show that RIN1 binds to activated RAS *in vivo*. The binding specificity is distinct, however, from that of RAF1 for RAS. Interestingly, the RBD of RIN1, like that of RAF1, can bind to 14-3-3 proteins. In addition, overexpression of the RIN1 RBD in mammalian cells leads to a block in RAS-mediated signaling. We also report that the amino-terminal domain of RIN1 contains sequences that can mediate interactions with the ABL tyrosine kinase and that RIN1 is itself tyrosine phosphorylated by c-ABL. These observations suggest that RIN1 is involved in multiple signal transduction pathways and might provide a direct link between RAS and tyrosine kinase-mediated signals.

MATERIALS AND METHODS

Plasmids and Viruses. Human *RIN1* cDNA clones were isolated from a U118-MG cell library (21). Rat *Rin1* clones were isolated from a genomic library (kindly provided by the late Robert Anderson, University of California, Los Angeles) and a brain cDNA library (Stratagene). PCR was used with human and rat 5′-RACE-Ready cDNAs (CLONTECH). Full-length *RIN1* cDNA was generated through PCR, digestions, and ligations (all PCR-generated material was sequenced). A *Sal*I site was added before the initiation codon of full-length

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Abbreviations: RBD, RAS binding domain; SH, SRC homology; HA, hemagglutinin; MBP, maltose binding protein; GST, glutathione *S*-transferase.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. for human and rat RIN1 are L36463 and U80076, respectively).

RIN1. A 0.8-kb *Kpn*I–*Bam*HI fragment from cDNA clone 99X4, which encodes the 62-codon deletion form, replaced the corresponding fragment of *RIN1* in *RIN1*Δ clones. pBTM117 (11) digested with *Sal*I and *Not*I was used to create LexA fusions. pLexA–*RIN1* was made from the 0.9-kb *Sal*I–*Kpn*I and the 1.6-kb *Kpn*I–*Not*I *RIN1* fragments. pLexA–*RIN1N* (RIN1 amino terminus) was made from the same 0.9-kb *Sal*I–*Kpn*I fragment and a *Kpn*I–*Not*I adaptor. pGST–*RIN1N* was made in the same way using a modified pGEX-2T (11). pGST–*RIN1N*Δ was created by digesting pGST–RIN1N with *Hin*dIII and *Kpn*I followed by ligation with an adaptor oligonucleotide. pcDNA3 (Invitrogen), digested with *Eco*RI and *Not*I, was ligated to the 0.9-kb *Eco*RI–*Kpn*I and the 1.64-kb *Kpn*I–*Not*I fragments of RIN1 from pLexA–*RIN1*. pcDNA3–*RIN1N* was made similarly but using a *Kpn*I–*Not*I adaptor oligonucleotide. pcDNA3HA–*RIN1C* was made by inserting a hemagglutinin (HA) epitope into the *Bam*HI site of pcDNA3 and then inserting the 1-kb *Nhe*I–*Bam*HI and 0.7-kb *Bam*HI–*Not*I fragments of pAD54–*RIN1C*, a yeast expression plasmid. To create retrovirus constructs, *Eco*RI linkers were added to the ends of the *Eco*RI–*Xba*I fragments of *RIN1*, *RIN1N*, and HA–*RIN1C* released from pcDNA3 constructs and cloned into pSRαMSVtkneo (22) using a unique *Eco*RI site. Retroviruses were made as described (23). *RIN1* was cloned into the *in vitro* transcription/translation vector pSP64–XβSN (11) with the same strategy used for cloning into pBTM117. Deletions of the RIN1 carboxyl terminus were made by PCR and cloning into pSP64–XβSN or pSP64–XβM (24). pMBP–14-3-3ε contains the epsilon isoform of murine 14-3-3 fused to maltose binding protein (MBP). It was made by inserting a *Bgl*II (Klenow blunted) to *Eco*RI fragment from the library isolate of 14-3-3ε into pPR997 (New England Biolabs) digested with *Sal*I (Klenow blunted) and *Eco*RI.

Two-Hybrid Interactions. Yeast strain L40 (25) was transformed with pLexA–*RIN1C* and a WEHI cell GAL4 activation domain cDNA fusion library (26) generously provided by Steven Goff (Columbia University). Transformants (6×10^6) were subjected to selection on synthetic media lacking tryptophane (pLexA–*RIN1C* marker), leucine (library marker), and histidine (two-hybrid reporter marker). This was followed by colony lifts and LacZ (two hybrid reporter) assays. From 3,000 His^+LacZ^+ colonies, 288 were examined by plasmid segregation and reanalysis following mating to cells with pBTM117 or pLexA–*RIN1C*. From 41 potential RIN1 RBD interactors, plasmid isolation and retesting yielded 4 groups (including 7 independent isolates of 14-3-3ε) and 4 individual clones. RAS effector mutant interactions were done in L40 with pGAD–RIN1 (11), –RAF1 (27) or –RalGDS (28) with pBTM116–RAS variants (27, 28).

Immunoprecipitation and Western Blotting. Immunoprecipitation of the RAS–RIN1 complex was done by a modification of previously described methods (29). To overexpress RIN1 proteins, NIH 3T3 cells with or without an activated RAS allele ($H\text{-}RAS^{61L}$, kindly provided by Adrienne Cox, University of North Carolina) were infected with RIN1-expressing retrovirus. Cells (3×10^5) were plated on each 100-mm dish 6–10 h before infection. Mock infections were done with virus collection medium (1× Iscove's medium/10% fetal bovine serum). Cells were grown 3 days in DMEM with 10% calf serum, rinsed with cold PBS and lysed in 1 ml cold Nonidet P-40 lysis buffer (29). Lysates were incubated 10–30 min on ice and centrifuged at $16{,}000 \times g$ for 15 min. Anti-Ras antibody Y13-238 (Oncogene Science) or Y13-259 (Calbiochem) was then added to the supernatants, and immune complexes were precipitated. In some cases, Y13-238 was neutralized by pre-incubation for 30 min with 20 μg GST-RAS^{V12} (Y238*). Anti-RAS and monoclonal anti-RIN1 (Transduction Laboratories, Lexington, KY) were used to detect RAS and RIN1, respectively, in Western blotting (It should be noted that a prominent 65-kDa band has been misidentified as RIN1 in Transduction Laboratory literature.) For "reverse" coimmunoprecipitation, 3T3 cells with $H\text{-}RAS^{61L}$ were infected with an HA-RIN1C expressing retrovirus. Monoclonal antibody to the HA epitope (12CA5) was conjugated to protein A agarose beads (Pierce) and used to precipitate the RIN1C–RAS complex. Immunoprecipitation of HA-RIN1C–14-3-3 complexes was done as described above using 12CA5. Antibody K-19 (Santa Cruz Biotechnology) was used to detect 14-3-3 proteins in Western blotting.

***In Vitro* Binding and Kinase Assays.** *In vitro* RIN1–RAS and RIN1–14-3-3ε binding experiments were done as described (11) except that the binding buffer for 14-3-3 was 125 mM NaCl, 0.5% Nonidet P-40, 50 mM Tris·HCL (pH 7.4), 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM DTT, and 1% dry milk. Wash buffer is binding buffer without dry milk. MBP and MBP–14-3-3ε proteins were purified as described (30). Purification of human c-ABL form 1b from insect cells was performed as described (31). For kinase assays, 0.9 μg c-ABL was immunoprecipitated with 10 μl ABL antiserum (pex-5). Precipitated c-ABL was incubated with 10 μCi [γ-^{32}P]ATP (1 Ci = 37 GBq) at 30°C for 10 min in kinase (+) buffer (10 mM Tris, pH 7.5/10 mM $MnCl_2$/0.1 mM Na_3VO_4/10 mM DTT) or kinase (−) buffer (10 mM Tris, pH 7.5/5 mM EDTA/0.1 mM Na_3VO_4). Reactions were terminated with 2× sample buffer and analyzed by SDS/PAGE and autoradiography. 0.7 μg RIN1N-6His, purified using Talon beads (CLONTECH), was used in c-ABL binding experiments with either kinase (+) buffer plus 1 mM ATP or kinase (−) buffer for 30 min at 30°C. Protein complexes were immunoprecipitated with ABL antiserum and subjected to *in vitro* kinase assays as described above. SH2 binding was done as described (22) using K562 cells and GST-RIN1NΔ bound to glutathione-Sepharose. Bound proteins were analyzed by SDS/PAGE, filter transfer and antiphosphotyrosine (Upstate Biotechnology, Lake Placid, NY) probing. For SH3 binding, pGST–PI3K–SH3, pGST–SRC–SH3, pGST–ABL–SH3 fusion plasmids were kindly provided by Richard Rickles (32). Glutathione *S*-transferase (GST) fusion proteins were purified on glutathione-Sepharose (Pharmacia), dialyzed with PBS, and concentrated. The peptide KSSPLSPPAVPPPPVPVLPGARRASLG (Genemed Biotechnologies, South San Francisco, CA) contains the putative SH3 binding site (underlined), five flanking RIN1 residues on either side, and a recognition site (RRASL) for bovine heart muscle kinase (HMK). A total of 2.5 nmol of peptide was labeled with 150 pmol [γ-^{32}P]ATP and 30 units of HMK for 50 min. A total of 250 pmol ^{32}P-labeled RIN1 peptide was incubated with 8 μg GST fusion protein and 10 μl 1:1 slurry of glutathione-Sepharose beads, in 250 μl PBS plus 2% dry milk for 1 h at 4°C with constant rotation. Beads were precipitated at 4°C and washed 5 times with unlabeled PBS before cpm measurement. A 100-fold excess of a competitor peptide AEPPPYPPPPIPGGK (32) or a control 26-mer random peptide was used as indicated.

Transfections and Luciferase Assays. Procedures for transfections and luciferase assays have been described (33). The total amount of DNA in each transfection was held constant by adjusting the quantity of vector DNA. All DNAs were purified by CsCl gradient. Experiments were performed in triplicate, and multiple such experiments were performed.

RESULTS

RIN1 Interacts with RAS in a Way That Is Distinct from RAF-RAS Binding. Previous RIN1 studies (11) employed a truncated human cDNA that encoded the carboxyl terminus of the protein. We isolated additional cDNA clones and constructed a full-length RIN1 message (Fig. 1*A*) that encodes a total of 783 aa, 293 residues more than the original isolate. One region of divergence within the RIN1 message results in a 186-nt (62 codon) deletion. This form of the message (RIN1Δ), while a minor component was observed in multiple human cDNA libraries and most likely results from an alter-

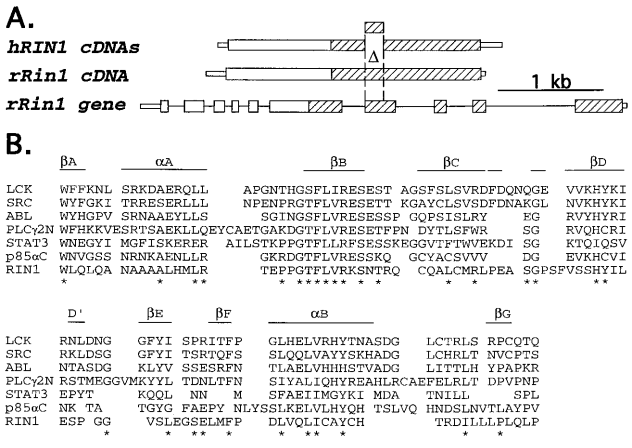

FIG. 1. RIN1 structure and sequence. (*A*) Human *RIN1* messages differ by an internal deletion, Δ (186 nt, 62 aa; residues 429–490). Hatched area (*RIN1C*) is the original RAS-interacting coding region (11). ORFs are indicated by open (or hatched) thick boxes. Untranslated regions are shown as thin open boxes, and introns are presented as solid lines. Broken lines indicate alignment at the alternate splice sites. A 1 kb scale bar is given. (*B*) Alignment of the RIN1 SH2 domain (residues 69–163) with known SH2 domains based on assignments of secondary structure (34) indicated at the top of figure. RIN1 residues identical to two or more others are marked with asterisks.

nate splicing event. This interpretation is supported by analysis of rat cDNA and genomic clones of RIN1. The 5′ end of the deleted sequence is located at a splice site in the genomic clone (Fig. 1*A*) and the 3′ end of the deletion is at a sequence that is similar to splice acceptor sites (35). When expressed in Rat1 cells, the common form of RIN1 (longest ORF, no deletion) was observed to comigrate with endogenous RIN1 protein from HeLa cells (data not shown).

We examined the ability of full-length RIN1 to interact with H-RAS in mammalian cells expressing both proteins. NIH 3T3 cells expressing an activated mutant H-RAS allele (36) were infected with a retrovirus encoding the RIN1 protein. When RAS is immunoprecipitated from these cells (using antibody Y238), RIN1 was also present (Fig. 2*A*). When the antibody was prebound to exogenous RAS, no RIN1 was detected. The RAS antibody Y259, which binds within the switch II region and blocks effector interactions (29, 37), eliminated RIN1 association. Also, in cells expressing RIN1 but only wild type RAS, RIN1 was not coimmunoprecipitated. The lack of association between RIN1 and wild-type RAS is consistent with the dependence of this interaction on RAS activation. A requirement for specific sequence and conformational determinants is a hallmark of RAS effector binding, and these results directly parallel what has been seen for RAF1 (29). The interaction of RAS with RIN1 was confirmed by copurification of activated RAS with an epitope tagged form of the carboxyl terminus of RIN1 (RIN1C) from cells expressing these proteins (Fig. 2*B*).

The structural determinants for effector binding are provided by several amino acids, many of which are localized to the "effector domain" of RAS. Some mutations in this sequence completely block RAS function (reviewed in ref. 38) while other mutations selectively inhibit the binding of some effector molecules without influencing others. These latter mutations have been used to distinguish multiple effectors (27) and the pathways they control (39). We tested the ability of RIN1 to interact with H-RAS effector domain mutants using the two-hybrid detection system (Table 1). RIN1, like RAF1, interacts well with both wild-type and the constitutively active mutant form of RAS (H-RAS^{V12}). Mutations at positions 35 (H-RAS^{V12S35}) or 40 (H-RAS^{V12C40}), however, block the RAS–RIN1 interaction. Interestingly, the position 35 mutation does not affect interactions with RAF1 (27). Conversely, a mutation at position 37 (H-RAS^{V12G37}) does not interfere with RIN1 binding but abolishes RAF1 binding. These data suggest that RIN1 and RAF1 employ both common and unique binding determinants on RAS. The putative RAS effector RalGDS had an interaction profile identical to RIN1 (Table 1; ref. 28).

The RAS-Binding Domain of RIN1 also Interacts with 14-3-3 Proteins. To determine the minimal RBD of RIN1, a series of deletions were assayed for *in vitro* binding to H-RAS. Both the originally isolated amino-terminal deleted form of RIN1 (RIN1C) and full-length RIN1 bind well to H-RAS (Fig. 3). Deletion of an additional 58 aa from the amino terminus of RIN1C reduced binding and deletion of 130 aa strongly blocked RAS interactions. At the carboxyl terminus, deletion of 56 aa had no inhibitory effect and actually enhance binding slightly. Additional deletions from the carboxyl terminus, however, eliminated binding. The naturally occurring 62-aa internal deletion form (RIN1Δ) showed significantly weaker RAS binding. The minimum RAS binding domain of RIN1, therefore, appears to lie within residues 294–727, and optimal binding requires some or all of the 62 aa that are missing in the internal splicing variant. We did not detect multiple independent Ras binding domains as seen for RAF1 (40).

The RAS effector RAF1 interacts through its RBD with other proteins that may facilitate signal transduction functions. We asked whether the RBD of RIN1 is also involved in other protein–protein interactions. Using the two hybrid procedure (41), we isolated eight distinct clones from a murine cDNA library. One of the most frequently isolated cDNAs encodes the epsilon isoform of 14-3-3. 14-3-3 proteins have been implicated in mitogenesis and oncogenic transformation,

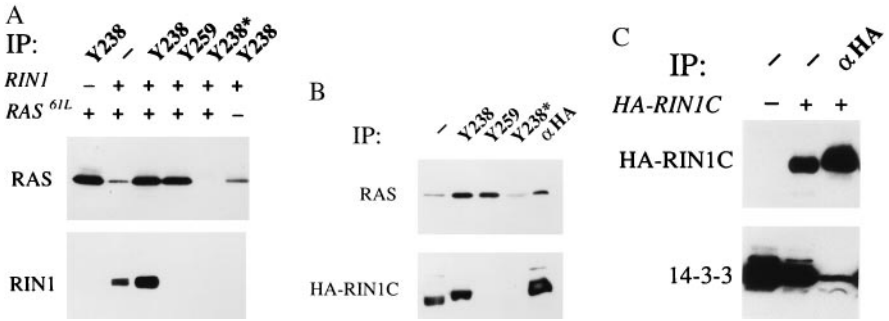

FIG. 2. RIN1 coimmunoprecipitations from NIH 3T3 cells. (*A*) Cells expressing H-RAS^{61L} (+) or wild-type cells (−) were infected with a RIN1 expressing retrovirus (+) or were mock infected (−). Antibodies Y238 or Y259 were used to immunoprecipitation (IP) RAS. Y238* indictates preincubation with GST–RAS protein. Whole cell lysate (no IP) was included (lane 2). Samples were analyzed by Western blot using antibody to RAS (RAS) or RIN1C (RIN1). (*B*) Cells expressing H-RAS^{61L} and HA-RIN1C were lysed and subjected to electrophoresis directly (−) or immunoprecipitated with RAS antibody (Y238, Y238*, Y259) or 12CA5 antibody (αHA) and analyzed by Western blot as in *A*. (*C*) RIN1 and 14-3-3 copurify in immunoprecipitations. Cells expressing (+) or not expressing (−) HA-RIN1C were analyzed as lysates (lanes 1 and 2) or as an IP with the 12CA5 antibody and examined by Western blot using 14-3-3 or RIN1C antibody as probe.

Table 1. Interaction of RIN1 with RAS effector domain mutants using two-hybrid analysis

RAS allele	Ras effector domain binders		
	RAF1	RIN1	RalGDS
H-RAS	+	+	+
H-RAS^{V12}	+	+	+
H-RAS^{V12S35}	+	–	–
H-RAS^{V12G37}	–	+	+
H-RAS^{V12C40}	–	–	–

A + indicates both *HIS3* expression (growth on selective media) and LacZ expression (β-galactosidase assay).

based on their interactions with signaling proteins. Interaction with 14-3-3 proteins has been linked to RAS-dependent activation of RAF1 (15–17), which may occur through a chaperon function that promotes RAS binding or through enhanced oligomerization of RAF1 (5).

We examined whether RIN1 binds to other isoforms of 14-3-3. Indeed, RIN1 interacted with the epsilon, beta and zeta isoforms of 14-3-3, although the zeta isoform showed markedly weaker binding (Fig. 4). The same pattern of interactions was seen with RAF1 (ref. 14 and data not shown) extending the similarity of RIN1 and RAF1 in their biochemical behavior. Deletion of the RBD of RIN1, or the naturally occurring 62-aa internal deletion, abolished binding to all 14-3-3 isoforms in this assay. Under *in vitro* binding conditions (Fig. 3), the carboxyl-terminal domain of RIN1 (RIN1C) binds to 14-3-3ε. Deletion of 121 aa from the carboxyl terminus reduced binding somewhat while deletions of 58 aa or greater from the amino terminus of RIN1C greatly reduced or abolished binding. The internal 62-aa deletion also caused a severe reduction in binding. The pattern of 14-3-3 binding capacity paralleled that for RAS binding, although the signal was somewhat lower.

The ability of RIN1 to interact with 14-3-3 *in vivo* was tested using NIH 3T3 cells expressing the carboxyl-terminal domain of RIN1 (RIN1C). These cells have significant levels of endogenous 14-3-3 protein. As shown in Fig. 2*C*, 14-3-3 proteins were indeed copurified in immunoprecipitations of RIN1C. Because a pan-14-3-3 antibody was used, the specific isoforms present cannot be discerned. RIN1, then, shares the 14-3-3 binding property that has been documented for other RAS-interacting proteins.

The RAS-Binding Domain of RIN1 Interferes with RAS-Mediated Signals in Mammalian Cells. The ability of RIN1 to interfere with RAS signaling in yeast, and the observed *in vitro* competition between RIN1 and RAF1 for RAS binding (11), suggested that expression of the RBD of RIN1 might alter signaling in mammalian cells. We tested whether RIN1C was

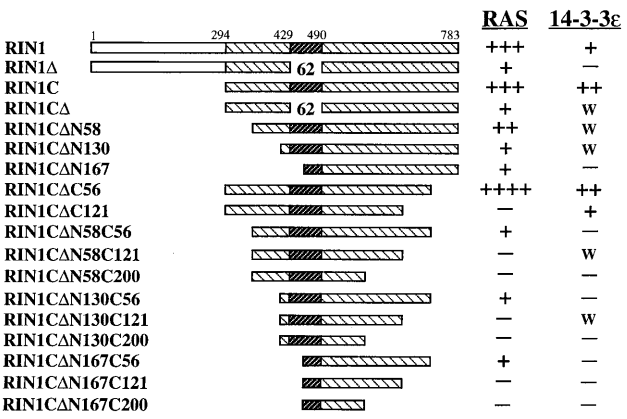

FIG. 3. Interaction of RIN1 with H-RAS and 14-3-3ε. Labeled RIN1 was analyzed separately for normalization. Bound protein was analyzed by SDS/PAGE and autoradiography. Binding above background (GST or MBP alone) was assessed. Multiple +'s indicate strong binding; "w" indicates weak binding (<2-fold above background).

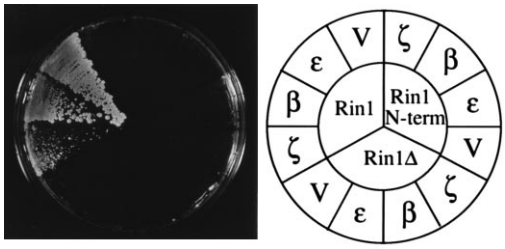

FIG. 4. Interactions of RIN1 with 14-3-3 isoforms. LexA-RIN1 and Gal4–14-3-3 constructs were expressed in L40 cells and *HIS3* reporter gene expression was assayed by growth on synthetic media without histidine. Rin1, full-length RIN1; Rin1Δ, 62-aa deletion form of RIN1; Rin1N-term, amino-terminal domain of RIN1; V, vector only; epsilon, beta, and zeta isoforms are indicated by Greek letters.

capable of blocking transcriptional activation that is dependent on functional RAS and downstream mitogen-activated protein kinase cascades. The expression system employed a luciferase reporter gene under the control of a prostaglandin synthase 2 (*PGS2*) promoter. This construct shows potent induction resulting from a RAS-mediated signal initiated by expression of activated SRC (33, 42). The response to RAS activation requires an ATF/CRE sequence within the promoter (42). Transfection with v-SRC produced a dramatic increase in expression of the PGS2-luciferase reporter (Fig. 5). When RIN1C was included in the transfection, there was a marked decrease in the expression level of the reporter gene and this effect was dependent on the amount of RIN1C used. Fig. 5 also demonstrates the effectiveness of a dominant negative mammalian RAS allele (43) in suppressing the v-SRC initiated signal. Full-length RIN1, however, showed no significant signal suppression in this assay (data not shown), although it is capable of blocking activated RAS in yeast as judged by a heat shock survival assay (11).

RIN1 Is a Binding Partner and Substrate for c-ABL. RIN1 contains a proline-rich sequence (residues 259–268) within the amino-terminal region. Such sequences can be critical determinants for binding to SH3 domains. In particular, the RIN1 sequence showed greatest similarity to peptides (32) and proteins (44) selected for binding to the SH3 domain of the tyrosine kinase c-ABL. Indeed, a synthetic peptide encoding the RIN1 proline-rich sequence gave a strong binding signal with the SH3 domain of c-ABL (Fig. 6). Some binding to SRC–SH3 was also observed, while binding to PI3 kinase–SH3 was close to background (GST only). Competition experiments demonstrated that RIN1 peptide binding to ABL–SH3 was specific; a known ABL–SH3 binding peptide present in 100-fold excess blocked binding, while an unrelated random peptide had no effect (Fig. 6).

To determine whether the amino terminal domain of RIN1 (RIN1N) can interact with c-ABL and serve as a substrate for tyrosine phosphorylation, we carried out coimmunoprecipitations and kinase assays. RIN1N could indeed be phosphorylated

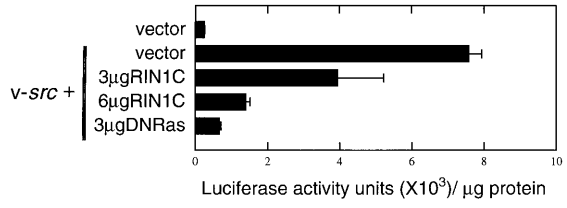

FIG. 5. The RBD of RIN1 interferes with RAS signaling. NIH 3T3 cells were transfected with *PGS2*-luciferase with or without v-*src*. 3 μg or 6 μg of pcDNA3HA–RIN1C (RIN1C) and 3 μg H-RAS^{N17} (DNRas) were used as indicated. pcDNA3HA was the control vector. The data represent experiments performed in triplicate. Multiple experiments were performed with the same result in each case.

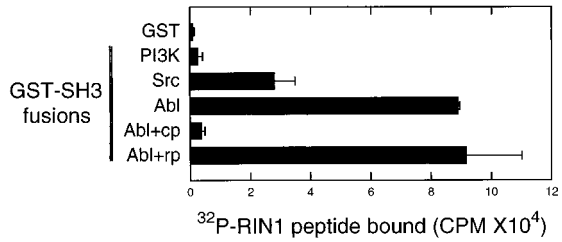

FIG. 6. RIN1 binding to SH3 domains. Labeled RIN1 peptide was incubated with purified GST (control) or GST fusion proteins with the SH3 domains of PI3 kinase, Src or c-Abl [with or without competitor (cp) or random (rp) peptides]. The mixture was then bound to glutathione Sepharose beads, washed extensively, and counted.

by c-ABL *in vitro* (Fig. 7, lanes 1 and 2). There are several tyrosine residues within RIN1N that could serve as phosphorylation substrates, and a low level of tyrosine phosphorylation can be detected on RIN1 overexpressed in Rat1 cells (data not shown). Autophosphorylated c-ABL protein was also detected. For c-ABL, ^{32}P-labeled incorporation may represent an exchange reaction since the c-ABL protein was tyrosine phosphorylated before the reaction while recombinant RIN1N was not (data not shown). When RIN1N and c-ABL proteins were mixed prior to immunoprecipitation with ABL antibody, RIN1N was detected in the precipitated material (Fig. 7, lane 5). In this *in vitro* system RIN1N tyrosine phosphorylation did not appear to be necessary, however, for some c-ABL interaction. This was addressed by performing the incubation of c-ABL with RIN1N and subsequent ABL immunoprecipitation under conditions that do not permit tyrosine phosphorylation of RIN1N to take place. Under these conditions, much of the RIN1N protein was still found to be associated with c-ABL when visualized by a subsequent kinase assay (Fig. 7, lane 7). However, these results do not rule out the possibility that tyrosine phosphorylation has some effect on binding affinity *in vivo*. Another basis for c-ABL/RIN1 binding may be the proline-rich sequence which can bind to the ABL–SH3 domain in isolation (Fig. 6) and/or other domains. It should also be noted that no change in c-ABL kinase activity was observed in the presence of RIN1N, as judged by the level of c-ABL autophosphorylation.

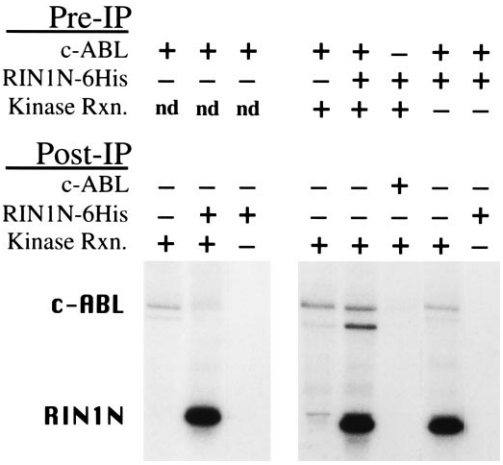

FIG. 7. RIN1N is a substrate and binding partner for c-ABL. Autoradiogram of c-ABL phosphorylation reaction products. For kinase reactions, + indicates use of kinase (+) buffer, − indicates use of kinase (−) buffer, and nd indicates no reaction. [^{32}P]ATP was used only in Post-IP (immunoprecipitation) reactions. Lanes 1–3: immunoprecipitated c-ABL protein without (lane 1) or with (lanes 2 and 3) RIN1N–6HIS protein present. Lanes 4 and 5: c-ABL was incubated without (lane 4) or with (lane 5) RIN1N–6HIS, immunoprecipitated, and used in a kinase reaction. No RIN1N–6HIS was immunoprecipitated in the absence of c-ABL protein (lane 6). Lanes 7 and 8: c-ABL and RIN1N–6HIS were incubated and immunoprecipitated from kinase (−) buffer followed by reactions in kinase (+) buffer (lane 7) or kinase (−) buffer (lane 8). Lanes 4–8 were run on a separate gel and do not perfectly align with lanes 1–3.

Experiments performed using extracts from mammalian cells expressing both c-ABL and RIN1 showed no coimmunoprecipitation (data not shown). This difference from the *in vitro* assay could reflect the fact that c-ABL is mostly, though not entirely, nuclear (45, 46). We have previously shown that a large proportion of RIN1 is itself associated with the plasma membrane (11) and may therefore be largely inaccessible for c-ABL binding under normal conditions.

RIN1 encodes another recognizable binding motif, an SH2 domain (Fig. 1*B*), near its amino terminus. Using a GST–RIN1NΔ fusion protein (and GST control), we have demonstrated the ability of the RIN1 SH2 domain to physically interact with multiple phosphotyrosine containing proteins from a whole cell extract *in vitro* (data not shown). However, it is not yet clear which, if any, of these interact functionally with RIN1 *in vivo*.

RIN1 Expression Shows Unequal Tissue Distribution. We examined the tissue distribution of *RIN1* expression to look for patterns that might reflect the physiological function of this gene. *RIN1* message was present in all tissues examined, although the level varied significantly (Fig. 8). The highest expression level was detected in brain tissue. Northern blot analyses on subtissues of the brain indicated that RIN1 expression was not uniform. Some tissues, such as hippocampus, showed notably higher expression compared with whole brain (data not shown).

DISCUSSION

RIN1–RAS Interaction. The *in vitro* properties of the physical interaction between RIN1 and RAS are strikingly consistent with the predictions for RAS effector molecules and with the observed properties of known and putative RAS targets. In particular, RIN1 binds specifically to the activated (GTP-bound) form of RAS. In addition, the *in vivo* association of RIN1 and RAS cannot be detected with a neutralizing (effector-competing) RAS antibody. These properties are shared with RAF1 (29) and are consistent with the observation that RIN1 and RAF1 bind competitively to RAS *in vitro* (11). Another property shared by RIN1 and other RAS targets is the ability to interact with 14-3-3 proteins. Even the 14-3-3 isoform binding preference is the same as that for RAF1, but the role of 14-3-3 in RIN1 function is not yet clear. In light of this convergence of consensus properties, however, there is a surprising disparity among the known RAS binding partners at the sequence level. To date, only one RBD structure, that of RAF1, has been determined (47). Conservation of higher order structure may yet be revealed by analysis of other RAS targets.

The ability of the RBD of RIN1 to block a RAS-mediated signal in mammalian cells is consistent with the binding properties

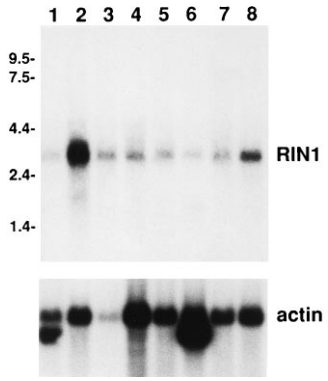

FIG. 8. Human tissue Northern blot analysis of *RIN1*. Lanes: 1, heart; 2, brain; 3, placenta; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, pancreas. (*Lower*) Actin probe results.

of RIN1. The observation that full-length RIN1 does not appear to be a signal suppressor in this system may reflect a stronger interaction between activated RAS and the truncated form of RIN1, although this is not the case *in vitro*. The expression level of RIN1 was somewhat lower than that of RIN1C, however, and this may account for some of the difference. Also, the ability of full-length RIN1 to interact with other proteins through its amino terminal binding motifs may, in part, account for the reduced effect in the RAS suppression assay if RIN1 is partially sequestered with other binding partners, resulting in a lower "effective" concentration. Alternatively, this difference might imply that full-length RIN1 has a positive signaling function that compensates for any reduction in mitogen-activated protein kinase cascade activation. That full-length RIN1 can suppress $Ras2^{V19}p$ in yeast may indicate that other partners, and other pathways, are not available in these cells.

In at least one important respect, RIN1 binding is distinct from RAF1 binding to RAS. The effector domain mutations at positions 35 and 37 clearly differentiate between RAF1 and RIN1. The biological properties of these RAS mutants (39, 48) suggest that the multiple effects resulting from the activation of RAS in mammalian cells can be attributed, at least in part, to the presence of multiple RAS effectors. There is also a diversity of responses to RAS activation depending on the cell type, suggesting that there are differences in the signaling "context." One possible molecular basis for signal context may be alterations in the palette of available RAS effectors. The observed variation in RIN1 expression levels among the tissues sampled suggests that it may be an important variable in the mix of RAS binding proteins. In addition, alternate RIN1 splicing may produce proteins with modified binding properties, as with the internal 62-aa deletion, providing another level of regulation.

RIN1–ABL Interaction. Binding to c-ABL involves the amino-terminal domain of RIN1 and is likely to be mediated, at least in part, through a proline-rich sequence that interacts with the ABL SH3 domain. One consequence of binding is the tyrosine phosphorylation of RIN1. But the interaction of RIN1 with c-ABL did not produce any significant change in c-ABL catalytic activity as judged by autophosphorylation levels. This does not rule out, however, a role in c-ABL-mediated signals through changes in substrate specificity or connections to downstream effectors.

We were unable to detect an *in vivo* interaction of RIN1 with the c-ABL protein. This likely reflects the fact that, under normal conditions, most c-ABL protein is localized to the nucleus and not available for binding to RIN1. Interestingly, mutations that lead to the oncogenic activation of c-ABL (amino-terminal truncations and fusions in the cases of murine *v-abl* and human BCR/ABL) result in increased cytoplasmic localization, along with elevated tyrosine kinase activity (reviewed in ref. 46), making it accessible for RIN1 binding. Indeed, RIN1 demonstrates both physical and functional interactions with BCR/ABL in mammalian cells (D.A., L.H., J. McLaughlin, A. Dhaka, O.N.W., and J.C., unpublished data). The specificity of the interaction between c-ABL and RIN1 and the subsequent tyrosine phosphorylation of RIN1, together with the *in vivo* data for BCR/ABL, suggest involvement of RIN1 in ABL signaling. There may be other endogenous interaction partners for the amino terminus of RIN1. The presence of a functional SH2 domain within RIN1 raises additional possibilities for participation in signaling pathways that may shed light on RIN1 function.

Taken together, the unique profile of RIN1 suggests that it participates in multiple signal transduction pathways in mammalian cells. It should be noted that the RIN1 interaction partners RAS and c-ABL are themselves key proteins that, when mutationally activated, are involved in tumorigenesis in humans. There is, as yet, no evidence of stable three-way complexes of RIN1 with RAS and c-ABL, and it remains a possibility that separate pools of RIN1 protein may be committed to either RAS interactions or SH3/SH2 interactions with c-ABL or a related protein.

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