

A novel partner for the GTP-bound forms of *rho* and *rac*

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Abstract Using the yeast two hybrid system and overlay assays we identified a putative *rho*/*rac* effector, *citron*, which interacts with the GTP-bound forms of *rho* and *rac1*, but not with *cdc42*. Extensive homologies to known proteins were not observed. This 183 kDa protein contains a C₆H₂ zinc finger, a PH domain, and a long coiled-coil forming region including 4 leucine zippers and the *rho*/*rac* binding site. We recently identified three others putative *rho* effectors characterized by a common *rho* binding motif. *Citron* does not share this motif and displays a distinctive protein organization, thus defining a separate class of *rho* partners.

Key words: Signal transduction; Small GTP-binding protein; Two hybrid system; Coiled-coil; Zinc finger; Pleckstrin homology

1. Introduction

RhoA, *rhoB*, and *rhoC* are three highly related proteins which have been implicated in cell shape regulation, cell adhesion, cell division, and have a drastic effect on actin structures, notably the stress fibers [1–3]. *Rac1*, *rac2*, *TC10*, *rhoG*, and *cdc42* constitute another family of highly related proteins which share approximately 50% identities with the *rho* sub-group. They also regulate actin structures, particularly membrane lamellipodia and filopodia [4,5]. In addition, *rhoA*, *rac1*, and *cdc42* have been recently reported to affect gene expression [6,7]. Like the other members of the p21 *ras* superfamily, these proteins are regulated through nucleotide binding, the GDP or GTP-bound forms representing the resting or the active state, respectively. These proteins are highly conserved and both *rhoA* and *cdc42* can functionally replace their *S. cerevisiae* counterparts, *RHO1* and *CDC42*, respectively [8,9].

Several *rho*-binding proteins have been described to date. Exchange factors, which induce release of GDP and subsequent GTP-binding, are implicated in *rho* activation. Conversely, GAP proteins, which stimulate GTPase activity of *rho*, are thought to act as negative regulators. GDI is a protein able to binds both *rho*-GTP or *rho*-GDP, and prevents nucleotide exchange. Several of these *rho* partners have been involved in oncogenesis [10–12].

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Abbreviations: p21, *ras*-related GTP-binding protein; PH, pleckstrin homology; aa, amino acid residue.

The nucleotide sequence of *citron* cDNA has been deposited in GenBank.

While two protein kinases and a phosphoinositide 3-kinase have been recently described as targets for activated *rac* and/or *cdc42* [13–15], there is no such report for the *rho* sub-group. Using the two hybrid system and overlay assays, we isolated a cDNA coding for a potential *rho* target. This report presents the isolation and sequence analysis of this cDNA as well as the binding properties of the expressed protein.

2. Materials and methods

2.1. Plasmids and expression in bacteria

Plasmid vector pBTM116 [16] cloning region was first modified from GAATTCCTCGGGGATCC to GAATTGGGGATCCGGGAATTC-CGATCC using an oligonucleotide strategy. The coding sequence of *rhoA* (from ATG to stop codon) was available as a 0.6 kb *Bam*HI-*Eco*RI fragment from pGEX-*rhoA* [17]. Similar *Bam*HI-*Eco*RI fragments containing *rhoA*^{Asn-19} or *rhoA*^{Val-14} will be described elsewhere. The same restriction sites were introduced by PCR into *rhoB* and *rhoC*. Deletion mutants were generated similarly, introducing the stop codon-*Eco*RI sequence after codon 181 in all three *rho*, removing CAAX box and poly-basic region. These *rho* cDNAs were inserted at the *Bam*HI and *Eco*RI sites of the modified pBTM116 vector in order to produce LexA fusion proteins. These plasmids were named pLexA- followed by the name of the gene of interest. The denomination D identifies the deletion mutants (i.e. pLexA-DrhoA). Sequence analysis of all the *rhoA* constructs demonstrated the absence of undesired mutations. Plasmid pLexA-lamin has been described [16].

The *Bam*HI-*Eco*RI fragments containing *rhoB* or *rhoC* were inserted at the same sites of pGEX-2T (Pharmacia Biotech). Plasmids pGEX-*cdc42* and pGEX-*rac1* were provided by Y. Takai and P. Polakis. The GST-p21 fusion proteins expressed by these plasmids were purified as described [17]. *Citron* cDNA (codons 674–870), obtained as a *Bam*HI-*Eco*RI fragment from the library clone pVP16-*citron*, was subcloned into pGEX-3X (Pharmacia Biotech) or pQE-9 (Qiagen) in order to produce GST-fusion or histidine-tagged *citron*, respectively. Bacterial cultures containing these plasmids were induced for 5 h at 37°C, centrifuged, and resuspended in Laemmli buffer at a bacterial concentration of 10 OD_{600nm} units per ml. Samples were boiled for 5 min, briefly sonicated, and analyzed by gel electrophoresis.

2.2. Two hybrid system and overlay assay

A two hybrid system kit was kindly provided by Stan Hollenberg, Rolf Sternglanz, Stan Fields and Paul Bartel. We used the same procedures, plasmids, library, and yeast strains as previously described, including the mating strategy [16]. However, the mating partner of L40, strain JC1, was replaced by strain AMR70 (*MAT α* , *his3*, *lys2*, *trp1*, *leu2*, *URA3::lexAop*), *lacZ*, *GAL4*). According to this method, the probe sequence is expressed as a protein fused with the DNA binding domain of LexA. Likewise, the cDNA library is expressed as peptides fused to the transcription activator domain of VP16. If the probe protein interacts with a library derived peptide, two LexA dependent genes present in L40 may express *HIS3* or *lacZ*. L40 bearing pLexA-*rhoC* was transformed with a mouse embryo (9.5 days and 10.5 days) cDNA library based in pVP16. From 3 × 10⁷ transformed yeasts, approximately 600 His⁺ colonies appeared, about half of them being also positive for β -galactosidase activity. Forty-five double positive clones were further analyzed. In 36 cases, the yeast clones could be cured from pLexA-*rhoA*, *TRP1* marked, while the library plasmids, *LEU2* marked, were retained. Mating these yeast strains with AMR70 containing a

probe plasmid results in diploid strains bearing both type of plasmid. AMR70 was transformed with the pBTM116 constructs coding for various *rho* or *rho* related proteins (see above). We used pLexA-lamin as a negative control. The diploids obtained by crossing AMR70 and L40 derived strains were assayed for β -galactosidase activity. Twenty clones, which appeared negative when tested against lamin and positive when retested against *rhoC*, were further analyzed.

Overlay assay was performed according to Manser et al. [14,18] using p21 loaded with [35 S]GTP γ S or [35 S]GDP β S (1000 Ci/mmol; New England Nuclear). After incubation with the probe (100 ng/ml), the blots were washed 5 times, 1 min each, in the described buffer [18].

2.3. Expression in mammalian cells and Western blot

A 5 kb *citron* cDNA was isolated, coding for a long splice variant. In this clone, which is truncated at its 5' end, the first 8 codons (according to Fig. 2) are replaced by the codons KELQDSQDKCHK, resulting in a protein closely mimicking the shorter variant presented in Fig. 2. This cDNA was subcloned into a pCMX derived vector driving expression of full-length *citron* tagged at its N-terminus with a 9 aa epitope from influenza hemagglutinin [19]. COS7 cells were transfected with this construct using lipofectamine (Gibco BRL) and were collected 48 h later. Western blot was performed with the monoclonal antibody 12CA5 (Boehringer) directed against hemagglutinin and visualized with a second antibody coupled to alkaline phosphatase.

3. Results and discussion

3.1. Isolation of a cDNA coding for a *rho* p21 partner

Using the yeast two hybrid system, a mouse embryo cDNA

library based in pVP16 was screened for potential *rhoC* targets. The probe was pLexA-*rhoC*, a pBTM116 derivative containing full-length *rhoC*. Twenty cDNA clones were identified, capable of transactivating LacZ when co-expressed with LexA-*rhoC*, but remaining negative when co-expressed with the LexA-lamin negative control.

Using a mating strategy, the 20 clones were tested against additional LexA-*rho* constructs. A single cDNA clone, provisionally termed *citron*, displayed a strong reactivity with *rhoA*^{Val-14}. Weaker signal was observed with full-length *rhoA* and no reactivity was detected with *rhoA*^{Asn-19} (Fig. 1A). The Val-14 mutation prevents GTP hydrolysis by *rho*, resulting in a protein predominantly in the GTP-bound state. Conversely, the Asn-19 mutation is thought to prevent nucleotide exchange, resulting in a *rho* protein predominantly in the GDP-bound conformation [7]. Thus, the data suggest that *citron* peptide recognizes *rho* proteins in their GTP-bound form. In addition, *citron* displayed an enhanced signal when tested against the C-terminal deletion mutants Δ *rhoC* or Δ *rhoA* (Fig. 1A). This effect may be attributed to the membrane localization signals present at the C-termini of *rho*. The library plasmid pVP16-*citron* contains a 0.6 kb insert.

The 19 remaining cDNA clones were negative against all the *rho* constructs, beside LexA-*rhoC*, suggesting the interaction requires the C-terminus of *rhoC*, probably the poly-basic

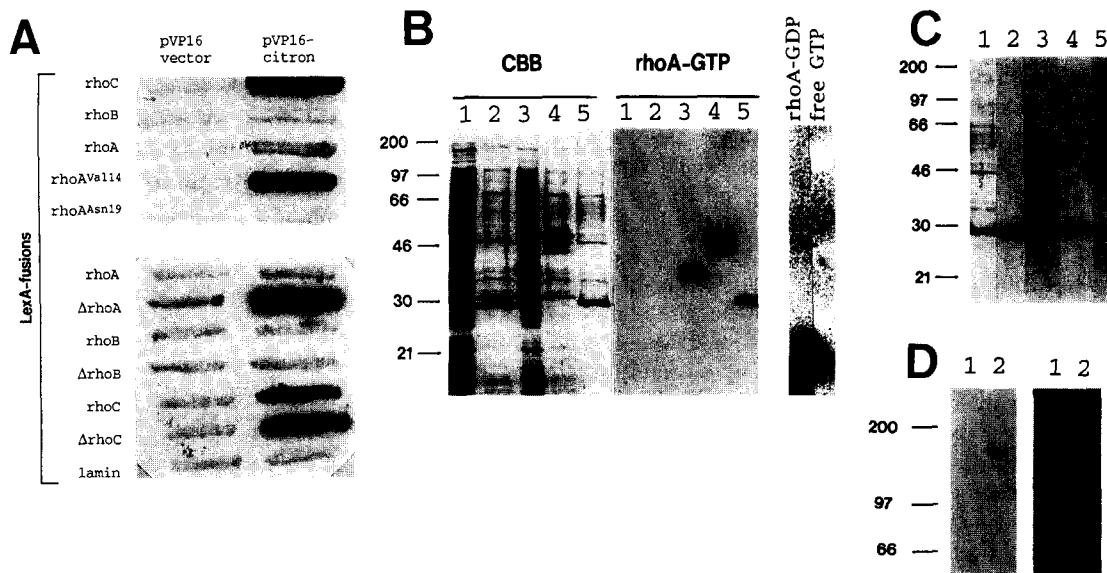


Fig. 1. Interaction of *citron* with p21 GTPases. (A) Yeast two hybrid system analysis of *citron* and *rho*. L40 strain bearing either the library plasmid pVP16-*citron* or the pVP16 vector without insert were mated with AMR70 strains bearing various type of pLexA-fusions, as indicated. The resulting diploid strains were plated as patches on selective medium for maintenance of both plasmids, transferred on cellulose filters (Whatman) and β -galactosidase activity was assayed in situ, as described [16]. *Rho* deleted after codon 181 are referred as *Drho*. (B) In vitro interaction of *citron* with *rhoA*, using overlay assay. Total lysate of bacteria expressing recombinant proteins were analyzed by electrophoresis using denaturing polyacrylamide gels. The amount loaded were adjusted so that 0.2–0.5 mg of the recombinant protein of interest was present in each lane, according to the protein stain of preliminary gels. A large excess of lysate from uninduced bacteria was used as a negative control. Proteins were either stained using Coomassie Brilliant Blue, or transferred to nitrocellulose filters and probed with GST-*rhoA* loaded with [35 S]GTP γ S. Uninduced bacteria (lane 1), GST (lane 2), rhotekin (another *rho*-binding protein) fused to GST (lane 3), *citron* fused to GST (lane 4), histidine-tagged *citron* (lane 5). On the right, control blots containing *citron* fused to GST were also probed with GST-*rhoA* loaded with [35 S]GDP β S or free [35 S]GTP γ S. (C) Parallel blots were probed with additional p21 (obtained as GST-fusion proteins) loaded with [35 S]GTP γ S. Lanes containing histidine-tagged *citron* were grouped as follows: protein stain (1), *rhoA* probe (2), *rhoB* probe (3), *rac1* probe (4), *cdc42* probe (5). (D) Expression of full-length *citron* in mammalian cells. COS7 cells were transfected with an expression plasmid driving the expression of full-length *citron* fused to an hemagglutinin epitope tag. Transfected cells (lanes 2) were lysed and analyzed side by side with untransfected COS7 cell lysates (lanes 1). Proteins were separated by gel electrophoresis, transferred to membranes and probed with either GST-*rhoA* loaded with [35 S]GTP γ S (right panel) or by the monoclonal antibody directed against the epitope tag (left panel).

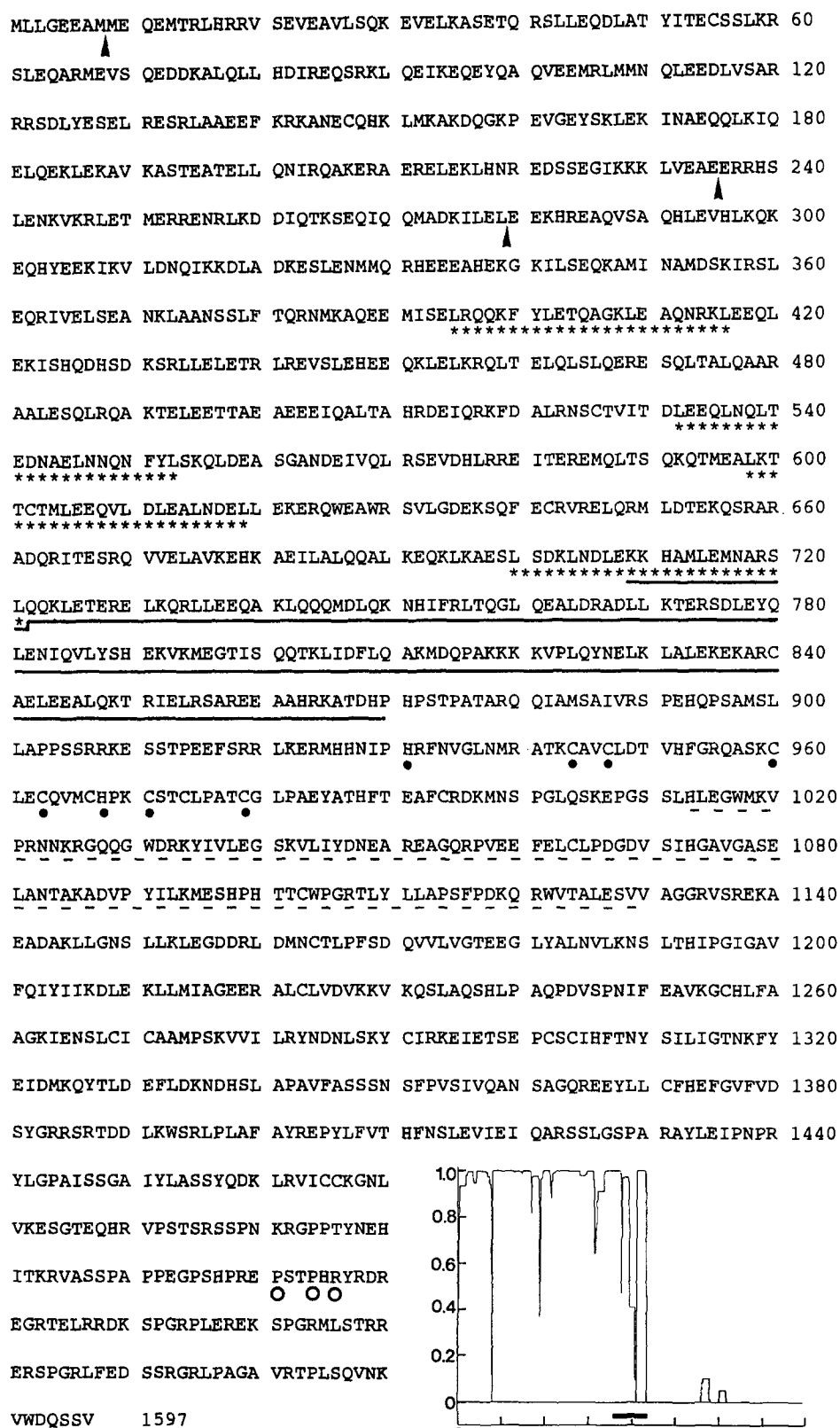


Fig. 2. Protein sequence of *citron*, deduced from cDNAs. Putative alternative splices (arrows) were each observed in several independent cDNA clones. Sequence was determined from both strands of several overlapping clones. The reconstituted sequence shown appeared consistent with the restriction enzyme pattern from one clone containing the complete coding sequence of this isoform. The sequence is translated from the first ATG of the open reading frame. The following domains are indicated: leucine zippers (stars); *rhofrac* binding region (solid line); PH domain (broken line); essential residues of the zinc finger (dots); putative SH3 binding site (open circle). The inset shows the probability of coiled-coil structure (vertical axis) for each position in *citron* (horizontal axis, *rho* binding site indicated by a bar) according to the algorithm of Lupas et al. [20], using a 28 residue-long window.

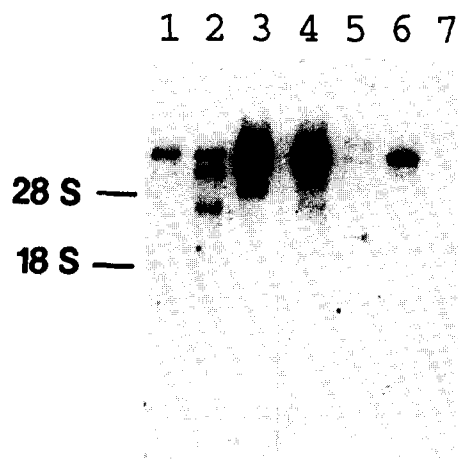


Fig. 3. Tissue distribution of *citron* mRNA. Two mg of poly(A)⁺ RNA isolated from each mouse organ were analyzed by Northern blot. The probe, labeled with ³²P, is a 5 kb cDNA containing full-length *citron* coding sequence. Kidney (1), spleen (2), testis (3), brain (4), heart (5), thymus (6), lung (7). Signal would be visible in lung with a longer exposure time.

region. In several instances, these cDNA appeared to code for transcription factors, as is often the case with two hybrid system false positives (data not shown).

3.2. *Rho* and *rac1* interact directly with *citron*, in vitro

In order to confirm the interaction detected with the two hybrid system, a *citron* peptide was expressed in *E. coli*. The 0.6 kb cDNA obtained from the yeast screen was transferred into pQE-9 or pGEX-3X vectors. Bacteria transformed with these plasmids produce *citron* peptide with a poly-histidine tag or as a GST-fusion protein, respectively. Lysates of *citron*-producing

bacteria, as well as control bacterial lysates, were analyzed by gel electrophoresis. Protein stain revealed the presence of the *citron* peptides, migrating at positions consistent with their predicted size, 26 kDa and 52 kDa, respectively (Fig. 1B). Proteins analyzed on a parallel gel were transferred onto nitrocellulose membrane and probed with GST-*rhoA* loaded with [³⁵S]GTPγS. As shown in Fig. 1B, *rhoA* efficiently detected *citron* as well as another *rho*-binding protein recently isolated in our laboratory, rhotekin (Tim Reid and Shuh Narumiya, manuscript in preparation). No signal was observed from uninduced bacteria, nor from bacteria producing GST alone. Parallel blots were probed with free [³⁵S]GTPγS or with *rhoA* preloaded with [³⁵S]GDPβS. In both cases no signal was observed. Overlay assays performed with additional p21 loaded with [³⁵S]GTPγS indicated that *citron* also binds to *rhoB* and *rac1*, but fails to interact with *cdc42* (Fig. 1C). Using a platelet extract as a positive control, *cdc42* could, however, detect a single band likely to represent p65^{PAK} [14], a *cdc42*-binding protein of similar size (data not shown). We were unable to produce *rhoC* as a GST-fusion protein. The interaction between *rhoB* and *citron* appeared much stronger in overlay assay than in two hybrid system. A possible explanation for this discrepancy is that the behaviour of *rhoB* in a yeast cell is not known. For example, *rhoB* might interact poorly with the exchange factors required for GTP-binding. In contrast, *rhoA* is capable to replace *RHO1* function in yeast [8] and, therefore, must interact essentially normally with the yeast cell machinery. This observation also justify the use of *rhoA*^{Val-14} and *rhoA*^{Asn-19} in a yeast environment.

In summary, both two hybrid system and overlay assay data indicate that *citron* binds to *rhoA* in a GTP-dependent manner. Using either technique, this property could be extended to *rhoB*, *rhoC*, and *rac1*. It is not excluded, however, that a tighter specificity for p21-binding occurs in vivo, under physiological conditions.

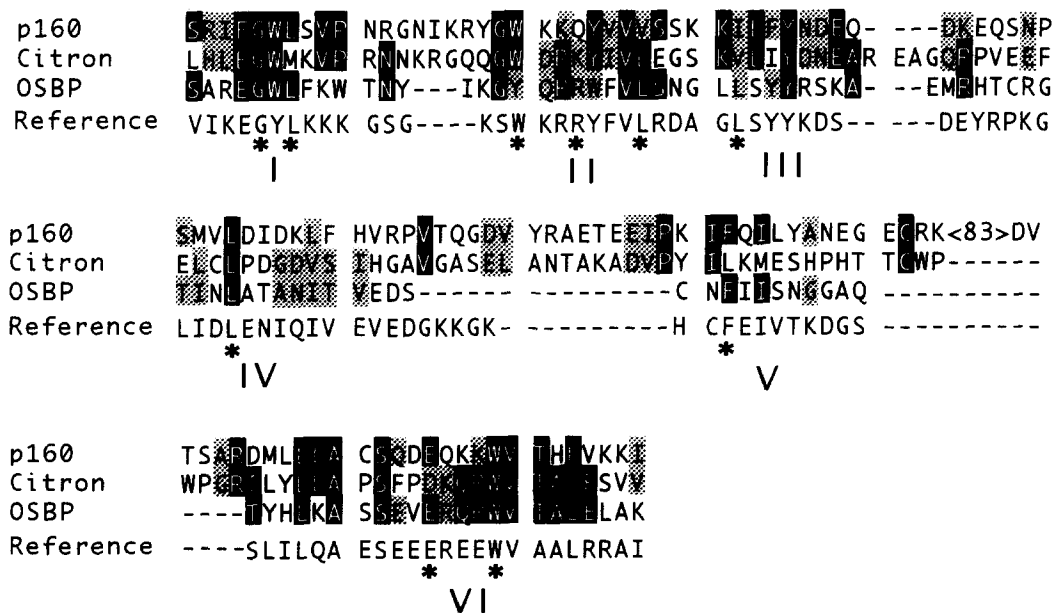


Fig. 4. PH domain in *citron*. *Citron* (aa 1012–1130) is aligned with the PH domains from oxysterol binding protein and p160^{ROCK}. Gaps are represented by dashes and a 83 aa insertion in p160^{ROCK} is indicated. Homologies between these three proteins are boxed, related residues being grouped as follows: EDQN; RKH; LVI; FYW; ST; GA. A consensus sequence for PH domains [23] is shown below; residues from this sequence conserved in at least 50% of the 71 reported PH domains [23,26] are marked by stars. The six PH sub-domains are indicated.

3.3 The predicted sequence of citron contains signal transduction motives

A cDNA library from adult mouse brain was screened successively with the 0.6 kb cDNA obtained from the two hybrid system. A common 5' end sequence bearing in frame stop codons was shared by several independent cDNA, implying that the coding sequence is complete. Twelve base pairs downstream of the 3' stop codon, an A-rich region is present. Full-length *citron* was expressed in COS7 cells and a protein of the predicted size could be detected specifically in transfected cells, using overlay assay with *rhoA* (Fig. 1D). Alternative splicing affects the N-terminal region of *citron*. The deduced protein sequence of one isoform, comprising 1597 aa and corresponding to a predicted molecular weight of 183 kDa, is displayed in Fig. 2. Alternative splicing may occur at codon 8 resulting in an N-terminal extension. Full size cDNA, however, was not obtained for this type of variant. Likewise, a short domain (codons 236–277) appears dispensable for both types of *citron*. In a Northern blot analysis, major signal was observed from testis and brain, but expression also was detectable in thymus, spleen, kidney, heart, and lung (Fig. 3).

Databases were searched for related proteins or consensus sequences. Beside a short human EST sequence (GenBank accession T87377, 94% identical over 100 aa), no protein closely related to *citron* was identified. Several regions, however, could be defined. Referring to the isoform presented on Fig. 2, the N-terminal domain (aa 1–845) presents a semi-repetitive structure and shares distant relationship with myosin and intermediate filaments proteins. These characteristics are typical of coiled-coil domains. Using the algorithm of Lupas [20], a very high probability for such conformation was indeed predicted for this area (inset in Fig. 2). This region also bears 4 leucine zippers (L-X₆-L-X₆-L-X₆-L), suggesting that *citron* interacts with additional proteins. The *rho/rac* binding site is defined by the cDNA isolated from the yeast screen (aa 674–870). In the course of performing additional two hybrid system screens using $\Delta\rho A$ as a probe, a slightly shorter *citron* cDNA was isolated (data not shown) delineating more accurately this binding site (aa 713–870). This region largely overlaps with the coiled-coil domain.

Two signal transduction signature motives are present in *citron*. First, a zinc finger (aa 922–986) comprising 6 cysteines and 2 histidines (C₆H₂) is located in the close vicinity of the *rho/rac* binding site. Highest homology was observed with the second zinc finger of protein kinase C from lower eukaryotes such as *Aspergillus niger* (39% over 75 aa, Genbank accession U10549). This motif, unlike classical zinc finger, is not involved in nucleic acid binding but is thought to bind lipid second messengers [21]. Several p21 GTPases partners also harbor this type of zinc finger, including the exchange factor homologs *vav* and *rotund*, the GAP-*rho* homologs *n-chimerin* and *myr5*, as well as the *raf* protein kinase. Usually, the C₆H₂ region lies next to the p21 binding site [22]. Another motif typical of signal transduction proteins, a PH domain, is present from aa 1012 to 1130. Comparison with a consensus sequence for PH domains [23] revealed 33% identities over 51 aa (PH sub-domains I, II, III, and VI). A single reported PH region (oxysterol binding protein, aa 89–180, Genbank accession M86917) scored better, displaying a block of 8 aa entirely conserved in *citron*. Another *rho* partner recently characterized in our laboratory, p160^{ROCK} (Toshimasa Ishizaki and Shuh Narumiya,

manuscript in preparation), also displays a PH domain significantly related to *citron* (Fig. 4).

The region 1509–1526 is relatively rich in proline residues and contains the consensus sequence P-X-X-P-X-R, a putative binding site for the SH3 of adapter proteins [24]. Finally, multiple potential phosphorylation sites are observed, namely, 25 sites for protein kinase C (S/T-X-K/R), 30 sites for casein kinase2 (S/T-X-X-D/E) and 5 sites for cAMP dependent protein kinase (K/R-K/R-X-S/T).

3.4 Model for citron activation and comparison to other putative rho targets

The most remarkable feature of *citron* sequence is its novelty. Contrary to several effectors of *ras*, *cdc42*, or *rac*, no homology was observed with catalytic domains of known proteins. If *citron* is indeed a *rho/rac* effector, it could be envisioned as a scaffold for a multimeric structure functioning through conformational changes and interactions with additional partners. The presence of several leucine zippers is consistent with such a model. Coiled-coil regions usually serve as dimerization domains, suggesting that native *citron* is a dimer. The p21 binding site lies in the middle of *citron*, near the end of the coiled-coil region. *Rac/rho* binding may partially open this structure, causing a major conformational change in *citron* dimer thereby affecting the properties of the scaffold. Activation of a protein involving a conformational change of its coiled-coil region has been established for myosin [25].

We have recently characterized several partners of GTP-*rho*. Three of them, including rhotekin and rhophilin (Go Watanabe, Tim Reid and Shuh Narumiya, manuscript in preparation), share a consensus sequence for *rho*-binding in their N-terminal regions, suggesting these proteins represent a particular class of *rho* effectors. *Citron*, however, does not contain this motif but interacts with *rho* through a coiled-coil structure. Moreover, zinc fingers and PH domains are not found in the rhotekin type of effectors. *Citron* features, however, may not be unique. Indeed, we have also identified another putative *rho* target, p160^{ROCK} (Toshimasa Ishizaki and Shuh Narumiya, manuscript in preparation), which displays a coiled-coil region as well as other characteristics found in *citron*, even though the primary sequences are widely diverged. Thus, we propose that these two molecules define a second class of *rho* effectors.

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