

Citron Rho-interacting Kinase, a Novel Tissue-specific Ser/Thr Kinase Encompassing the Rho-Rac-binding Protein Citron*

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We have identified a novel serine/threonine kinase belonging to the myotonic dystrophy kinase family. The kinase can be produced in at least two different isoforms: a ~240-kDa protein (Citron Rho-interacting kinase, CRİK), in which the kinase domain is followed by the sequence of Citron, a previously identified Rho/Rac binding protein; a ~54-kDa protein (CRİK-short kinase (SK)), which consists mostly of the kinase domain. CRİK and CRİK-SK proteins are capable of phosphorylating exogenous substrates as well as of autophosphorylation, when tested by *in vitro* kinase assays after expression into COS7 cells. CRİK kinase activity is increased severalfold by coexpression of constitutively active Rho, while active Rac has more limited effects. Kinase activity of endogenous CRİK is indicated by *in vitro* kinase assays after immunoprecipitation with antibodies recognizing the Citron moiety of the protein. When expressed in keratinocytes, full-length CRİK, but not CRİK-SK, localizes into corpuscular cytoplasmic structures and elicits recruitment of actin into these structures. The previously reported Rho-associated kinases ROCK I and II are ubiquitously expressed. In contrast, CRİK exhibits a restricted pattern of expression, suggesting that this kinase may fulfill a more specialized function in specific cell types.

Small GTPases of the Rho family, including Rho-A, -B, and -C; Rac-1 and -2; and CDC42, have been intimately connected with dynamic control of cell structure, as well as downstream transcriptional events (1–3). Activated Rho GTPases have been shown to trigger distinctive kinase cascades. In particular, the Rho-binding serine/threonine kinase (ROCK)¹ binds to Rho (5),

and its kinase activity is moderately stimulated (2–5-fold) by this association (6). Moreover, activated Rho has been reported to promote translocation of the ROCK kinase to the membrane (7), and this in turn may facilitate recognition of specific substrates. In addition, Rho can function as a bridge between ROCK and selected substrates, such as the myosin-binding subunit of the myosin light chain phosphatase, and the myosin light chain itself (8, 9).

A number of other proteins have been identified which associate specifically with the activated form of Rho (10–13). Some of these molecules may also be effectors of Rho function, while others may modulate function of bona fide effectors (such as ROCK). One of these molecules is Citron (10), which was identified using the yeast two-hybrid system and overlay assays, as a specific interactor of Rho and Rac but not CDC42. Citron was found to share a significant degree of structural homology with ROCK. However, it contained no kinase domain, leaving open the question of its biochemical function.

We report here the identification of a novel serine/threonine protein kinase of the myotonic dystrophy kinase family, CRİK (Citron Rho-interacting kinase, which can be produced into two forms as the result of differential splicing, a smaller size protein kinase, with a region of homology with the myotonic dystrophy and PK428 kinases extending beyond the kinase domain, and a much larger protein, in which most of the shorter form of the kinase is fused to all of Citron except its first 8 amino acids. Unlike ROCK, CRİK is expressed in a restricted number of tissues, suggesting that this kinase may serve a more specialized function in specific cell types.

MATERIALS AND METHODS

cDNA Cloning—The KK-1 clone was obtained by PCR with degenerate oligonucleotides for protein kinases from a cDNA library from mouse primary keratinocytes, essentially as described by Holzman *et al.* (14). Both 5'- and 3'-directed rapid amplification of cDNA ends (RACE)-PCR were performed with two nested amplification primers using as template a spleen Marathon-Ready™ cDNA (CLONTECH), according to the manufacturer's specifications. In every case the clones obtained were sequenced on both strands using the Thermo-Sequenase DNA sequencing kit (Amersham Pharmacia Biotech). Sequence similarity searches were performed using the Blastnt and Blastx programs at the NCBI on the Non Redundant Data base. Alignment of the kinase domain sequences was obtained using the CLUSTAL method.

RNA Analysis—Total and poly(A)⁺ RNA were extracted and analyzed by Northern blotting as described previously (15, 16). Radioactive probes corresponded to nucleotides 810–2260 of CRİK-SK (short kinase) and nucleotides 1610–2240 of the published mouse Citron cDNA sequence (10).

For RT-PCR analysis, 100 ng of total RNA were reverse transcribed with an RNase H-Moloney murine leukemia virus enzyme (Promega) after random priming. The kinase domain-specific forward oligonucleotide (KS) corresponded to nucleotides 1849–1873, while the three different Citron-specific reverse oligonucleotides corresponded to nucle-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF086824 for CRİK cDNA and AF086823 for CRİK-SK cDNA.

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¹ The abbreviations used are: ROCK, Rho-binding serine/threonine kinase; CRİK, Citron Rho-interacting kinase; SK, short kinase; KD, kinase domain; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RT, reverse transcription; KS, kinase-specific; CS, Citron-specific; PAGE, polyacrylamide gel electrophoresis; kb, kilobase pair(s).

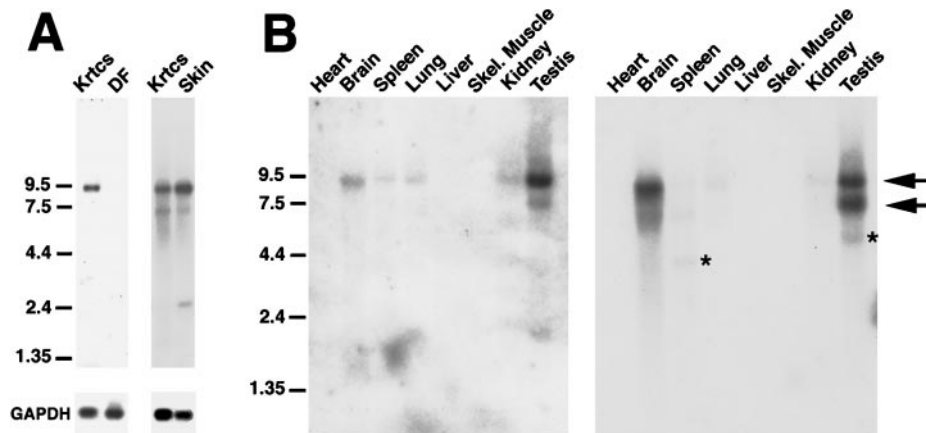


FIG. 1. **CRIK mRNA expression.** Panel A, 2 μg of poly(A)⁺ RNA from primary mouse keratinocytes (*Krtcs*), dermal fibroblasts (*DF*), and skin were analyzed by Northern blotting with a probe specific for the kinase domain of CRIK. Equal loading was verified by hybridizing the same blot with a glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) probe. The blot on the right had a higher exposure time to enable detection of minor bands. Panel B, a Northern blot of poly(A)⁺ RNA from the indicated mouse tissues was hybridized with a probe specific for the kinase domain of CRIK (left panel). The same blot was stripped and hybridized with a Citron-specific probe (right panel). Arrows point to the two major bands referred to in the text, while the minor bands detected only with the Citron probe are indicated by asterisks. The position of molecular size markers (in kilobases) is indicated.

tides 1289–1264 (CS1), 1755–1729 (CS2), and 2715–2692 (CS3) of the Citron sequence.

Expression Constructs—For expression of CRIK-SK, a fragment of the CRIK-SK cDNA from nucleotide 810 to 2260 was amplified from the full-length CRIK-SK and cloned in frame with a FLAG epitope in the pcDNA3 expression vector (Invitrogen), to give the plasmid FLAG-CRIK-SK. The amino acid substitution K126A was introduced by PCR using the oligonucleotides GACGTCTATGCCATGGCAATCAAGAA-GAAG and CTTCTTCTTGATTGCCATGGCATAGACGTC, to give the plasmid FLAG-CRIK-SK-KD>. For expression of full-length CRIK, the coding sequence of mouse CRIK was amplified by PCR from a spleen Marathon-Ready™ cDNA (CLONTECH) using the same forward oligo as above and a reverse oligonucleotide corresponding to nucleotides 4983–4957 of Citron and including a *NotI* restriction site. The plasmids FLAG-CRIK and FLAG-CRIK(KD>) were then generated by replacing the *BamHI-NotI* fragment of FLAG-CRIK-SK and FLAG-CRIK-SK-KD>, respectively, with the corresponding *BamHI-NotI* derived from the full-length CRIK cDNA. In every case the amplifications were performed using the Advantage™ KlenTaq polymerase mix (CLONTECH), and the recombinant plasmids were verified by sequencing.

Protein Analysis—Subconfluent COS7 cells in 10-cm dishes were transiently transfected by the DEAE-dextran/chloroquine method with 10 μg of empty vector or recombinant FLAG-CRIK plasmids. In case of cotransfection experiments, cells were transfected either with 25 μg of empty vector alone, or with a combination of 20 μg of CRIK expression vector plus 5 μg of either empty vector or of an expression vector for the Rho V14 or Rac V12 mutants. Cells were harvested 48 h after transfection. Immunoblotting was performed as described previously, and cells were probed with anti-FLAG M2 antibodies (Eastman Kodak Co.). Blots were developed using horseradish peroxidase-conjugated secondary antibodies and ECL detection system (Amersham Pharmacia Biotech).

Immunoprecipitations of COS7 cells or primary mouse keratinocytes, cultivated in medium at low calcium concentrations (16), were performed as described previously (16), by overnight incubation at 4 °C with either anti-FLAG (Kodak) or anti-Citron S-20 and C-20 antibodies (Santa Cruz Biotechnology). *In vitro* kinase assays were performed as described previously (16) by incubating immune complexes in 50 ml of kinase buffer (50 mM HEPES, pH 7.4, 5 mM MgCl₂, 3 mM MnCl₂, 1 mM dithiothreitol), in the presence or absence of 5 mg of histone H1 or myelin basic protein, plus 0.1 mM ATP and 10 mCi of [γ -³²P]ATP (6000 Ci/mM, NEN Life Science Products) for 30 min at 30 °C. The products were analyzed by 5% or 12.5% SDS-PAGE followed by autoradiography. For immunoprecipitation of metabolically labeled proteins, primary keratinocytes were incubated with 0.1 mCi/ml [³⁵S]methionine (Expres³⁵S; NEN Life Science Products) for 4 h in methionine-free medium in the presence of serum (16). Immunoprecipitated proteins were separated on a 5% SDS-PAGE gel and visualized by autoradiography.

Immunofluorescent Staining of Keratinocytes—Mouse primary keratinocytes plated on collagen-coated glass coverslips were transfected with FLAG-tagged CRIK expression vectors. Cells were fixed in 2% paraformaldehyde in phosphate-buffered saline, permeabilized with

0.5% Triton X-100 in phosphate-buffered saline, and blocked in 5% goat serum in phosphate-buffered saline. The cells were incubated with anti-FLAG M2 monoclonal antibodies for 1 h at room temperature, followed by fluorescein isothiocyanate-conjugated anti-mouse secondary antibodies (Southern Biotechnology Associates Inc., Birmingham, AL). For actin staining, Bodipy 581–591-conjugated phalloidin (Molecular Probes) was added during the incubation with the secondary antibodies. For labeling of the endosomal/lysosomal compartments, cells were incubated in serum-free medium with Fluorescein-conjugated dextran (10,000 *M_r*, anionic; Molecular Probes) for either 5 or 10 min, prior to paraformaldehyde fixation (17). Slides were analyzed by confocal microscopy using a TCS4D scanner (Leica) connected to an inverted LEITZ DM IRB microscope.

RESULTS

Cloning of a New Serine/Threonine Kinase Which Is Specifically Expressed in Mouse Primary Keratinocytes—In order to identify new kinases specifically expressed in mouse primary keratinocytes, a degenerate oligonucleotide-based PCR approach was undertaken to screen a keratinocyte c-DNA library (14). The resulting amplification products were cloned and sequenced. A BLAST sequence similarity search revealed that one of the clones (KK-1) encoded a new serine/threonine kinase domain. Northern blot analysis showed that the corresponding mRNA is specifically expressed in mouse primary keratinocytes and not dermal fibroblasts, and that KK-1 mRNA levels in cultured keratinocytes were comparable to those in total skin (Fig. 1A). The nucleotide sequence assembled from a series of overlapping cDNAs (obtained by screening a keratinocyte cDNA library with a KK-1 probe) consists of 2373 base pairs, with a large open reading frame extending from nucleotides 772 to 2253, and coding for a 495-amino acid protein (GenBank™ accession no. AF086824). The predicted amino acid sequence contains a complete serine/threonine kinase catalytic domain that extends from amino acids 96 to 359 (18, 19). This domain shares a 76% homology with the myotonic dystrophy protein kinase (20) (50% identity), 79% homology with PK428 (21) (49% identity), 74% homology with p160-ROCK I (22) (43% identity), 76% homology with *Caenorhabditis elegans* LET-502 (23) (45% identity), 72% homology with *Drosophila* tumor suppressor protein kinase *warts* (24) (43% identity), and 65% homology with protein kinase C α (25) (32% identity) (Fig. 2A). Outside the highly conserved kinase domain, KK-1 shared a lower but still significant homology with the myotonic dystrophy protein kinase (31% identity, 61% homology), and PK428 (27% identity, 49% homology) proteins in their C-terminal region.

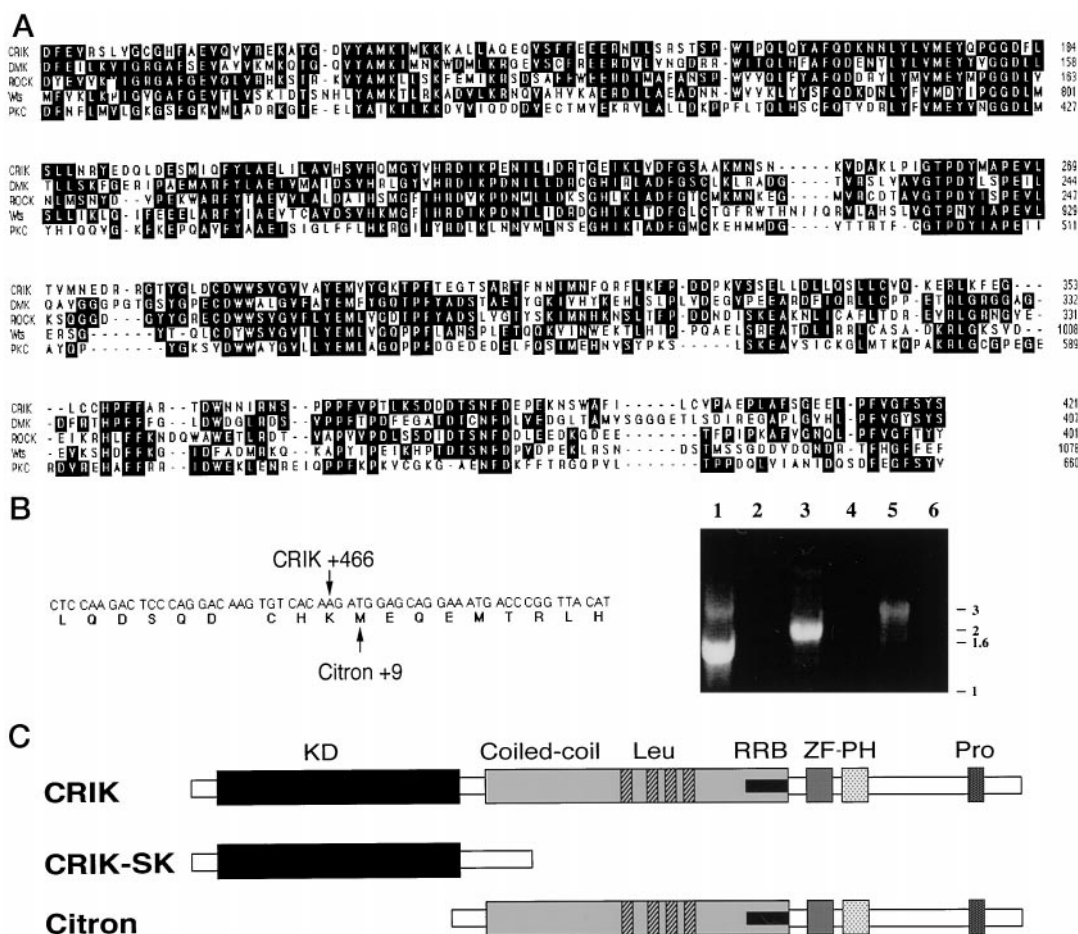


FIG. 2. Structure of CRIK and related gene products. *Panel A*, alignment of the CRIK kinase domain with the sequences of human myotonic dystrophy kinase (*DMK*, accession no. L08835), mouse ROCK I (*ROCK*, accession no. U58512), *Drosophila warts* (*Wts*, accession no. L39837), and mouse protein kinase C_c (accession no. M25811). Areas of amino acid identity are highlighted in *black*. *Panel B*, left, sequence of the junction between the amino-terminal part of the protein, common to both CRIK and CRIK-SK, and the Citron moiety, present only in CRIK. The arrows point to the site of alternative splicing that produces the full-length form of CRIK. *Right*, the CRIK/Citron splicing product as assessed by RT-PCR. Total RNA from primary mouse keratinocytes was analyzed by RT-PCR, using an oligonucleotide corresponding to the kinase domain-part of CRIK as common forward primer (KS), and three different oligonucleotides (CS-1, -2, and -3) corresponding to Citron-specific sequences as reverse primers (see "Materials and Methods" for details). The expected molecular size for RT-PCR products with oligonucleotide pairs KS-CS1 (*lane 1*), KS-CS2 (*lane 3*), and KS-CS3 (*lane 5*) were 1,412, 1,872 and 2,833 base pairs, respectively. Negative controls with PCR performed without reverse transcriptase were also included (*lanes 2, 4, and 6*). The position of molecular size markers is indicated (kb). *Panel C*, schematic representation of the three possible products of the CRIK gene. Indicated are the KD, the coiled coil region (*Coiled-coil*), the Rho/Rac binding domain (*RRB*), the zinc finger region (*ZF*), the plekstrin homology domain (*PH*), the proline-rich, putative SH3 binding domain (*Pro*).

Identification of KK-1 as an Alternative Form of the Rho/Rac Interacting Protein Citron—The overlapping KK-1 cDNA clones obtained in our initial screening corresponded to ~2.2 kb of sequence, which is substantially less than the entire length of the major transcript detected by Northern blotting (~9.5 kb) (Fig. 1). Additional bands of smaller size were also evident on the KK-1 RNA blots after prolonged exposures. This raised the possibility that the transcripts coding for the KK-1 kinase may exist in multiple forms, as a result of differential splicing and/or alternative polyadenylation. For this reason, we decided to expand our KK-1 cDNA cloning using RACE-PCR, with a set of nested oligonucleotides extending toward either the 5' or 3' direction. Myotonic dystrophy-related kinases can be subdivided into two subfamilies, depending on whether the catalytic domain is located at the amino-terminal (as in myotonic dystrophy protein kinase, ROCK, PK428) or carboxyl-terminal (as in *Drosophila warts*) part of the protein (20–22, 24). The 5'-directed RACE-PCR of KK-1 transcripts did not result in any significant extension of the coding sequence. In contrast, sequencing of clones obtained by 3'-directed RACE-PCR, revealed the existence of an alternative coding sequence beyond position 2169, corresponding to codon 466 of KK-1 (Fig. 2B).

The sequence 3' of that point extended for an additional 4770 nucleotides of open reading frames, and was found to be identical to that of the Rho/Rac interacting protein Citron (10). A possible site of alternative splicing at codon position +8 of Citron was noted (10). Alternative splicing at this position is now supported by our 3'-RACE-PCR experiments, which produced cDNAs with an in frame fusion of the KK-1 coding region to codon +9 of Citron (Fig. 2B).

To confirm the existence of these KK-1/Citron transcripts, total RNA from primary keratinocytes was directly analyzed by RT-PCR, using a common oligonucleotide corresponding to bases 1807–1832 of KK-1 as a forward primer, and a set of nucleotides at different positions of Citron as reverse primers. Products of the expected molecular weight were generated in all cases (Fig. 2B, right panel), which were cloned and found to have the predicted nucleotide sequence (not shown). Using this approach, the existence of transcripts coding for the short form of this kinase was also confirmed (not shown).

Henceforth, we will refer to the full-length KK-1/Citron product as CRIK, and to its short kinase form as CRIK-SK. In keeping with the literature, we will still use the name of Citron for the already reported form, devoid of kinase domain.

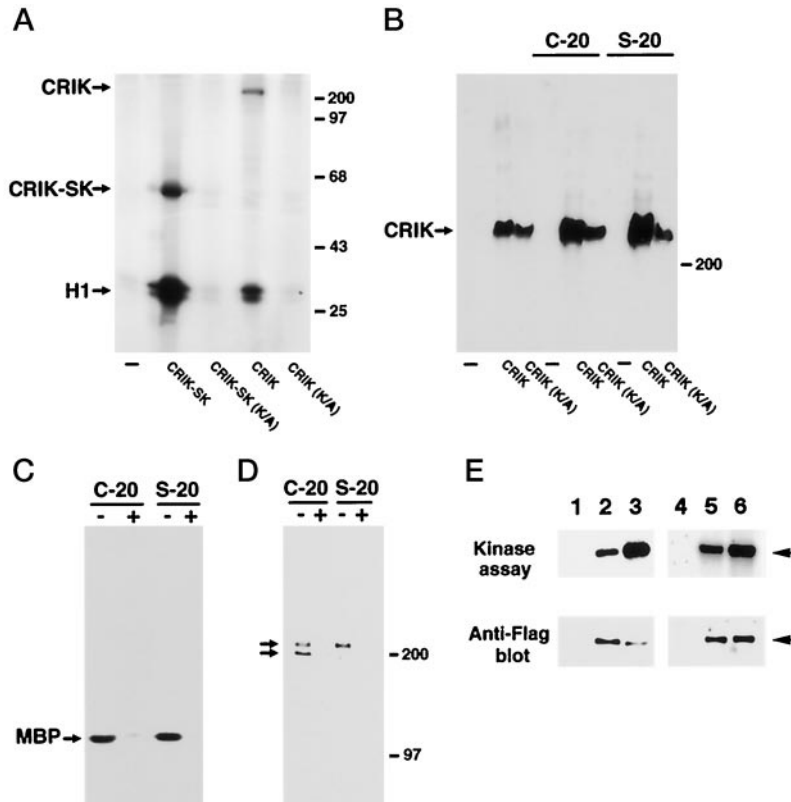


FIG. 3. Expression and kinase activity of CRIK proteins. *Panel A*, COS7 cells were transfected with 10 μ g of empty vector (–), alongside expression plasmids for either wild type CRIK and CRIK-SK, or corresponding mutant proteins with a Lys to Ala substitution at position 126 (K/A). Total cell lysates were immunoprecipitated with anti-FLAG antibodies and tested by *in vitro* kinase assay with histone H1 as exogenous substrate. Samples were analyzed by 12.5% SDS-PAGE and autoradiography. The expected positions of CRIK and CRIK-SK autophosphorylated proteins, and of the molecular mass standards (kDa) are indicated. *Panel B*, total cell lysates prepared from COS7 cells transfected with empty vector (–) or the indicated FLAG expression plasmids were either analyzed as such, or immunoprecipitated with polyclonal antibodies C-20 and S-20, directed against two distinct carboxyl-terminal peptides of Citron. Proteins were separated by 5% SDS-PAGE and immunoblotted with anti-FLAG monoclonal antibodies. The position of the CRIK protein and of the 200-kDa molecular mass marker is indicated. *Panel C*, total cell extracts were prepared from growing primary mouse keratinocytes and immunoprecipitated with either C-20 or S-20 antibodies, in the absence (+) or the presence (–) of the corresponding competing peptides. *In vitro* kinase assays were performed as in the experiment of *panel A*, except that myelin basic protein (MBP) was used as an exogenous substrate. *Panel D*, total cell extracts from 35 S-labeled primary mouse keratinocytes were immunoprecipitated with either C-20 or S-20 antibodies, in the absence (+) or the presence (–) of the corresponding competing peptides, as in the experiment of *panel C*. Immunoprecipitates were analyzed by 5% SDS-PAGE and fluorography. The positions of the molecular mass markers, and of the two proteins specifically immunoprecipitated by the anti-Citron antibodies are indicated. *Panel E*, COS cells were transfected with equal amounts of empty vector (1, 4), vector for FLAG-tagged CRIK (2, 5) and vectors for either FLAG-tagged CRIK plus Rho V14 (3) or FLAG-tagged CRIK plus Rac V12. Total cell extracts were immunoprecipitated with anti-FLAG antibodies. Immune complexes were subjected to *in vitro* kinase assay in the absence of exogenous substrate. Samples were analyzed either by autoradiography (*upper panel*) or by anti-FLAG immunoblot (*lower panel*). Densitometric analysis revealed an 8-fold increase of CRIK kinase activity in the presence of Rho V14, after normalization for CRIK protein amounts, while CRIK kinase activity increased less than 1.5-fold in the presence of Rac V12.

Tissue-specific Pattern of Expression of CRIK and Citron—To examine whether the different CRIK/Citron isoforms exhibit a similar pattern of expression, Northern blots from different mouse tissues were sequentially hybridized with a probe corresponding to the catalytic kinase domain, found in CRIK and CRIK-SK but not Citron, and with a second probe within the coiled-coil region of Citron, found in CRIK and Citron but not in CRIK-SK. As in primary keratinocytes and skin, a major 9.5-kb mRNA band was found to hybridize with the kinase domain probe in brain, spleen, lung, kidney, and, as an especially strong signal, in testis (Fig. 1*B*, *left panel*). A second ~7-kb band was also detectable in the testis and, after longer exposures, in spleen, kidney, and brain (not shown). No expression was detectable in heart, liver, and skeletal muscle. When the same blot was rehybridized with the Citron probe, the two 9.5- and 7-kb bands were also detected, which showed a similar but not identical hybridization pattern as with the kinase domain probe (Fig. 1*B*, *right panel*). In particular, the RNA derived from testis again yielded the strongest signal, but the relative signal intensity of the 9.5- versus 7-kb bands was reversed with the Citron versus kinase domain probe. Similarly, in brain

RNA, the signal intensity of the 9.5-kb band was much stronger with the Citron probe and, in testis and spleen, two weaker bands of approximately 5.5 and 4.4 kb, respectively, were detected only with this probe (Fig. 1*B*).

Kinase Activity of CRIK and CRIK-SK—To demonstrate that the CRIK gene encodes functional kinases, full-length cDNAs for both CRIK and CRIK-SK were fused to an amino-terminal FLAG epitope and cloned into a cytomegalovirus mammalian expression vector. Mutant forms of these cDNAs were also constructed, with a lysine to alanine (K/A) substitution at position 126, in the predicted ATP binding pocket (18). These mutations were expected to generate CRIK and CRIK-SK forms devoid of kinase activity. Total cell extracts were prepared from COS7 cells transfected with the various expression plasmids, and immunoprecipitated with anti-FLAG antibodies. An *in vitro* kinase assay was then performed with histone H1 as exogenous substrate. Elevated kinase activity toward histone H1 was found in cells transfected with the expression constructs for wild type forms of both CRIK and CRIK-SK (Fig. 3*A*). Myelin basic protein also functioned as an efficient substrate (not shown). In addition to exogenous substrates, two *in*

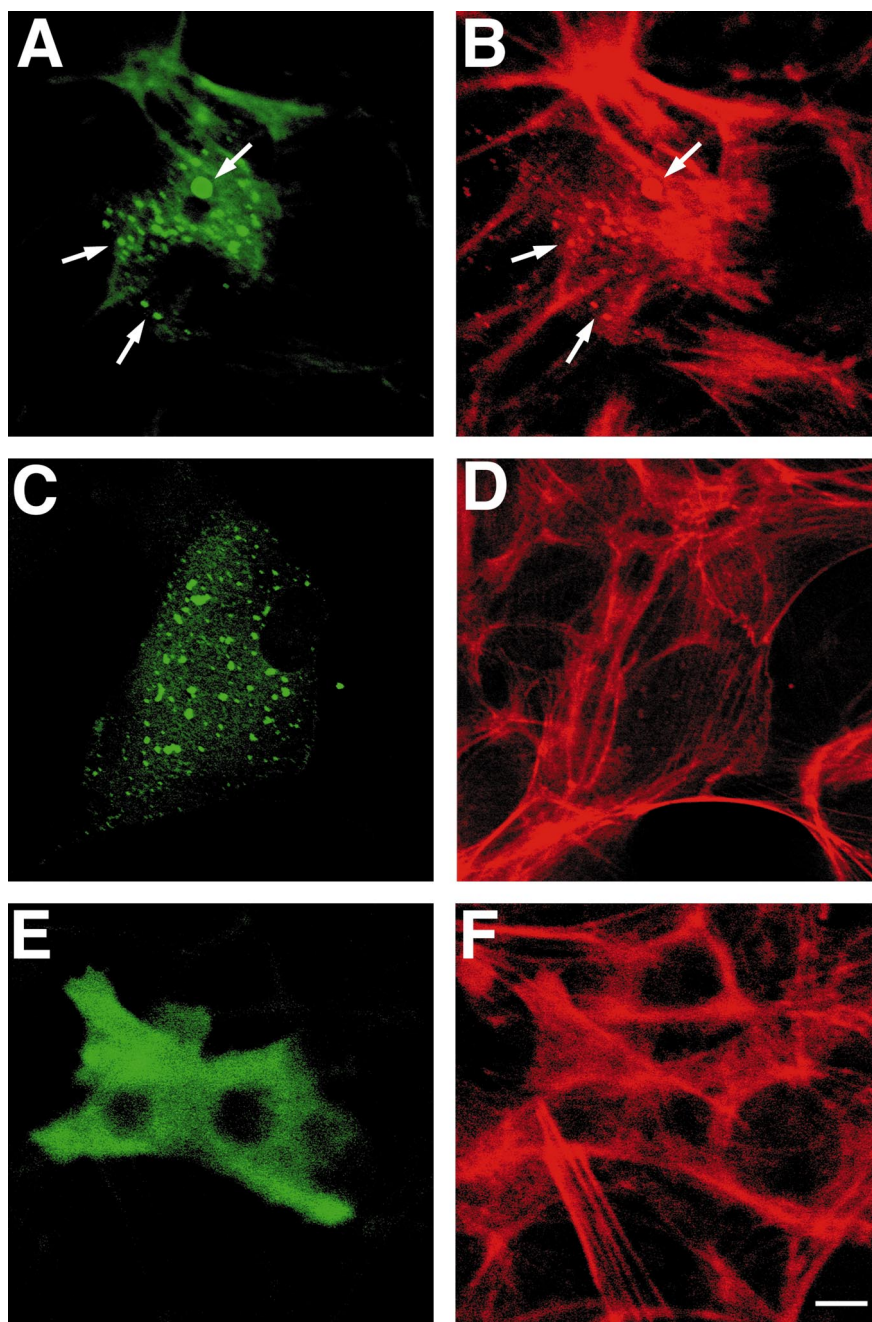


FIG. 4. Intracellular localization of CRIK and CRIK-SK in keratinocytes, and their effects on the actin cytoskeleton. Primary mouse keratinocytes grown in low calcium medium were transfected with expression vectors for FLAG-tagged CRIK (A and B), FLAG-tagged CRIK in its kinase dead mutant form (C and D), or CRIK-SK (E and F). 24 h after transfection, cells were switched to high calcium medium for additional 24 h. Cells were then fixed in 2% paraformaldehyde and double stained with anti-FLAG monoclonal antibodies (green, left panels) and phalloidin (red, right panels). Samples were analyzed by confocal microscopy. The nucleus is not evident because of a lower confocal plan. Arrows indicate the dotted structures in which CRIK and actin colocalize. The results are representative of what was observed in three independent experiments. In each case, an average of 20 transfected cells from a total of 5–10 different fields was examined. Bar, 4 μ m

in vitro phosphorylated proteins of the expected molecular weight for CRIK and CRIK-SK were found in the respective immunoprecipitates, consistent with the two proteins being capable of autophosphorylation (Fig. 3A). In contrast, none of these *in vitro* kinase activities was found in cells transfected with the mutants of CRIK and CRIK-SK harboring the Lys to Ala (K126A) substitution. Western blot analysis with anti-FLAG antibodies indicated that the wild type and mutated forms were expressed at similar levels in the transfected cells (Fig. 3B and data not shown). Activity of the ROCK kinase has been shown to be increased a few folds by coexpression of an activated form of Rho (6). Similarly, cotransfection of cells with expression vectors for CRIK and Rho(V14) resulted in an 8-fold stimulation of CRIK kinase activity, while coexpression of activated Rac (Rac(V12)) led only to a 1.5-fold stimulation (Fig. 3E).

Two different polyclonal antibodies, raised against peptides corresponding to amino acids 1547–1576 (S-20) and 1578–1597 (C-20) of the previously published Citron sequence were found

to immunoprecipitate the exogenously expressed full-length form of CRIK (Fig. 3B). Untransfected primary keratinocytes were immunoprecipitated with these antibodies, to test whether an endogenous kinase activity amenable to CRIK could be recovered. *In vitro* kinase activity with myelin basic protein as an exogenous substrate was found in the immunoprecipitates with these antibodies, and recovery of this activity was effectively competed in immunoprecipitates performed in the presence of blocking peptides (Fig. 3C). Immunoprecipitation experiments with 35 S-labeled keratinocytes revealed that two endogenous proteins were specifically immunoprecipitated by the C-20 antibodies, with molecular weights consistent with those of CRIK and Citron (Fig. 3D). Interestingly, the other antibodies immunoprecipitated only the larger of these proteins, which is likely to correspond to CRIK, both on the basis of size and on the similar recovery of kinase activity in the C-20 and S-20 immunoprecipitates (Fig. 3, C and D). Under our conditions, immunoblotting with the C-20 and S-20 antibodies

generated no detectable bands above background, thus preventing further analysis with this technique.

Subcellular Localization of CRIK and CRIK-SK in Keratinocytes and Their Effects on the Actin Cytoskeleton—In parallel with their different structure, CRIK and CRIK-SK may differ significantly in their intracellular localization and/or their effects on the actin cytoskeleton. This possibility was investigated by transfecting keratinocytes with epitope tagged forms of CRIK or CRIK-SK, followed by double immunofluorescence with epitope-specific antibodies and fluorescein isothiocyanate-conjugated secondaries (green), and BODIPY-conjugated phalloidin (red). Growing keratinocytes in medium at low calcium concentrations (0.05 mM) have a high nuclear/cytoplasmic ratio that hampers detailed analysis of their cytoplasmic organization. For this reason, cells were analyzed 24 h after growth in extracellular calcium (2 mM), which causes flattening of cell morphology, with a well ordered disposition of the actin cytoskeleton. As shown in Fig. 4, A and B, full-length CRIK showed a discrete pattern of intracellular localization, into dotted cytoplasmic structures to which F-actin was also recruited. Interestingly, this corpuscular pattern of intracellular organization was maintained by the kinase-dead mutant form of CRIK, while colocalization of actin in these structures was reduced or absent (Fig. 4, C and D). Expression of an activated Rho protein in keratinocytes did not induce the changes caused by full-length CRIK, nor additional effects were observed by overexpression of the two proteins (not shown). In contrast to full-length CRIK, CRIK-SK showed diffuse localization throughout the cytoplasm, and had little if any effect on the actin cytoskeleton (Fig. 4, E and F). Additional experiments were performed where keratinocytes were transfected with an expression vector for the epitope-tagged full-length form of CRIK and loaded with fluorescein-conjugated dextran prior to fixation. Immunofluorescence analysis with antiepitope antibodies and Texas Red-conjugated secondaries indicated that the discrete subcellular localization of CRICK does not coincide with the endosomal compartment as detected by the fluorescein isothiocyanate-dextran-labeling technique (17).

DISCUSSION

Among the downstream effectors of Rho function, ROCK proteins are probably the best characterized (5, 6, 26, 27). The activated form of Rho appears to directly associate with ROCK. Consequently, the ROCK kinase is moderately activated (6) and translocates to peripheral membranes where it may interact with some of its substrates (8, 9). Citron was reported to share many of the structural features of ROCK, including a domain mediating direct association with Rho (10). However, no biochemical activity was attributed to Citron. We have shown here the existence of a new Ser/Thr kinase, CRICK, which can be expressed in at least two forms, one of which encompasses the previously reported form of Citron in almost its entirety. Besides cDNA cloning, RT-PCR and Northern data indicate that CRIK and Citron are part of the same gene. This conclusion is further supported by our cloning of the human homologue of the CRIK kinase domain, and the finding that it maps to chromosome 12q24 1-3,² which is in excellent agreement with the map position of the human homologue of Citron (*Homo sapiens* PAC clone 127H14, GenBankTM accession no. AC002563).

Like ROCK (6), CRIK kinase activity was found to be increased a few folds by coexpression of an activated form of Rho. Interestingly, activated Rac had little or no effect, pointing to a more specific involvement of Rho in control of CRIK activity.

The ROCK kinases are ubiquitously expressed (22). By contrast, CRIK expression exhibits a striking pattern of cell type specificity, suggesting that this kinase may serve as a downstream mediator of Rho function in these cells. In keratinocytes, full-length CRIK was found to localize into corpuscular structures which do not correspond to the endosomal compartment, and whose identity remains to be established. Actin was recruited into these structures in a manner that was dependent on CRIK activity, as such recruitment was not observed after expression of a kinase dead form of CRIK. Actin recruitment was not further increased by activated Rho expression, indicating that the basal level of CRIK activity in cells overexpressing this kinase is by itself sufficient for this effect. The subcellular localization of full-length CRIK is likely to depend on one or more of the multiple domains present in the Citron moiety of the protein, as it was not observed with the CRIK-SK isoform, containing only the kinase region. Future studies will have to dissect the function of CRIK and its multiple isoforms in the specific cell types where these molecules are normally expressed.

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Encompassing the Rho-Rac-binding Protein Citron**
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