Interaction of the Calcium-sensing Receptor and Filamin, a Potential Scaffolding Protein*

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In many cases, the biologic responses of cells to extracellular signals and the specificity of the responses cannot be explained solely on the basis of the interactions of known signaling proteins. Recently, scaffolding and adaptor proteins have been identified that organize signaling proteins in cells and that contribute to the nature and specificity of signaling pathways. In an effort to identify proteins that might organize the signaling system(s) activated by the extracellular Ca²⁺ receptor (CaR), we used a bait construct representing the intracellular C terminus of the human CaR and the yeast two hybrid system to screen a human kidney cDNA library. We identified a clone representing the C-terminal 1042 amino acids (aa) of the cytoskeletal protein filamin. Analysis of truncation and deletion constructs of the CaR C terminus and the filamin cDNA clone demonstrated that the CaR and filamin interact via regions containing aa 907–997 of the CaR C terminus and aa 1566–1875 of filamin. Interaction of the two proteins in mammalian HEK-293 cells was demonstrated by co-immunoprecipitation and colocalization of them using immunofluorescence microscopy. The functional importance of their interaction was documented by transiently expressing the CaR in M2 melanoma cells that lack filamin, or in A7 melanoma cells that stably express filamin, and demonstrating that the CaR activated ERK only in the presence of filamin. Co-expression of the CaR with a peptide derived from the region of the CaR C terminus that interacts with filamin reduced the ability of the CaR to activate p42ERK in a dose-dependent manner, but did not inhibit the ability of the ET₂ receptor to activate ERK. The fact that filamin interacts with the CaR and other cell signaling proteins including mitogen-activated protein kinases and small GTPases, indicates that it may act as a scaffolding protein to organize cell signaling systems involving the CaR.

G protein-dependent signaling systems are composed of three basic sets of proteins: receptors, heterotrimeric G proteins, and effector molecules that may be enzymes, RGS proteins, or ion channels (1). The traditional model of signaling by these systems suggests that the various components, receptors, membrane-associated G proteins, and effector proteins, interact preferentially in their activated states, and that the specificity of these interactions is determined primarily by the structures of the proteins. However, this model cannot explain the specificity or character of these signaling systems that is found in vivo (2). For example, in the renal collecting duct, both vasopressin (AVP) and β-adrenergic receptors are coupled to adenyl cyclase via Go₃, but only AVP receptors stimulate H₂O transport. In intact platelets, AVP and platelet-activating factor activate phospholipase C (PLC), but do not inhibit stimulation of adenyl cyclase, whereas in platelet membranes, AVP and platelet-activating factor activate PLC and inhibit stimulation of adenyl cyclase (3). These data suggest that cell structure or additional proteins such as “scaffolding” proteins may place constraints on cell signaling systems that contribute to their specificity (4, 5).

In eukaryotic cells, signaling complexes can be organized by proteins that make use of structural motifs to recognize and position components of signaling cascades (2, 6). Signaling pathways involving receptor tyrosine kinases utilize multiple structural motifs and adaptor proteins to generate complex signals (7). Examples of organization of signaling pathways by structural proteins include STE-5, which coordinates members of the MAP kinase cascade in Saccharomyces cerevisiae; INAD, a pentaavental PDZ domain-containing protein in the Drosophila retina that coordinates rhodopsin, PLC, protein kinase C (PKC), and Ca²⁺ channels; NHE-RF, a PDZ domain-containing protein that couples the β-adrenergic receptor to NHE-3; Homer, a protein that couples the M5 metabotropic glutamate receptor to intracellular Ca²⁺ stores; and Jip-1 (Jun kinase interacting protein) that coordinates members of the Jun kinase cascade in mammalian cells (5, 6, 8–10).

The cytoskeletal actin-binding protein filamin (actin-binding protein 280, ABP-280, FLN1) may act as a scaffolding protein for signaling cascades because it binds a number of cell surface receptors and intracellular signaling molecules, and disruption of the interactions of these proteins with filamin interferes with signaling by them (11–14). Filamin is a ubiquitously expressed homodimer composed of 2647 aa (280 kDa) monomers, each of which contains 24 immune globulin-like repeats, two hinge regions, a dimerization domain at the C terminus, and an actin-binding domain at the N terminus (15). Filamin cross-links actin in orthogonal arrays and directly links cell surface receptors to the cytoskeleton. Filamin interacts directly and specifically with the dopamine D₂ receptor (a G protein-coupled receptor), the Fcy receptor, platelet glycoprotein Iba, β₁ and β₂ integrins, tissue factor, MAP kinases, the tumor necrosis factor receptor-associated factor-2 (TRAF2), Rhe GTPases, and Smad proteins (12, 13, 16–21). Filamin also interacts directly with caveolin, a protein component of caveolae, structures that contain many signaling molecules including the CaR and G proteins (22, 23). Filamin is involved in protein trafficking and

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contributes to localization and cycling of proteins in the cell (14, 24). These results suggest that filamin may serve as a scaffolding protein to co-localize and organize signaling molecules from a variety of signaling systems.

The extracellular calcium-sensing receptor, a G protein-coupled receptor that responds to extracellular Ca$^{2+}$ and other polycations, was recently cloned by expression (25, 26). This receptor is expressed in parathyroid cells where it is the Ca$^{2+}$ sensor that regulates PTH secretion, the kidney where it contributes to Ca$^{2+}$, Na$^{+}$, Cl$^{-}$, and H$_2$O balance, and in the brain, gastrointestinal tract, skin, and other epithelial tissues where its role is less clear (27). Recent studies indicate that the CaR may participate in paracrine signaling (28, 29). In various tissues, the CaR regulates numerous messengers and cellular activities (30, 31). However, these second messenger systems are not sufficient to explain all of the biologic functions of the CaR (32).

In an effort to identify proteins that interact with the CaR and that could potentially organize the signaling proteins through which it acts, we used the yeast two-hybrid cloning approach with the C terminus of the CaR as bait to screen an adult kidney cDNA library. We identified a number of interacting proteins, one of which was filamin.

**EXPERIMENTAL PROCEDURES**

**Materials**—Chemicals were purchased from Sigma or Fisher Scientific unless specified otherwise. Tissue culture medium and serum were obtained from Life Technologies, Inc., plasticware was from Falcon, the HEK-293 cells were from the American Tissue Culture Collection, restriction and DNA-modifying enzymes were purchased from Promega, and Superfect and plasmid preparation kits were from Qiagen. The cDNA coding for the human CaR was a gift from Drs. E. M. Brown and M. Bai (Harvard University, Boston, MA), and the full-length filamin cDNA was from Dr. John Hartwig (Harvard Medical School, Boston, MA). The M2 and A7 melanoma cells, originally described by Cunningham et al. (33), were from Dr. Fred Southwick (University of Florida, Gainesville, FL).

**Yeast Two-hybrid Cloning**—A cDNA representing the C-terminal 219 as of the human calcium receptor (aa 861–1078) in the bait plasmid pAS2.1 was used to screen a human adult kidney cDNA library. We identified a number of interacting proteins, one of which was filamin.

** Interaction of the Calcium Receptor with Filamin**

**Antibodies**—The monoclonal antibody against the CaR was produced in mice by standard techniques in the University of Florida hybridoma core facility. A synthetic peptide (WHSSAYGPDQRAQ) that corresponds to amino acids 15–29 in the extracellular N terminus of the CaR was synthesized by the University of Florida biotechnology core facility and injected into mice, and hybridomas were prepared. Supernatants were screened by enzyme-linked immunosorbent assay for reactivity against the peptide. Positive clones were tested against membranes from HEK 293 cells that express an HA-tagged CaR and membranes prepared from the medulla and cortex of mouse kidneys. The monoclonal antibody to the CaR (6D4) identified specific bands of 125 and 140 kDa that were also identified by the anti-HA antibody in the membranes from the cells that expressed the HA-tagged CaR.

**Immunoprecipitation**—Cells that expressed the CaR were grown to ~80% confluence on polystyrene-coated coverslips. The cells were washed with PBS and fixed in 4% paraformaldehyde for 20 min, washed three times in PBS, quenched for 30 min in PBS with 50 mM glycine, and washed again in PBS. The cells were blocked and permeabilized by incubating them for 30 min in PBS containing 3% BSA, 0.1% Triton X-100. The cells were incubated with primary antibodies (10 μg/ml CaR monoclonal) and 1 μg/ml rhodamine phalloidin in PBS containing 3% BSA, 0.5%, or 1% Triton X-100 for 2 h at room temperature. The slides were then washed three times with PBS and incubated in secondary antibody (1:200 FITC-conjugated goat anti-mouse IgG (H+L) in PBS containing 3% BSA, 5% BSA, and 0.1% Triton X-100 for 2 h at room temperature. The cells were then washed in PBS and attached to slides with a solution that contained 4.6-diamidino-2-phenylindole to visualize nuclei. Images of the cells were obtained with a Zeiss Axiopt fluorescence microscope, and images were stored using a digital Spot camera (Diagnostic Instruments, Inc.).

**Expression of cDNAs in Cell Lines**—cDNAs were transfected into HEK293 cells using the CaPO$_4$ co-precipitation technique (35). A total of 3 μg of DNA (a combination of cDNAs coding for proteins of interest with empty vector and carrier DNA) were added to 60-mm dishes and allowed to precipitate. The medium was changed after 18–20 h, and experiments were performed 48 h after transfection. For stable expression of the CaR in HEK293 cells, stable clones were selected with G-418. cDNAs were transfected into M2 and A7 melanoma cells using Superfect according to the manufacturer’s instructions. Experiments were performed 48 h after transfection.

**Immunoprecipitation**—Cells were lysed on ice in immunoprecipitation (IP) buffer containing 125 mM NaCl, 62.5 mM Na$_2$HPO$_4$, pH 7.2, 0.625% C$_4$H$_8$EO$_3$ (Lubrol), and protease inhibitors. The lysates were centrifuged at 13,500 rpm in a microcentrifuge for 30 min at 4°C and incubated with an equal volume (500 μl) of the hybridoma supernatant from clone 6D4 (anti-CaR monoclonal antibody) or the anti-filamin corresponding to aa 987–1078 was produced using a sense primer (TTG GAT CCG CAG CCT CCT CC) that contained an NdeI site, and an antisense primer (GCC CAG TCG ACT CCT CCT ATT) that contained a SalI site. The PCR fragments were cloned into the TA cloning vector pCR2.2-TOPO (Invitrogen) and then subcloned in frame into pAS2.1 (Invitrogen) using the NheI/SalI sites. The sequences of these fragments were verified by dideoxy sequencing.

The filamin constructs were produced in a similar manner by PCR using the human cDNA clone as a template (15). The fragment corresponding to aa 1566–1579 was produced using the sense primer containing EcoRI/SalI sites (GCC GAA GTA TTC GTC GAG TGG TGC TGC TCC ATC GCT) and the antisense primer containing an XbaI site (TTT TCT CCA TAG CCT ATC AA) that contained XhoI and BglII sites. The fragment corresponding to aa 2021–2647 was produced using the sense primer that contained BamHI/KpnI sites (CAG GTC ACC AGC AGG ATG GCC TCG ATG GTG TTG AT), and the antisense primer (CCC AGA TCT CGA GGT TCA ACA CCA TGA ACT GGA A) into which XhoI and BglII sites had been incorporated. The fragment corresponding to aa 2402–2647 was produced using a sense primer containing BamHI and KpnI sites (ACC GCG ATC CCT GGT ACC CCC TTC AAC AAT CT) and an antisense primer containing an XbaI site (TTT TCT CCA TAG CCT ATC). The PCR products were cloned into pCR2.2-TOPO, amplified, and then subcloned into pACT2 in frame. The sequences were verified using dideoxy sequencing. For the mammalian expression studies, the fragment representing aa 907–1024 was subcloned from pBluescript into pCruzyMyB using the KpnI and BglII sites.

**Dedication**—We thank John Aris (University of Florida, Gainesville, FL) and maintained expression of the CaR in HEK-293 cells, stable clones were selected with G-418. cDNAs were transfected into M2 and A7 melanoma cells using Superfect according to the manufacturer’s instructions. Experiments were performed 48 h after transfection.

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antibody (45 μg) at 4 °C by slow rotation overnight. The samples were then incubated with 7.5 μl of rabbit anti-mouse antibody for 30 min at 4 °C. The precipitations were performed by incubating the extracts with Pansorbin cells (S. aureus, Calbiochem) for 30 min on ice and then centrifuging them at top speed in a microcentrifuge at 4 °C. The pellet was resuspended in 1× IP buffer, centrifuged at top speed, and processed for immunoblotting with the antibody indicated.

Preparation of Cell Extracts and Immunoblotting, and Measurement of ERK Activity—HER-293, M2, and A7 melanoma cells were serum-deprived overnight before experiments. At the time of experiments, the medium was replaced with a solution containing 150 mM NaCl, 5 mM KCl, 10 mM HEPES, pH 7.4, 0.5 mM CaCl₂, and 0.5 mM MgCl₂. At time 0, reagents were added at the concentrations indicated and incubated with the cells at 37 °C for the times indicated. Commonly, the cells were exposed to activators of the receptor for 5 min, at which time the reactions were stopped by rinsing the cells at 4 °C in buffer containing 50 mM NaF, 100 mM NaCl, 0.1 mM sodium orthovanadate, and 20 mM NaH₂PO₄, pH 7.4–7.5, and placing the dishes on a dry ice and ethanol bath. The cells were scraped in ice-cold buffer that contained 50 mM Tris, pH 7.5, 50 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM β-glycerophosphate, 50 mM NaF, 50 mM okadaic acid, 5 mM sodium pyrophosphate, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 40 mM p-nitrophenyl phosphate, 4 μg/ml pepstatin, 4 μg/ml aprotinin, 4 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The cell lysates were centrifuged at 15,000 × g for 10 min in a refrigerated microcentrifuge. Triton-soluble extracts were normalized for protein, size-fractionated using SDS-PAGE, and processed for immunoblotting. Proteins were detected by enhanced chemiluminescence.

Identification of Proteins That Interact with the C Terminus of the CaR in Yeast—In order to identify proteins that interact with the CaR and that could act as scaffolding proteins to organize the signaling molecules regulated by the CaR, we used the C-terminal 219 aa (aa 861–1078) of the human CaR representing the cytoplasmic tail as bait in the pAS2 vector (Invitrogen) to screen a human adult kidney cDNA library in pACT2 (Invitrogen) using the yeast two-hybrid system (36). We used the yeast strain PJ69-4A, which offers triple genetic selection and a reduced rate of false positive clones (34). The bait plasmid and the cDNA library were co-transformed into the yeast using the lithium acetate/single-stranded DNA/polylethylene glycol method of Geitz et al. (37), and plated on medium deficient in Leu, Trp, and His. The plates were replicated on media lacking His, Trp, Leu, and adenine, and the surviving colonies were tested for β-galactosidase activity. Positive clones were sequenced from either end. Four positive clones were isolated and sequenced, one of which was filamin 1 (ABP-280). The filamin clone was 3326 nucleotides in length and represented the C-terminal 1082 aa (aa 1566–2647), which includes the C-terminal 61 aa of repeat 14, repeats 15–26, and the two hinge regions (15, 38).

Further analysis of the CaR C terminus in yeast (Fig. 1) demonstrated that the first 48 aa of the C terminus distal to the seventh membrane-spanning domain (aa 860–908), a region of many receptors that interacts with G proteins and that is required for signaling by the CaR, does not interact with filamin (39, 40). The interacting region includes aa 907–997, but may also include additional amino acids because the full-length C terminus interacts with filamin more strongly based on growth and β-galactosidase assays (Table I). The interacting 91-aa region is rich in glutamine and proline residues but does not contain sequences that correspond to functional domains that mediate protein-protein interactions such as Src homology regions 2 and 3, or PDZ domains (Fig. 1, bottom).

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**RESULTS**

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**Interaction of the CaR and Filamin in Mammalian Cells**—In order to determine if the CaR and filamin interact in mammalian cells, we communoprecipitated the two proteins using...
ovals represent hinge regions in the filamin protein. The 

TABLE I
Interaction of CaR C-terminal constructs with two filamin constructs

| CaR construct | Filamin 1566–2647 growth | Filamin 1566–1647 growth | Filamin 1566–1647 growth | Filamin 1566–1647 growth |
|---------------|-------------------------|-------------------------|-------------------------|-------------------------|
|               | β-Gal units             | β-Gal units             | β-Gal units             | β-Gal units             |
| Vector (pAS2.1) | –                       | –                       | –                       | 4.2                     |
| 860–1078      | +                       | +                       | +                       | 15.5                    |
| 860–991       | +                       | +                       | +                       | 11.0                    |
| 860–908       | +                       | +                       | +                       | 5.1                     |
| 907–997       | +                       | +                       | +                       | 12.3                    |
| 987–1078      | –                       | –                       | –                       | 6.2                     |

Fig. 2. Map of filamin cDNA clone obtained from the human kidney cDNA library and filamin deletion and truncation constructs and their interactions with the CaR C terminus (aa 860–1078) and the minimal interacting region of the CaR (aa 907–997). The CaR and filamin constructs were co-transformed into the yeast strain PJ69 4A, and assayed for growth on medium lacking His, Leu, and adenine. + represents interaction of the constructs indicated based on growth on selective medium and stimulation of β-galactosidase activity (49). – represents failure of growth and the absence of a protein-protein interaction, and no stimulation of β-galactosidase activity. The numbers along the top line refer to nucleotide positions in the human sequence with TGA representing the stop codon. The numbered boxes represent repeats, and the ovals represent hinge regions in the filamin protein. The numbers at the beginning and end of the lines refer to aa positions in the human full-length filamin sequence (15).

TABLE II
Interaction of filamin constructs with two CaR C-terminal constructs

| Filamin construct | CaR 860–1078 growth | CaR 860–1078 growth | CaR 907–997 growth | CaR 907–997 growth |
|-------------------|---------------------|---------------------|--------------------|--------------------|
|                   | β-Gal units         | β-Gal units         | β-Gal units        | β-Gal units        |
| Vector (pACT2)    | –                   | –                   | –                  | 6                  |
| 1566–2647         | +                   | 18.2                | +                  | 12.1               |
| 1566–1875         | + +                 | 21.3                | +                  | 13.8               |
| 2021–2647         | –                   | 8                   | –                  | 7                  |
| 2021–2029         | –                   | 6                   | –                  | 8.2                |
| 2402–2647         | –                   | 4.9                 | –                  | 5.9                |

either the anti-CaR antibody, 6D4, or the anti-filamin antibody. Fig. 3 shows co-immunoprecipitation of the CaR and filamin from HEK-293 cells that stably express the CaR. G418-resistant HEK293 cells (expression vector, V) were used as controls. In the top panels, 6D4 was used to immunoprecipitate the CaR and its associated proteins. The IP pellet was immunoblotted with either the anti-filamin antibody (left panel) or an anti-HA antibody that recognizes the HA-tagged CaR (right panel). In the bottom panels where the anti-filamin antibody was used for immunoprecipitation, the IP pellet was immunoblotted with the anti-filamin antibody (left) or the anti-HA antibody (right). Filamin is present in the pellet immunoprecipitated with 6D4, and the CaR is present in the pellet immunoprecipitated with the anti-filamin antibody. These results demonstrate that the CaR and filamin are present in the same complex in mammalian cells.

Immunocytochemistry to Co-localize the CaR and Filamin—If the CaR and filamin interact in mammalian cells, they should co-localize in HEK-293 cells that stably express the CaR. Since both of our antibodies are mouse monoclonal proteins, we chose to co-localize each of them with actin because filamin and actin are associated in vivo (41). In the top three panels of Fig. 4, cells were stained with the anti-CaR antibody, 6D4 (green top left), and rhodamine-phalloidin (red center panel) and the images were merged in the top right panel. Areas of overlap of the two proteins are seen as orange or pale green, indicating co-localization of actin (rhodamine phalloidin) and the CaR. In the bottom three panels, cells were stained with the anti-filamin antibody, (green bottom left), and rhodamine-phalloidin (red center panel) and the images were merged in the top right panel. The bottom right panel demonstrates that filamin and actin co-localize in the HEK-293 cells. The finding that both the CaR and filamin co-localize with actin demonstrates that these two proteins co-localize with each other in the region of the plasma membrane. These results together with the co-immunoprecipitation data indicate that the CaR...
and filamin co-localize in mammalian cells.

**Functional Interaction of the CaR with Filamin**—To test for a functional interaction of the CaR and filamin, we expressed the CaR transiently in M2 melanoma cells that lack filamin, and in A7 cells (M2 cells in which filamin has been stably expressed; Ref. 33), and compared CaR-stimulated ERK activity in the two cell types. We documented transient expression of the CaR and filamin in the A7 cells by immunoblotting (Fig. 5, top panels) or the anti-filamin antibody (bottom panels). In the top panels (IP 6D4), the immunoprecipitation pellet was immunoblotted with either the anti-filamin antibody (left, 1:2000) or the monoclonal antibody 12CA5, which recognizes the HA-tagged CaR, (right, 1:500). In the bottom panels (IP anti-filamin), the immunoprecipitation pellet was immunoblotted with the anti-filamin antibody (left) or 12CA5 (right). Blots were visualized with ECL using a horse-radish peroxidase-conjugated mouse IgG (1:10,000).

Fig. 3. Reciprocal co-immunoprecipitation of filamin and the CaR. HER-293 cells that stably express pcDNA3 (V) or the HA-tagged CaR (CaR) were extracted and incubated with either the anti-CaR antibody 6D4 (top panels) or the anti-filamin antibody (bottom panels). In the top panels (IP 6D4), the immunoprecipitation pellet was immunoblotted with either the anti-filamin antibody (left, 1:2000) or the monoclonal antibody 12CA5, which recognizes the HA-tagged CaR, (right, 1:500). In the bottom panels (IP anti-filamin), the immunoprecipitation pellet was immunoblotted with the anti-filamin antibody (left) or 12CA5 (right). Blots were visualized with ECL using a horse-radish peroxidase-conjugated anti-mouse IgG (1:10,000).

To specifically disrupt the CaR-filamin interaction and test for effects on signaling, we overexpressed a Myc-tagged peptide corresponding to the 116-aa region of the C terminus of the CaR (aa 907–1022) that interacts with filamin (Fig. 1) together with the CaR and measured endogenous ERK activation in response to 5 mM Ca$^{2+}$ (5.0 mM) does not activate ERK. However, in the A7 cells that do express filamin, 5 mM Ca$^{2+}$ activated ERK. These results demonstrate that filamin is required for activation of ERK by the CaR. However, because filamin has many functions in the cell, complete absence of filamin could disrupt signaling by the CaR for reasons unrelated to the CaR-filamin interaction (11).

To specifically disrupt the CaR-filamin interaction and test for effects on signaling, we overexpressed a Myc-tagged peptide corresponding to the 116-aa region of the C terminus of the CaR (aa 907–1022) that interacts with filamin (Fig. 1) together with the CaR and measured endogenous ERK activation in response to 5 mM Ca$^{2+}$. As shown in Fig. 6, expression of the CaR peptide 907–1022 reduced activation of ERK in a dose-dependent manner by approximately 50% at the maximum amount of peptide expressed. Fig. 6 also shows that expression of increasing amounts of cDNA coding for the peptide resulted in increasing amounts of peptide expression in the cells. In control cells, the CaR and Myc-tagged β-galactosidase were co-expressed. Fig. 7 demonstrates that the effects of the CaR-C-terminal peptide (aa 907–1022) are specific for signaling by the CaR. The peptide inhibits activation of ERK by the CaR, but not the ET$_4$ receptor. Although these results do not define the precise mechanism by which the CaR peptide inhibits signaling, they demonstrate that the peptide interferes with an essential function of the receptor presumably by competing with the CaR for binding to filamin.

**DISCUSSION**

The factors that determine the specificity and character of G protein-dependent signaling systems are not fully understood, but involve more than just the specificity conferred by the interactions of the signaling proteins themselves. Despite the fact that many receptors such as those for vasopressin, acetylcholine, Bradykinin, thrombin, and extracellular Ca$^{2+}$ act via similar mechanisms (Go, Gi, phospholipase C, intracellular Ca$^{2+}$, protein kinase C, and the MAP kinases), they have distinct biologic effects, and responses are different in whole cells and in disrupted cells (3, 42). One mechanism by which individual receptors could control unique signaling pathways is through interaction with a unique combination of proteins that is determined by the receptor and structural proteins of the cell. These structural proteins could include cytoskeletal proteins or proteins that are particular to a cell compartment or cell type. These proteins and the receptor in combination could then determine which additional proteins would participate in a signaling complex. The CaR and filamin could be the basic components of such a complex.

Our results demonstrate that the CaR interacts with filamin, a cytoskeletal protein that has functions beyond acting as a structural protein that simply cross-links actin. The interaction of filamin and the CaR is important for normal signaling by the CaR. Our results are consistent with those of others who have demonstrated that filamin directly binds a number of signaling proteins, and that to the extent that it has been studied, the interaction of filamin and the other signaling proteins is important functionally. The other proteins that interact with filamin include the D$_2$ dopamine receptor, SEK-1 (MKK-4, a member of the Jun kinase cascade), TRAF-2 (TNF receptor-associated factor-2), the small GTPases Rac, Rho, CDC42, and RaLA, and Smad proteins (12–14, 18, 21). In the absence of filamin (M2 melanoma cells), the ability of the D$_2$ dopamine receptor to inhibit forskolin-stimulated cAMP production was reduced (14). The studies by Marti et al. demonstrated that SEK-1 interacted with filamin, and that activation of SEK-1, MAPK, and p38 by TNF and lysophosphatidic acid were reduced in M2 melanoma cells that lack filamin but that the responses in A7 cells that express filamin were intact (12). Related studies by Leonardi et al. (13) demonstrated that TRAF-2, a TNF receptor-associated protein, interacts with filamin, and that in filamin-deficient M2 melanoma cells, activation of stress-activated protein kinase and NF-κB via TRAF-2 is abolished. In the absence of filamin, activation of Smad 5 by transforming growth factor-β is defective (21). The functional significance of the interaction of filamin with the small GTPases (Rac, Rho, CDC42, and RaLA) has not been established, although these proteins contribute to the structure of the actin cytoskeleton and are involved in many signaling pathways (18).

Filamin also participates in protein trafficking and may contribute to localization and cycling of cell signaling complexes in the cell (14, 24). The distribution of D$_2$ receptors on the cell surface is altered in cells that lack filamin (14). In one well-defined experimental system, filamin tethers furin, a protease that interacts with it, on the cell surface reducing its rate of internalization. Filamin is required for efficient sorting of furin to the trans-Golgi network, and is also required for appropriate localization of late endosomes and lysosomes in the cell. However, the absence of filamin does not affect the cycling of proteins that traffic independently of the microfilament cytoskeleton such as the transferrin receptor (24). Although the exact
mechanism is not defined and the biologic significance is not understood, oxygenation-reoxygenation injury and H$_2$O$_2$ cause translocation of filamin from the region of the plasma membrane to the cytosol (43). These findings indicate that filamin has multiple functions in cells that include acting as a structural element, acting as a scaffolding protein for signaling systems and acting as a protein that participates in protein trafficking.

Our data from the yeast two-hybrid analysis demonstrate that the CaR interacts with a region of filamin that corresponds to aa 1566–1875, or most of repeat 14, repeat 15, a hinge region, and repeat 16. The D$_2$ receptor, another G protein-coupled receptor that inhibits adenylyl cyclase, interacts with the same general region of filamin as the CaR (14). This region of filamin may be important for localizing G protein-coupled receptors that inhibit adenylyl cyclase with other proteins in their signaling pathways. Furin is another protein that interacts with the same general region of filamin as the CaR. Furin is a protease that cleaves and activates a number of prohormones including pro-PTH, pro-PTHrP, pro-ET-1, pro-insulin-like growth factor-1, and pro-transforming growth factor-$eta$1 (44). Placing a prohormone processing enzyme near a group of signaling molecules would localize additional components of cell signaling systems. Finally, the fact that filamin interacts with caveolin, a protein that interacts with and colocalizes with a number of signaling proteins, and that has itself been suggested to be a scaffolding protein, further indicates that filamin participates in the organization of signaling complexes in the cell (23).

The CaR interacts with filamin through a 91-aa region (aa 907–997) located in the mid portion of the intracellular C terminus of the receptor. The interaction of the filamin constructs is stronger with the full-length CaR C terminus than the shorter aa 907–997 constructs based on growth and -galactosidase assays (Tables I and II). These results suggest that additional regions of the CaR C terminus, possibly more C-terminal, also contribute to the interaction of these two proteins. However, the aa 907–997 peptide appears to contain the essential binding activity for the interaction because the fragment containing aa 987–1078 does not interact with filamin (Fig. 1 and Table I). Based on the data from the yeast two-hybrid cloning system, the interaction is direct. Supporting data from mammalian cells are co-immunoprecipitation of the two proteins (Fig. 3) and co-localization of them in HEK-293 cells by immunofluorescence (Fig. 4). This interaction is important for the function of the CaR because the CaR did not signal normally in cells that do not express filamin, and co-expression of a peptide corresponding to the region of the receptor that interacts with filamin that would be expected to interfere with the CaR-filamin interaction reduced the ability of the receptor to activate ERK (Figs. 5–7).

Studies by others indicate that the intracellular C terminus of the CaR has functional importance. Two groups found that mutation or loss of the C terminus distal to the seventh transmembrane domain (constructs beginning at aa 866 or 886) reduced signaling by the CaR seen as either a reduction in cooperativity and a reduction in sensitivity to Ca$^{2+}$, or loss of signaling when intracellular Ca$^{2+}$ (Ca$^{2+}$i) or phosphoinositide

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**Fig. 4.** Colocalization of filamin and the CaR in HEK-293 cells that stably express the CaR. Top left, CaR; top middle, actin; top right, overlay CaR and actin. Bottom left, filamin; bottom middle, actin; bottom right, overlay filamin and actin. The CaR was identified with the CaR antibody 6D4 and FITC-labeled anti-mouse IgG, filamin was identified with the anti-filamin monoclonal antibody and FITC-labeled anti-mouse IgG, and actin was identified with rhodamine-phalloidin. Cells were fixed in paraformaldehyde, incubated with the antibodies indicated, and secondary antibodies conjugated to FITC. Rhodamine-phalloidin was added with the primary antibody. Images were obtained with a Zeiss Axioshot fluorescence microscope and stored using a digital Spot camera (Diagnostic Instruments, Inc.). (Original magnification, ×40.)
and replete (A) melanoma cells. The interaction of the CaR with filamin may be indicated that the CaR does not activate ERK in the absence of ERK activation, that may depend on the interaction of component(s) of the ERK kinase cascade with filamin (12). Our data indicate that the CaR does not activate ERK in the absence of filamin (Fig. 5). The interaction of the CaR with filamin may be important for activation of ERK, and relatively unimportant for regulation of PLC and Ca\(^{2+}\). The levels of expression or localization of the truncated receptor constructs within the cells may have affected their function. The construct of Ray et al. (39), truncated at aa 903, exhibited a higher level of cell surface expression than the wild type receptor, and Gama and Breitwieser’s (40) progressive truncations from aa 908 to 868 appear to lead to progressively more membrane expression. The increased cell surface expression could compensate for less efficient signaling by individual receptors resulting from failure to interact with filamin. Finally, loss of the CaR C terminus from aa 903 or 908 could have multiple effects on receptor function that could obscure the effect of loss of the CaR-filamin interaction.

The region of the CaR that interacts with filamin, aa 907–997, does not contain regions of homology to other known proteins based on a search of GenBank\(^{38}\). The sequence is rich in glutamine residues, containing 22, with several regions of two to four glutamine residues separated by one to five other amino acids. The high content of glutamine residues is interesting in the context of neurodegenerative diseases caused by proteins with expanded glutamine sequences (45, 46). The interacting region of the CaR C terminus does not contain the

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**Fig. 5. Activation of ERK by the CaR in filamin-deficient (M2) and replete (A7) melanoma cells.**

A, CaR-stimulated ERK activity. B, immunoblot demonstrating expression of the CaR in M2 and A7 cells transiently transfected with either the CaR in pcDNA3 or pcDNA3. C, immunoblot demonstrating absence of filamin in M2 cells and its expression in A7 cells. M2 and A7 melanoma cells were transiently transfected with the pcDNA3 expression vector or the CaR, as indicated using Superfect. After 48 h, the cells were stimulated with 5.0 mM Ca\(^{2+}\) for 5 min, extracts were prepared, and ERK activity was measured as -fold stimulation over basal using an anti-active ERK antibody and densitometry. The values shown represent four separate transfections and are shown as mean ± S.D. In B and C, cell extracts were prepared, size-fractionated by SDS-PAGE, and immunoblotted with antibodies to the CaR (B) or filamin (C).

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**Fig. 6. Inhibition of CaR-stimulated ERK activity by expression of a peptide derived from the CaR C terminus.** A, ERK activity in cells transiently cotransfected with increasing amounts of the Myc-tagged C-terminal peptide (aa 907–1022), and decreasing amounts of Myc-tagged β-galactosidase. B, expression of the Myc-tagged C-terminal peptide. C, expression of Myc-tagged β-galactosidase. The CaR was transiently coexpressed with the Myc-tagged 116-aa region of the CaR C terminus that interacts with filamin (aa 907–1022) and Myc-tagged β-galactosidase in HEK-293 cells. In the control cells, Myc-tagged β-galactosidase was used in place of the CaR C-terminal peptide. Increasing amounts of cDNA coding for the CaR C-terminal peptide were used along with decreasing amounts of c-Myc-β-galactosidase so that the total amount of cDNA coding for peptides was constant (3 μg). Forty eight hours after transfection with CaPO\(_4\), ERK activity was measured in response to treatment with 5 mM Ca\(^{2+}\) with an anti-active ERK antibody and densitometry. Immunoblots were probed with the anti-active ERK or 9E10 (anti-Myc). This figure is representative of three separate experiments.
region just distal to the seventh transmembrane domain that is required for signaling and that characteristically interacts with G proteins (4, 40).

The C terminus of the CaR contains three potential PKC consensus sites for phosphorylation, Thr<sup>989</sup>, Ser<sup>995</sup>, and Ser<sup>997</sup> that appear particularly important for PKC-mediated inhibition of CaR signaling in parathyroid and HEK-293 cells (47). The region of the CaR C terminus that interacts with filamin (aa 907–997) contains one of these sites, Ser<sup>997</sup>. Phosphorylation of a PKC consensus site in the third cytoplasmic loop of the D<sub>2</sub> dopamine receptor, the region of the D<sub>2</sub> receptor that binds filamin, reduces the affinity of it for filamin (14). Phosphorylation of the third PKC consensus site (Ser<sup>997</sup>) in the C terminus of the CaR could inhibit CaR signaling by reducing its affinity for filamin.

Overexpression of the peptide derived from the CaR C terminus that interacts with filamin (aa 907–1022) reduces activation of ERK by the CaR by ~50%, while ERK activation in the M2 melanoma cells that lack filamin is completely inhibited (Figs. 5–7). This apparent discrepancy in results could arise for several reasons. Filamin is probably not the only structural protein that organizes the component proteins of the CaR-regulated signaling pathway. In the presence of filamin and with expression of the CaR C-terminal peptide, the interaction of the CaR with filamin would be blocked. However, the CaR would still be able to interact with many other proteins in the complex due to interactions with them that are independent of filamin. In this case, the interaction of the CaR with filamin would be "catalytic" in that it would increase the efficiency with which the CaR activates signaling molecules. Complete absence of filamin, such as in the M2 cells, could result in loss of CaR-stimulated ERK activity because filamin interacts with many components of the CaR-dependent signaling pathway. Loss of filamin would result in complete disorder of the signaling proteins in the cell, and loss of their ability to interact efficiently enough to signal. Alternatively, the 50% inhibition of CaR-dependent stimulation of ERK activity seen in Figs. 6 and 7 could result from the fact that the CaR and peptides were transiently cotransfected into the cells, and endogenous ERK activity was measured. If the receptor was expressed in some cells without inhibitory peptide, submaximal inhibition of stimulated ERK activity would be observed. Finally, the expressed peptide may be an imperfect antagonist of the CaR-filamin interaction, allowing some level of association between the two proteins to take place under all conditions. At this time, we cannot distinguish among these possibilities.

Filamin may contribute to the organization of signaling complexes at numerous levels by organizing their interactions and processing by cells. Many proteins that interact with filamin including the CaR, tissue factor, and integrins also associate with caveolin, a protein that is characteristically associated with caveoli, cell structures that are associated with many signaling molecules (22, 48). Since filamin is expressed ubiquitously, by itself, it is unlikely to provide all of the specificity necessary for the many signaling systems found in cells. Filamin with the CaR and possibly other proteins such as caveolin could form the basic components of a signaling scaffolding system in which they provide localization information for some signaling proteins. In this model, the receptor would provide the additional information giving each receptor-stimulated signal its unique character.

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