Filamin-A Binds to the Carboxyl-terminal Tail of the Calcium-sensing Receptor, an Interaction That Participates in CaR-mediated Activation of Mitogen-activated Protein Kinase*

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The G protein-coupled, extracellular calcium-sensing receptor (CaR) regulates parathyroid hormone secretion and parathyroid cellular proliferation as well as the functions of diverse other cell types. The CaR resides in caveolea-plasma membrane microdomains containing receptors and associated signaling molecules that are thought to serve as cellular “message centers.” An additional mechanism for coordinating cellular signaling is the presence of scaffold proteins that bind and organize components of signal transduction cascades. With the use of the yeast two-hybrid system, we identified filamin-A (an actin-cross-linking, putative scaffold protein that binds mitogen-activated protein kinase (MAPK) components activated by the CaR) as an intracellular binding partner of the CaR’s carboxyl (COOH)-terminal tail. A direct interaction of the two proteins was confirmed by an in vitro binding assay. Moreover, confocal microscopy combined with two color immunofluorescence showed co-localization of the CaR and filamin-A within parathyroid cells as well as HEK-293 cells stably transfected with the CaR. Deletion mapping localized the sites of interaction between the two proteins to a stretch of 60 amino acid residues within the distal portion of the CaR’s COOH-terminal tail and domains 14 and 15 in filamin-A, respectively. Finally, introducing the portion of filamin-A interacting with the CaR into CaR-transfected HEK-293 cells using protein transduction with a His-tagged, Tat-filamin-A fusion protein nearly abolished CaR-mediated activation of ERK1/2 MAPK but had no effect on ERK1/2 activity stimulated by ADP. Therefore, the binding of the CaR’s COOH-terminal tail to filamin-A may contribute to its localization in caveolea, link it to the actin-based cytoskeleton, and participate in CaR-mediated activation of MAPK.

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The G protein-coupled, extracellular calcium (Ca\textsuperscript{2+})\textsuperscript{-}sensing receptor (CaR) (1) plays a central role in calcium homeostasis by recognizing and responding to (e.g. “sensing”) small changes in Ca\textsuperscript{2+} and, in turn, appropriately modulating the functions of CaR-expressing cells so as to normalize the level of Ca\textsuperscript{2+} (for review, see Ref. 2). In the parathyroid gland, the CaR mediates low Ca\textsuperscript{2+}-evoked increases in secretion of the Ca\textsuperscript{2+}-elevating hormone, parathyroid hormone, as well as in parathyroid cellular proliferation (2). The CaR also mediates homeostatically appropriate changes in renal function, increasing urinary calcium excretion when Ca\textsuperscript{2+} is high and reducing it when Ca\textsuperscript{2+} is low (2). The receptor is expressed in several other tissues and cell types involved in Ca\textsuperscript{2+}, homeostasis, such as intestine and bone, as well as in numerous other cells seemingly uninvolved in mineral ion metabolism, but its biological functions in these different cells and tissues are not fully understood (3).

Recent studies have shown that the CaR regulates a variety of intracellular signaling pathways thought to mediate its diverse biological actions in the cells and tissues expressing it. The receptor stimulates phospholipases C, A\textsubscript{2}, and D (4), inhibits adenylate cyclase through pertussis toxin-sensitive and -insensitive mechanisms (2) and activates several mitogen-activated protein kinase (MAPK) cascades (5–8). The latter include the p42/44 (ERK1/2) (5, 8) and p38 MAPK (6) cascades as well as the c-Jun NH\textsubscript{2}-terminal kinase pathway (7).

In the cells in which its subcellular localization has been studied, such as the parathyroid chief cell, the CaR is located predominately in caveolea (9). The latter are small flask-shaped structures on the cell surface that are thought to serve as cellular “message centers” owing to their capacity to concentrate diverse signaling molecules coupled to both G protein-coupled as well as other classes of cell surface receptors (e.g., receptor tyrosine kinases) (10, 11). Thus, caveolea may play important roles in ensuring the specificity with which various receptors couple to their downstream effector systems.

Scaffold proteins are another mechanism that can contribute to the specificity of receptor-effector coupling. These are multidomain proteins that bind and spatially organize functionally related proteins (for review, see Ref. 12). In the present studies, we utilized the yeast two-hybrid screen to identify proteins, in addition to heterotrimeric G proteins, which interact directly with the CaR. Our studies reveal that the CaR interacts with...
domains 14 and 15 of filamin-A, which is a large actin-binding, putative scaffold protein that also binds caveolin-1 (13, 14), a key structural protein of caveolae (11), as well as several components of MAPK signaling cascades (e.g. mitogen-activated protein kinase/extracellular signal-regulated kinase kinases 1/2 and stress-activated protein kinase/ERK kinase-1/mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase4 (Ref. 15)). Moreover, interferring with the CaR-filamin-A interaction nearly abrogates CaR- but not ADP-mediated activation of ERK1/2, suggesting that the specific interaction between the CaR and filamin-A could play an important role in the CaR’s coupling to activation of MAPK.

EXPERIMENTAL PROCEDURES

**Plasmid and cDNA Library Constructs—pBCaRt and pCD1-7 refer to cDNAs encoding the full COOH-terminal intracellular tail of the bovine CaR or various fragments thereof, or the respective cDNAs encode amino acid residues 860–1085, 869–913, 913–973, 913–1031, 972–1034, and 1031–1065 (1). The various cDNAs were obtained by polymerase chain reaction and subsequently cloned between the NdeI and SalI sites of the yeast two-hybrid bait vector, pPA2–1 (CLONTECH, Palo Alto, CA). For the cDNA library construct, the yeast two-hybrid prey vector, pCT2 (CLONTECH), was modified by introduction of randomized cDNA sequences in a non-conditional manner. This was done by first digesting the two existing HindIII sites in pACT2 and thereafter filling in the four resultant sticky ends of the DNA with T4 DNA polymerase. The ends of the two DNA fragments were then ligated back together in their original orientation, giving a vector, pACT2m, in which the two endogenous HindIII sites are destroyed. Thereafter, a pair of complementary synthetic oligonucleotides containing a new HindIII site was ligated between the EcoRI and XhoI sites in the cloning box of pACT2 m. This created pACTGH, which was used in the cDNA library construction (see below). Vectors pPI1, pP12, and pPI3 correspond to the cDNAs encoding amino acid residues 1212–1355, 1344–1514, and 1534–1719 of bovine filamin-A, respectively, and were introduced into the EcoRI and HindIII sites of pACTGH using PCR. To permit ready transfer of the cDNA inserts of clones from the yeast two-hybrid prey vector, pACTGH, into vectors used for bacterial fusion protein expression in the correct reading frame, the following plasmid derivative was constructed. The EcoRI site in the vector, pQE-32 (Qiagen, Valencia, CA), a bacterial expression vector that produces proteins of interest with a His_6 tag (referred to hereafter as a His tag) into the NH2-terminal part of the fusion protein, was destroyed with HindIII. Thereafter, pQTFI3 was re-cloned into the EcoRI and HindIII sites in the cloning box. This new vector was named pQEGH. To permit production of bacterial fusion proteins for use in protein transduction experiments (16), complementary synthetic oligonucleotides were ligated between the BamHI and HindIII sites of pQEGH to create the BamHI site to be mutated. These oligonucleotides also introduced after the His tag of this vector derivative, named pQTAT, a cDNA sequence encoding the Tat peptide (16), YGRKKRRQRRR, followed by new BamHI, EcoRI, SalI, and SauI sites. To create pQTFI3, the cDNA insert of pPI3 was subcloned into the EcoRI and HindIII sites of pQTAT. The insert of clone pBCaRt was also subcloned into the pQTAT vector, creating pQBCt. For production of a glutathione S-transferase (GST) fusion protein, the cDNA insert of pPI3 was subcloned into the EcoRI and SalI sites of the bacterial expression vector, pGEX-5X-2 (Amersham Pharmacia Biotech, Uppsala, Sweden), creating pXFI3.

**cDNA Library Construction—**The OrienteX cDNA kit (Novaga-


den, Madison, WI) was used for CDNA synthesis from poly(A)_+ RNA purified from total RNA extracted from bovine parathyroid glands via two rounds of oligo(DT)-cellulose chromatography (Oligotex mRNA kit; Qiagen). The random primed cDNA was unidirectionally ligated into the EcoRI and HindIII sites of pACTGH. Part of the resultant ligated DNA was electroproted into DH10b bacteria (Life Technologies, Inc.). Plasmid library DNA was obtained using the plasmid mega kit (Qiagen).

**Yeast Two-hybrid System—**Saccharomyces cerevisiae Y190 yeast contin-

taining the vector, pCBaRt, were transformed with the bovine parathy-

rroid cDNA plasmid library described above using the lithium acetate method as detailed elsewhere (17) and incubated at 30 °C with SD/


t-Leu/_+Trp/>-His_/_+3A-1,2,4-triazole media. After 5–10 days of incubation, rapidly growing yeast colonies were picked and re-screened under identical media. Subsequently, this process was carried out on these re-grown colonies to identify those that responded by turning blue. Plasmid DNA, containing the cDNA library insert, was purified from yeast that were positive for both HIS3 and lacZ reporter gene activation and were re-transformed into Y190 yeast containing either pBCaRt or the negative control vectors, pAS2–1 and pVA3–1 and pCD1. A cDNA clone was considered to be positive in the yeast two-hybrid system when the transformed cells grew on media lacking histidine and gave a blue color in the β-galactosidase assay, but failed to induce the two reporter genes with the negative control plasmids. For deletion mapping, pCTI-7-containing Y190 yeast were transformed with pYC3 and evaluated to determine if they were positive for HIS3 and lacZ reporter gene activation. pCDS-containing yeast were subsequently transformed with either pPI1, pP12, or pPI3, and reporter gene activation was again analyzed.

**DNA and Protein Sequence Analysis—**DNA sequencing was performed using dRhodamine Terminatce chemistry (PerkinElmer Life Sciences) at the University of Maine DNA Sequencing Facility (Orono, ME), using an ABI model 373A Stretch DNA sequence with the XL upgrade (PerkinElmer Life Sciences). DNA and protein sequences were analyzed using the EditView (PerkinElmer Life Sciences) and MacVec- tor (Genetics Computer Group, Madison, WI) software packages.

**Northern Blot Analysis—**Total RNA was obtained from bovine para-

yroid glands using the Trizol reagent (Life Technologies, Inc.), and poly(A)_+ RNA was purifed using the Oligotex kit (Qiagen). Five μg of the resultant poly(A)_+ RNA was separated on a 2% agarose gel in 2.2 μl formaldehyde together with an RNA ladder (Life Technologies, Inc.) and transferred to a nylon membrane (Duralon, Stratagene, La Jolla, CA). The membrane was prehybridized in 50% formamide, 4X Den-



hardt’s solution (5X Denhardt’s solution contains 5 g of Ficoll, 5 g of polyvinylpyrrolidone, and 5 g of BSA/50 ml), 5X SSPE (20X SSPE contains 2.98 μl NaCl and 0.02 EDTA in 0.1 μl phosphate buffer, pH 7.0), 10% dextran sulfate, 200 μg/ml calf thymus DNA, 250 μg/ml yeast tRNA, and 0.5% SDS. The membrane was then incubated with a rad-



om primed 32P-labeled cDNA insert from the pYC3 clone. After washing, the membrane was exposed to a phosphorimager screen, and signal intensity was quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**Bacterial Production of Fusion Proteins and in Vitro Binding—**An overproducing strain of TOP10F+ (Invitrogen) carrying the pQBCt plasmid was diluted 1/15 and grown for 90 min at 37 °C. Isopropyl-1-thio-



β-galactopyranoside (18) was added to a final concentration of 1 μl, and the bacteria were incubated for another 5 h at 37 °C. Bacteria were subsequently resuspended in buffer A (8 μl, 1X phosphate-buffered saline (PBS) (157 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4), 20 ml imidazole, 0.2% Triton X-100, and 0.5% SDS). The sample was applied to a nickel-NTA column, washed with 2.5 mM histidine and gave a blue color in the β-galactosidase assay, but failed to induce the two reporter genes with the negative control plasmids.

Preparation of TAT fusion proteins for protein transduction was performed essentially as described elsewhere (16), but with some minor modifications. In brief, isopropyl-1-thio-β-galactopyranoside-induced bacteria transformed with pQTFI3 were resuspended in 0.5 ml 20X SSPE (0.2 M NaCl, 0.01 M EDTA at pH 8.0, 1% Triton X-100, and 2.5 mM β-mercaptoethanol, lysed by sonication, and sedimented. Imidazole and NaCl were added to the filtered supernatant to concentrations of 10 and 250 mM, respectively. The sample was applied to a nickel-NTA column, washed with buffer without Triton X-100, and proteins remaining bound to the column were then eluted with 0.5 M imidazole. The eluted proteins were subsequently applied to PD-10 columns pre-equilibrated with 4 μl urea,
A His-tagged Tat-peptide, RGSHGYYYGRKKRRQRRRGG, was synthe-
sized at the Biopolymers Laboratory (Harvard Medical School, Bos-
ton, MA). This peptide corresponds to amino acid residues 2–25 in
pQAT and was used as a negative control in the protein transduction
experiments.

Immunohistochemistry—Dispersed bovine parathyroid cells were
prepared by digestion of neonatal bovine parathyroid glands with
collagenase and DNase as described previously (16), seeded on glass
coverslips, and allowed to attach for 1 h. HEK-293 cells stably transfect-
ed (4) with the human CaR cDNA (19) or non-transfected HEK-293 cells
(which do not express the CaR) were grown on glass coverslips in
DMEM (without sodium pyruvate) containing 10% fetal bovine serum.
Attached cells were washed with PBS and fixed for 5 min with 2%
formaldehyde in PBS. After washing again with PBS, the cells were
permeabilized by incubation for 10 min with 0.3% Triton X-100 in PBS.
The slides were then incubated with 0.1 μg/ml Alexa 488-conjugated goat
anti-mouse IgG (Molecular Probes) for 30 min at 37 °C, washed with
PBS, and mounted with anti-fading mounting medium. The slides were
then incubated with goat anti-rabbit IgG coupled to Alexa-568 (Molecu-
lar Probes). They were subsequently washed as above, incubated with a polycy-
chal secondary antibody (denoted 4G6, raised to a peptide containing residues
345–359 of the human CaR), goat anti-rabbit IgG, and goat anti-mouse IgG
coupled to Alexa-568 (Molecular Probes). After washing again with PBS,
slides were incubated with 0.1 μg/ml Alexa 488-conjugated goat anti-rabbit
and Alexa 568-conjugated goat anti-mouse IgGs (Molecular Probes) for
30 min at 37 °C, washed, mounted with Vectashield, and fluorescence
images were obtained as above.

Assay for ERK1/2 MAPK—A rabbit polyclonal antibody specific for the
phosphorylated forms of ERK1/2 (p42/44MAPK) (Th202/Tyr204,
respectively) was purchased from New England Biolabs, Inc. (Beverly,
MA). Preparation of cellular extracts and Western blot analyses were
carried out according to the manufacturer’s instruction and as de-
scribed below. Subconfluent, CaR-transfected HEK-293 cells in 12-well
plates were incubated overnight in 0.5 mM Ca2+ in DMEM without
serum but containing 0.2% BSA and 4 mM glutamine, and with either
CaR filamin-A fusion protein (10–100 nM) or the His-
tagged Tat peptide (100 nM) as a negative control. The cells were then
treated in the same medium with 0.5 or 3.0 mM Ca2+ (see Results
section for details). The cells were subsequently washed once with
ice-cold PBS, and then lysed with 100 μl of SDS sample buffer contain-
ing 50 mM dithiothreitol.

To perform Western analysis, each well was washed twice with 1 ml
EDTA in PBS, and the cells were lysed with 175 μl of lysis solution (25
mM HEPES, pH 7.5, 5 mM MgCl2, 5 mM EDTA, 2 mM phenylmethyl-
sulfonyl fluoride, 1% Triton X-100, 10 μg/ml leupeptin, and 10 μg/ml
pepsin A). The viscosity of the samples was reduced by brief sonica-
tion, and insoluble material was removed by centrifugation in a micro-
centrifuge for 5 min. Aliquots of 50 μg of protein from the resultant
whole cell lysates were denatured in the lysis buffer, to which had been
added 100 mM freshly prepared dithiothreitol, and were then heated at
90 °C for 5 min. The proteins were resolved electrophoretically on 10%
SDS-PAGE gels. Proteins were then transferred electrophoretically to
nitrocellulose blots at 240 mA for 1 h in transfer buffer containing 19
mM Tris, 150 mM glycine, 0.015% SDS, and 20% methanol (v:v). The
and twice with water, and then mounted with anti-fading mounting
fluid (Vectashield; Vector Laboratories, Burlington, CA). Fluorescence
images were obtained using the 100× objective of a Bio-Rad
MRC1024/2P multi-photon microscope equipped with krypton and ar-
on lasers at the Brigham and Women’s Hospital Confocal Microscopy
Core facility.

Slides with attached HEK-293 cells (transfected or non-transfected)
were treated similarly, except that they were first incubated with
the His-tagged Tat-filamin and Tat-filamin peptides
at 4 °C with a monoclonal anti-CaR antibody at a 1:50 dilution (denoted
LRG), raised against a peptide containing residues 374–391 of the
human CaR; a generous gift of Drs. Kimberly Rogers and Edward
Nemeth), washed as above, and then incubated at 37 °C for 30 min
with goat anti-mouse IgG coupled to Alexa-568 (Molecular Probes).
They were subsequently washed as above, incubated with a polycy-
chal secondary antibody (denoted 4G6, raised to a peptide containing residues
345–359 of the human CaR), goat anti-rabbit IgG, and goat anti-mouse IgG
coupled to Alexa-568 (Molecular Probes) for 30 min at 37 °C, washed, mounted with Vectashield, and fluorescence
images were obtained as above.

FIG. 1. Schematic representation of the filamin-A protein and of the CaR’s COOH-terminal intracellular domain as well as of the
sites of interaction between the two proteins based on deletion mapping. A detailed description of each clone can be found under
“Experimental Procedures” and “Results.” The encoded protein sequence of each cDNA clone has been aligned to its corresponding position in
the two proteins. A, schematic drawing of the 280-kDa human filamin-A protein, illustrating its NH2-terminal actin-binding domain and 24 repetitive
domains (13). Shown below is the bovine filamin-A clone pYC3 reported herein, and also indicated are the three cDNA clones, pF11, pF12,
and pF13, that were used for deletion mapping (part C). B, representation of the last 226 amino acid residues of the bovine CaR protein, containing
the COOH-terminal cytoplasmic domain. Clones pCD1–7 were used for mapping the interaction sites of the CaR with filamin-A. The amino acid
residue numbers at the NH2 and COOH termini of the protein fragments encoded by the various constructs of the CaR
protein are indicated. C, several cDNA clones corresponding to different parts of the CaR and filamin-A that were used to test activation of the
lacZ and HIS3 reporter genes in the yeast two-hybrid system. + stands for a negative response in the two reporter gene assays. ++ + indicates strong
positive blue staining in the β-galactosidase assay and robust growth on histidine-deficient media, whereas + + represents a somewhat weaker
response. β-gal, β-galactosidase activity; His, HIS3 reporter gene activation.

20 mM ethanolamine, pH 9.5, and 2.5 mM β-mercaptoethanol. After this
change in buffer, the proteins were applied to a Q Sepharose High
Performance column (Amersham Pharmacia Biotech). Unbound pro-
teins were removed with 20 mM ethanolamine, pH 9.5, containing 2.5
M β-mercaptoethanol, and the remaining bound proteins were eluted
with 1.5 M NaCl. This eluted protein sample was desalted on a PD-10
column (Amersham Pharmacia Biotech) pre-equilibrated with DMEM,
and subsequently flash-frozen in liquid N2 and stored at −70 °C until
use. As assessed by PAGE with Coomassie staining, the bacterially
produced Tat-filamin protein sample was about 50% pure, with two
major protein bands, one of which had the apparent molecular mass
expected of the Tat-filamin-A fusion protein and was immunoreactive
on Western blotting with an anti-His antibody. The other major band
was non-immunoreactive and was presumably a contaminating bacte-
rial protein.

A His-tagged Tat-peptide, RGSHGLGGYGRKKRRQRRRGG, was synthe-
sized at the Biopolymers Laboratory (Harvard Medical School, Bos-
ton, MA). This peptide corresponds to amino acid residues 2–25 in
pQAT and was used as a negative control in the protein transduction
experiments.
blots were blocked overnight at 4 °C in a buffer containing PBS with 0.5% Triton X-100, 0.5% BSA, and 5% skimmed milk (blocking solution). The following day, the blots were incubated with a blocking solution that was identical except for containing a reduced content of skimmed milk (1%) at 4 °C overnight with phospho-ERK1/2-specific antibody (New England Biolabs). A 1:1000 dilution of the stock provided by the manufacturer was utilized for the primary antibody. Following incubation with the primary antibody, blots were subsequently washed three times with PBS containing 0.1% Triton X-100 (washing solution) at room temperature for 30 min each. The blots were further incubated with a 1:2000 dilution of horseradish peroxidase-coupled, goat anti-rabbit IgG (Sigma) in blocking solution for 1 h at room temperature. The blots were then washed three times with the washing solution at room temperature for 30 min each, and specific protein bands were detected using enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech).

**Statistical Analyses**—The data for MAPK activation are presented as the means ± S.E. of the indicated number of experiments. Statistical analyses were carried out using analysis of variance together with a Scheffe F test. A p value of <0.05 was considered to indicate a statistically significant difference.

**RESULTS**

Filamin-A Is an Intracellular Binding Partner of the CaR—To identify intracellular proteins, other than heterotrimeric G-proteins, interacting with the bovine CaR (1), the full carboxyl-terminal, cytoplasmic domain of the receptor was used as a bait to screen a bovine parathyroid cDNA library using the yeast two-hybrid system. Of the 5.2 × 10⁸ transformants screened, one clone, pYC3, was found to activate the HIS3 nutritional reporter gene in yeast and to be positive in the β-galactosidase assay. Re-transformation of pYC3 into yeast with either the empty vector, pAS2–1, or pVA3–1, which encodes part of the murine p53 protein, gave negative responses. BLAST search of the NCBI data bases with the pYC3 sequence revealed 90% nucleotide sequence identity with the human actin binding protein, ABP-280/filamin-A (13). The deduced protein sequence encoded by pYC3 shares greater than 99% amino acid identity with the corresponding region of human filamin-A and spans amino acid residues 1212–1719 of the latter. The two amino acid differences between the bovine and the human sequences are replacements of the Arg at position 1312 and of the Met at position 1316 in the human protein with Cys and Thr, respectively, in the bovine protein. The protein encoded by pYC3 extends from repetitive domain 10 of filamin-A through part of domain 15 (Fig. 1A; Ref. 13).

To narrow down further the regions of interaction between the CaR and filamin-A, various plasmid constructs were prepared for the yeast two-hybrid screen, which were called pCD1–7 for the CaR and pFI1–3 for filamin-A. The inserts of these plasmids encode various fragments from within the regions of the two proteins that interacted in the original yeast two-hybrid screen (Fig. 1, A and B). Using this approach, three constructs with partially overlapping sequences that encoded smaller portions of the CaR’s COOH-terminal tail were found to bind to filamin-A. As can be seen in Fig. 1C, the interacting site of the CaR resides within a 60-amino acid stretch that corresponds to amino acid residues 972–1031 in the distal portion of the receptor’s COOH-terminal tail, just distal to the highly Glu/Pro-rich region of the latter (1). The smallest part of filamin-A that was capable of binding to the CaR comprised amino acid residues 1534–1719, which contains repetitive domain 10 and nearly all of domain 15 (clone pFI3 in Fig. 1, A and C; Ref. 13).

A direct interaction between the relevant regions of the CaR and filamin-A was further confirmed by an in vitro binding assay using purified fusion proteins produced in bacteria. A fusion protein comprising a His tag followed by the CaR’s COOH-tail that had been bound to nickel-NTA beads was incubated with either GST or a GST-FI3-filamin-A fusion protein. Proteins that remained bound to the beads after extensive washing were then separated by SDS-PAGE and visualized with colloidal Coomassie stain. Fig. 2A shows that GST alone did not bind to the His-tagged CaR fusion protein, whereas there was a clear interaction between the CaR and the GST-filamin-A fusion protein.

Bovine parathyroid cells are known to express the CaR at high levels (1). In order to determine whether bovine parathyroid cells also express filamin-A, we performed Northern blot analysis of mRNA extracted from bovine parathyroid gland, which showed a single transcript of ~8 kilobases using the bovine filamin-A cDNA in clone pYC3 as a probe (Fig. 2B). Immunolocalization of CaR and Filamin-A—The cellular distributions of filamin-A and the CaR and the extent to which the two proteins are colocalized were investigated using confocal two-color immunofluorescence microscopy in bovine parathyroid cells, which express both proteins endogenously, and either non-transfected HEK-293 cells or those stably transfected with the human CaR cDNA. HEK-293 cells have been previously shown to express filamin-A endogenously (15). In parathyroid cells, the CaR was localized both along the periphery of the cells as well as in a cytoplasmatic and perinuclear distribution (Fig. 3A). Filamin-A exhibited a more localized intracellular distribution, with its highest density within the cytoplasm immediately beneath the points of contact between
parathyroid cells (Fig. 3B). The two proteins were most extensively colocalized in the latter region (Fig. 3C). In CaR-transfected HEK-293 cells, the two proteins both showed similar patterns of distribution close to and immediately beneath the plasma membrane (Fig. 3, D and E), with extensive regions of colocalization immediately beneath the plasma membrane based on confocal, two-color fluorescence imaging (Fig. 3F). In non-transfected HEK-293 cells, in contrast, there was no CaR immunoreactivity (Fig. 3G). Therefore, despite the presence of filamin-A in a distribution similar to that in the CaR-transfected HEK-293 cells (Fig. 3H), there was no visible colocalization (Fig. 3I).

**Transduction of CaR-transfected HEK-293 cells with a TAT-Filamin-A Fusion Protein Inhibits CaR- but Not ADP-mediated Activation of MAPK—**High levels of Ca\(^{2+}\) activate ERK1/2 (Fig. 4A) in CaR-transfected HEK-293 cells (4, 20). In order to determine whether the binding interaction between the CaR and filamin-A is functionally significant with regard to high Ca\(^{2+}\)-evoked, CaR-mediated activation of MAPK, we utilized the protein transduction technique recently described by Nagahara et al. (16). Coupling a protein of interest to the Tat peptide (amino acid residues YGRKKRRQRRR within the HIV Tat protein) confers upon the resultant fusion protein the capacity to translocate across the membranes of essentially all mammalian cell types in culture and even the tissues of an animal with high efficiency (for review, see Ref. 21). The Tat fusion protein reached an intracellular concentration that may approach its extracellular concentration within 15 min. This technique has been employed to successfully “transduce” several cell types with a large variety of proteins of up to 120 kDa in size (21). Although the mechanism by which Tat-tagged proteins traverse lipid membranes is unknown, it does not appear to involve receptor-, transporter-, or endocytosis-mediated processes (21).

We used a Tat-filamin-A fusion protein to introduce the portion of filamin-A shown to interact with the CaR’s COOH-terminal tail (amino acid residues 1534–1719) into CaR-transfected HEK-293 cells as a means of disrupting the CaR-filamin-A binding interaction, while a His-tagged Tat peptide served as a negative control. Fig. 4A shows a representative Western blot with a phospho-ERK1/2-specific antibody, demonstrating that high (3 mM) Ca\(^{2+}\) produces a robust stimulation of the phosphorylation of ERK1/2 that is inhibited by the Tat-filamin fusion protein (100 nM) but not by the same concentration of the His-tagged Tat peptide. In pooled data from three similar experiments, the Tat-filamin peptide (100 nM) produced a statistically significant (p < 0.05), greater than 80% inhibition of ERK1/2 activity at 3 mM Ca\(^{2+}\) (Fig. 4B). Neither the His-tagged Tat peptide nor the His-tagged Tat-filamin peptide had any effect on the basal activity of ERK1/2 (e.g. at 0.5 mM Ca\(^{2+}\)) (Fig. 4, A and B). In order to document the capacity of the His-tagged, Tat-filamin-A fusion protein to be transduced intracellularly, we used the immunoperoxidase technique with an antibody against the His tag to show the presence of the His-tagged protein within fixed and permeabilized bovine parathyroid cells (data not shown).

We then assessed whether the inhibition of CaR-stimulated ERK1/2 activity by the Tat-filamin peptide was specific for this G protein-coupled receptor or could also be observed with another G protein-coupled receptor that stimulates ERK1/2 in HEK293 cells. Fig. 5A shows a representative Western blot documenting that the His-tagged, Tat-filamin-A fusion protein inhibits high Ca\(^{2+}\)-evoked activation of ERK1/2 in a concentration-dependent manner. There was 40%, 79%, and 92% inhibition of high Ca\(^{2+}\)-evoked, CaR-mediated activation of ERK1/2 activity by 10, 30, and 100 nM, respectively, of the Tat-filamin peptide in pooled data from three experiments (Fig. 5B). In contrast, a 100 nM concentration of the Tat-filamin
tion of ERK1/2 by 3 mM Ca\(^{2+}\) without serum and either with or without the His-tagged Tat or Tat-filamin peptides (100 nM). The cells were then exposed for 10 min at 37 °C to either 0.5 (lanes A–C) or 3 mM Ca\(^{2+}\) (lanes D–F), with the same concentration of either the His-tagged Tat (lanes B and E) or Tat-filamin peptides (lanes C and F), and the activities of ERK1/2 were determined as described under “Experimental Procedures” using Western blotting with phosphospecific antibodies, with equal loading of proteins in all lanes. B, results of pooled data from three similar experiments showing little, if any, effect of the His-tagged Tat or His-tagged Tat-filamin peptides at low Ca\(^{2+}\), whereas the His-tagged Tat-filamin but not the His-tagged Tat peptide produces a significantly significant (p < 0.05), 80% or more inhibition of the stimulation of ERK1/2 by 3 mM Ca\(^{2+}\). Letters A–F in panel B refer to the following: A, 0.5 mM Ca\(^{2+}\) alone; B, 0.5 mM Ca\(^{2+}\) + 100 nM His-Tat; C, 0.5 mM Ca\(^{2+}\) + 100 nM Tat-filamin; D, 3 mM Ca\(^{2+}\) alone; E, 3 mM Ca\(^{2+}\) + 100 nM His-Tat; F, 3 mM Ca\(^{2+}\) + 100 nM Tat-filamin.

peptide had little or no effect on ADP-stimulated ERK1/2 activity. ADP (1 µM) increased ERK1/2 phosphorylation by 11.4 ± 2.9-fold (n = 4) over that observed under basal conditions (0.5 mM Ca\(^{2+}\)), which was similar to the degree of stimulation observed in cells exposed to the same concentration of ADP in the presence of 100 nM Tat-filamin (13.6 ± 2.9-fold, n = 4) (Fig. 5B). Thus, the inhibitory effect of the Tat-filamin was specific for CaR-elicted as opposed to ADP-evoked ERK1/2 activation.

### DISCUSSION

The G protein-coupled CaR plays key roles in mineral ion metabolism and also modulates the functions of various cell types not clearly involved in Ca\(^{2+}\) homeostasis. It regulates numerous cellular processes, including hormonal secretion, cellular proliferation, chemotaxis, apoptosis, ion transport, and the activity of various ion channels (2). It is thought to do so by modulating a variety of intracellular signaling pathways, including activating phospholipases C, A\(_2\), and D (4), inhibiting adenylate cyclase (2), and activating the p42/44 (5, 8, 20) and p38 MAPK cascades (6) as well as the c-Jun NH\(_2\)-terminal kinase pathway (7). In many cases, however, the molecular mechanisms by which the CaR regulates these intracellular second messenger pathways and the details of how the latter are coupled to various CaR-mediated biological responses remain to be fully elucidated.

The CaR is located primarily in caveolae in parathyroid cells (9). Caveolin-1, a major structural protein of caveolae (11), binds to the actin-cross-linking protein, filamin-A (14), which also binds various MAPK components (15). Filamin-A is a 280-kDa protein (ABP-280, non-muscle filamin-A; Ref. 13) that contains an NH\(_2\)-terminal actin-binding site as well as 24 repeated domains of ~96 amino acids each (13). Domains 1–15 and 16–23 are separated by a small hinge region containing a calpain cleavage site. The last, slightly shorter, repeat is preceded by a second hinge. This last repeat domain is also responsible for the capacity of filamin-A to form a homodimer, by directly interacting with the 24th domain of a second filamin-A molecule. Electron microscopy has shown the dimer to form a leaf springlike structure (13).

Recent studies have identified numerous molecular binding partners of filamin-A and have provided increasing evidence that this nearly ubiquitously expressed protein may serve as a scaffold protein in addition to its known capacity to cross-link actin, i.e. filamin-A binds to a substantial number of transmembrane and intracellular proteins known to be involved in rearrangement of the actin cytoskeleton as well as in numerous other cellular processes. For example, the non-homeostatic role of tissue factor in cell adhesion and migration is dependent...
upon its interaction with filamin-A (22). In platelets, filamin-A anchors the actin cytoskeleton to the plasma membrane by binding to the GPIbα subunit of the von Willebrand factor receptor complex (23). Other proteins that interact directly with filamin-A include the stress-activated protein kinase/ERK kinase-1, mitogen-activated protein kinase/extracellular signal-regulated kinase kinases 1/2, and p38 kinases (15), Ras-related GTPases (e.g., Rac, RhoA, Cdc42, and RalA; Ref. 24), β3-integrin (25), furin (26), caveolin-1 (14), tumor necrosis factor receptor-associated factor 2 (27), and the dopamine D2 receptor (28). Mutations in the human FLN1 gene, encoding filamin-A, cause periventricular heterotopia, an X-linked dominant disorder where subsets of neurons fail to migrate into the developing cerebral cortex and persist as nodules lining the ventricular surface (29).

The CaR can now be added to the list of proteins that interact directly with filamin-A. We demonstrated this interaction in several ways. We initially utilized the yeast two-hybrid system to screen a bovine parathyroid cDNA library and found that filamin-A (13) interacts with the CaR's COOH-terminal tail. Subsequent use of the yeast two-hybrid system to perform deletion mapping localized the sites of interaction to a 60-amino acid stretch between residues 972 and 1031 in the distal portion of the CaR's COOH-tail and a region of filamin-A containing amino acid residues 1534–1719, which includes repetitive domain 14 and nearly all of domain 15. We then utilized an in vitro binding assay to confirm a direct interaction between the two proteins. There was clear binding of a bacterially produced, GST-filamin fusion protein containing the binding site defined in the yeast two-hybrid system to the His-tagged, COOH tail of the CaR. A less than equimolar amount of the GST-filamin fusion protein appeared to bind to the immobilized COOH-terminal tail of the CaR, suggesting that the interaction may not be of high affinity. It is also possible, however, that protein production in bacteria, which included exposure to 8 M urea during one step of the procedure, did not permit the resultant protein fragments of the CaR and filamin-A to assume the native conformations of the respective full-length proteins as they exist in vivo, thereby compromising their capacity to interact with a normal affinity in vitro. To confirm that the two proteins colocalize in intact bovine parathyroid and CaR-transfected HEK-293 cells, we combined confocal imaging and two-color immunofluorescence. The results showed extensive colocalization in the latter and more confined regions of intracellular colocalization in the former. In contrast, as expected, non-transfected HEK-293 cells lacked CaR immunoreactivity and, therefore, exhibited no colocalization between CaR and filamin-A immunoreactivities, thereby providing a useful negative control.

It is of interest that the CaR and filamin-A colocalize in the regions of cellular contact between adjacent parathyroid cells, which in bovine parathyroid glands form pseudoacinar structures where the secretory poles of individual parathyroid chief cells reside at sites of cellular attachment. It is possible, therefore, that the binding of filamin-A to both the CaR and β3-integrin (25) may contribute to the colocalization of the two proteins in this region of the parathyroid cell. We have also shown in recent preliminary studies that the CaR and caveolin-1 colocalize in the same region of parathyroid cells, perhaps because of the interaction of both the CaR and caveolin-1 with filamin-A (and, in turn, β3-integrin).3

The preceding results, showing immunocolocalization of the CaR and filamin-A in both parathyroid and CaR-transfected HEK-293 cells, indicate that the two proteins are in close physical proximity to one another in intact cells, although they do not prove the existence of a direct binding interaction in vivo. Nevertheless, taken together, the results in the yeast two-hybrid screen, the in vitro binding assay, and the immunocolocalization in intact cells provide cogent evidence for a direct interaction between the two proteins.

What are the functional implications of the CaR's interaction with filamin-A? We previously showed that the CaR in bovine parathyroid cells is located predominantly in caveolae (9). Furthermore, it is possible to co-immunoprecipitate the CaR from detergent extracts of parathyroid cells using anti-caveolin-1 antibodies (9). Since filamin-A has been shown to interact directly with caveolin-1 (14), it is possible that binding of the CaR to filamin-A contributes, at least in part, to the receptor's preferential localization in caveolae. The receptor's interaction with filamin-A could also link it to the actin-based cytoskeleton, although the potential functional consequences of such an interaction have not been investigated to date.

Filamin-A, by virtue of its capacity to bind signal transducing molecules (e.g., low molecular weight G proteins and MAPK components), appears to serve not only as an actin-cross-linking protein but also as a scaffold protein for certain signaling pathways. Indeed, transduction of CaR-transfected H9253 cells with a His-tagged, Tat-filamin-A fusion protein nearly obliterated CaR-mediated activation of ERK1/2, whereas the control, His-tagged Tat peptide had no such effect. Moreover, the Tat-filamin-A fusion protein had no effect on ERK1/2 activity stimulated by another G protein-coupled receptor, that for ADP. Although we are not aware of any data regarding the possible interaction of the ADP receptor with filamin-A, these results strongly suggest that the direct interaction of the CaR's COOH-terminal tail with filamin-A participates in CaR-mediated activation of this MAPK cascade. By placing the CaR in close spatial proximity to its downstream effector elements in the ERK1/2 MAPK cascade, filamin-A may facilitate CaR-mediated activation of this pathway. Further studies are needed, however, to investigate the mechanisms upstream of ERK1/2 through which the CaR activates this MAPK cascade. It will also be of interest to determine whether the interaction between the CaR and filamin-A also contributes to CaR-mediated activation of p38 and c-Jun NH2-terminal kinase pathways, since components of these two pathways also bind to filamin-A.

In addition to its coupling to various MAPK pathways, the CaR can modulate several additional signaling pathways within any given cell type, e.g., the receptor not only activates ERK1/2 (8, 20) but also phospholipases C, A2, and D (6) in CaR-transfected H9253 cells. It will also be of interest, therefore, to determine the extent to which the interaction between the CaR and filamin-A determines the specificity of the CaR's coupling to these various signaling cascades within different regions of a given cell as well as in the diverse cell types in which it is expressed. As can be seen in Fig. 3, for instance, the co-localization of CaR and filamin-A is far from complete in bovine parathyroid cells. Thus, a subpopulation of the CaR in parathyroid cells could potentially couple to the MAPK pathway, at least in part, through its interaction with filamin-A, whereas the receptor in other subcellular locations might interact with as yet unidentified, additional scaffold proteins that enable it to modulate other signaling pathways. In the case of the G protein-coupled D2 dopaminergic receptor, for instance, filamin-A binds to the third intracellular loop of the receptor (28), and this interaction increases the coupling efficiency of the receptor to activation of adenylate cyclase.

In summary, the CaR interacts directly with the actin-cross-linking protein, filamin-A, in the yeast two hybrid system as well as in an in vitro binding assay, and the two proteins colocalize

3 O. Kifor and E. M. Brown, unpublished observations.
in bovine parathyroid cells and CaR-transfected HEK-293 cells. The binding of the CaR to filamin-A may contribute to the known localization of the CaR in caveolae in parathyroid cells and could also provide a physical link between the receptor and the actin-based cytoskeleton. Finally, filamin-A likely plays a key role as a scaffold protein for CaR-mediated activation of ERK1/2 and may, therefore, provide a mechanism for determining the specificity of CaR-mediated signaling in the diverse cell types in which this receptor is expressed.

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Filamin-A Binds to the Carboxyl-terminal Tail of the Calcium-sensing Receptor, an Interaction That Participates in CaR-mediated Activation of Mitogen-activated Protein Kinase
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