Numerous alternatively spliced transcripts are generated from the gene for the G protein-coupled calcitonin receptor, and some of the splice variants show differences in receptor-mediated signaling events. This study showed that the Δe13 splice variant of the rabbit calcitonin receptor is expressed together with the more common C1a in osteoclast-like cells. Since other G protein-coupled receptors form homo- or heterodimers, we examined whether heterodimerization of the calcitonin receptor splice variants occurs and, if so, whether it affects the function of the receptor. Homodimers of both isoforms and Δe13/C1a heterodimers were detected by co-immunoprecipitation and fluorescence resonance energy transfer analysis. In contrast to the C1a isoform, the Δe13 isoform was not efficiently transported to the cell surface. When co-expressed with the C1a splice variant, the Δe13 isoform colocalized with the C1a isoform within the cell but not at the cell surface. Furthermore, the overexpression of the Δe13 variant led to a significant reduction of the C1a surface expression and consequently a reduction of the cAMP response and Erk phosphorylation after ligand stimulation. We therefore suggest that the Δe13 variant of the rabbit calcitonin receptor acts to regulate the surface expression of the C1a isoform.

Calcitonin (CT) is a 32-amino acid polypeptide that was originally identified as a hypocalcemic factor present in bovine parathyroids (1). CT acts on bone and kidney to maintain calcium homeostasis and is also present in the central nervous system, where it has anorexic and analgesic effects (2). CT acts on bone and kidney to maintain calcium homeostasis and is also present in the central nervous system, where it has anorexic and analgesic effects (2). Of the cells present in bone, osteoclasts are the main target of CT. It inhibits motility and induces marked cellular retraction of osteclasts, two effects that are thought to be essential to the CT-induced inhibition of bone resorption (3, 4).

The calcitonin receptor (CTR) belongs to the class B of G protein-coupled receptors (GPCRs) and was first cloned in 1991 (5). It couples to multiple heterotrimeric G proteins, resulting in activation of the effector proteins adenyl cyclase and phospholipase Cβ (6–9). We have reported that the rabbit CTR also stimulates Shc tyrosine phosphorylation and Erk1/2 activation, primarily via G1i and Gq-dependent signaling pathways (10). Recent data indicate that in the presence of "receptor activity-modifying proteins," the CTR functions as a receptor for amylin, and therefore the complexes of the CTR with receptor activity-modifying protein 1, 2, or 3 are called AMY1-, AMY2-, and AMY3-receptor, respectively (11).

Numerous alternatively spliced transcripts of the CTR have been described in different species. The most common isoform in all species corresponds to the sequence originally cloned from porcine cells (5) and the rodent C1a isoform (12). In humans, the less abundant insert-positive form shows a loss of Gq-mediated responses and attenuation of Gi-mediated signaling and also decreased internalization (13). In rodents, the C1b splice variant has an insertion of 37 amino acids in the first intracellular loop and shows only a weak interaction with human CT (12, 14–16). Cloning of the rabbit CTR by our group revealed both the C1a transcript and a splice variant with a deletion of exon 13, designated CTRΔe13, which encodes much of the seventh transmembrane domain (8). The Δe13 variant failed to induce the production of inositol phosphates or to mobilize intracellular calcium and also showed a decreased cAMP response to salmon and human CT stimulation (8, 17). Interestingly, the exon deleted in the CTRΔe13 isoform is highly conserved in the class B GPCRs. Receptor-spliced variants resulting in deletion of the homologous 14 amino acids as in the CTRΔe13 variant have been described for two other class B GPCRs, the CRH-R1 (18) and the parathyroid hormone/parathyroid hormone-related protein receptors (19).

Recent reports suggest that many GPCRs form homodimers and/or heterodimers (20–22). The functional relevance of GPCR dimerization was strikingly shown for the GABAB receptor. Several groups simultaneously reported that co-expression of the GBR1 and GBR2 isoforms is a prerequisite for the formation of functional GABA_B receptors at the cell surface (23–25). Subsequent studies demonstrated that the GBR1 isoform bears an endoplasmic reticulum retention signal that is blocked by dimerization with the GBR2 isoform, allowing endoplasmic reticulum export and plasma membrane targeting (26). Others reported that effects of GPCR dimerization include changing ligand selectivity and altered signal transduction (27).

Since there are numerous alternatively spliced transcripts of...
the CTR gene product, and some of the splice variants show considerable differences in their trafficking or receptor function, dimerization of these splice variants could result in profound changes in the biology of the CTR. We show here that the Δ13 variant accumulates within the cell and is only minimally expressed on the cell surface, whereas the C1a variant of the rabbit CTR is highly expressed on the cell surface. We also show that different splice variants express variations in the same cell, they form both homo- and heterodimers, via interaction of the C-terminal tail but also via at least one other domain of the receptor. Co-expression of the Δ13 and C1a isoforms results in a marked decrease in the surface expression of the C1a isoform. Moreover, the reduced surface expression of the C1a isoform was accompanied by diminished CAMP production and Erk phosphorylation after ligand stimulation, suggesting that the dimerization of the Δ13 isoform with the C1a isoform serves to down-regulate CT-induced signaling by retaining the C1a splice variant in an intracellular compartment.

**EXPERIMENTAL PROCEDURES**

*Reagents and Antibodies*—Salmon calcinin (sCT) was purchased from Peninsula Laboratories, Inc. (Belmont, CA). The monoclonal anti-β-ACT antibody (7D10) and the monoclonal anti-β2-microglobulin (M2) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); the monoclonal anti-GFP antibody and the monoclonal anti-RFP antibody were from Sigma. Enhanced chemiluminescence solutions and nitrocellulose membranes were from Amersham Biosciences and Schleicher & Schuell, respectively.

**Cell Culture and Transient Transfections**—Dulbecco's modified Eagle's medium and fetal bovine serum were purchased from Invitrogen. Minimal essential medium, α modification (α-MEM) was from Sigma. All media were supplemented with 100 μg/ml streptomycin and 100 units/ml penicillin. HEK 293 cells were cultured as described before (28). For transient transfections, cells were grown to 60–70% confluence and then transfected with Fugene 6 (Roche Molecular Biochemicals) according to the protocol of the manufacturer. When not otherwise described (i.e., Fig. 2A), experiments that involved transfecting the CTR isoforms alone or in combination were performed with constant concentrations of each cDNA and adding empty vector DNA when only one isoform was expressed to keep the total amount of DNA constant.

**Production of Rabbit Osteoclast-like Cells**—Rabbit osteoclast-like cells (OCLs) were produced as described before (29). Briefly, long bones (femur and scapulae) from 4-day-old rabbits were isolated and dissected from the CTR isoform was expressed to keep the total amount of DNA constant. The forward primer (5'-GGTGGCAGCTCTGGTGGTCAAT) corresponded to nucleotides 1545–1549 of the rabbit C1a cDNA (8). The reverse primer (5'-GATGCGACAGCTCGTGGTGCAT) corresponded to nucleotides 1529–1545 of the rabbit C1a cDNA (8). The PCR was done in the presence of 1.5 mm MgCl₂ and 0.3 μm each primer by performing 40 cycles consisting of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C.

**DNA Constructs**—cDNAs encoding the rabbit C1a and Δ13 variants of the CTR were generated by PCR and cloned in the Kpn I/HindIII sites of the p3XFLAG-CMV-15 vector (Sigma) to obtain CTRs with a C-terminal 3-fold FLAG tag. The same PCR products were cloned in the Kpn I/HindIII site of pEGFP-N1 (Clontech) to obtain C-terminal GFP-tagged CTR splice variants. The PCR fragments were also cloned in the Kpn I/HindIII site of pDsRed2-N1 (Clontech) to obtain C-terminal RFP-tagged CTR splice variants. The GFP constructs were published before and compared with the wild type CTR in respect to ligand binding, trafficking after ligand stimulation, and CAMP generation and found to be indistinguishable (30). For this study, we also compared the cellular localization of the GFP- and RFP-tagged CTR constructs with the FLAG-tagged constructs by immunocytochemistry. They were found to be indistinguishable (data not shown). A fragment spanning the complete receptor including the seventh transmembrane domain but lacking the intracellular C-terminal tail of the receptor (amino acids 1–397) was cloned in the Kpn I/HindIII site of the pEGFP-N1 vector and designated C1a397-GFP. We also cloned these fragments in the Kpn I/HindIII site of the p3XFLAG-CMV-13 vector. Constructs expressing each of the two splice variants with three tandem Myc epitope tags at the C terminus were generated as described before (30). All constructs contained an HA tag in the extracellular N terminus of the receptor (after amino acid 29 of the original sequence). We also generated a C-terminally GFP-tagged Δ13 construct containing a Myc tag instead of an HA tag in the extracellular N terminus by mutagenesis using the QuikChange mutagenesis kit (Stratagene, Cedar Creek, TX) according to the instructions of the manufacturer. The ligand binding and CAMP production of all full-length CTR constructs were measured and were found to be indistinguishable from the wild type CTR (data not shown). A fragment spanning the C-terminal tail of the CTR (amino acids 397–474) was cloned between the HindIII and Kpn I sites of the p3XFLAG-CMV-13 vector after generation by PCR. A PCR-derived fragment spanning the same region was cloned in the Kpn I/HindIII sites of the pEGFP-N1 vector (Amersham Biosciences) to obtain a GST-C-terminus fusion protein called GST-C-tail. The C-terminally RFP-tagged V1a vasopressin receptor was a generous gift from Dr. M. Nathanson (Yale University). All PCR-derived constructs were sequenced by the Yale Sequencing Facility.

**Co-immunoprecipitation and Western Blotting**—Cells were lysed in mRIPA buffer (50 mm Tris-HCl, pH 7.5, 150 mm NaCl, 0.1% IGEPAL, 1% sodium deoxycholate, 10 mm NaF, 1 μg/ml pepstatin, and 1 mm phenylmethylsulfonyl fluoride) and incubated at 4 °C for 30 min. Lysates were then centrifuged for 30 min at 4 °C, 16,000 × g, and the protein concentrations were measured with the BCA protein assay kit (Pierce), and equal amounts of protein were used for immunoprecipitation. 30 μl of protein A-Sepharose typically were suspended in 500 μl of PBS and incubated for 1 h at 4 °C. The beads were washed three times in mRIPA buffer, and then 500 μl of protein lysate and bovine serum albumin (0.2% w/v) were added, and the mix was incubated for 2 h at 4 °C. The immune complexes on the beads were washed four times with washing buffer containing 300 mm NaCl and 0.1% Triton X-100 and once with PBS. Beads were boiled in 2% SDS-PAGE buffer, and samples were electrophoresed on precast 10% SDS-PAGE gels (Invitrogen). Proteins were transferred to nitrocellulose membranes, and the transfer was verified by staining with 0.2% Ponceau S in 3% trichloroacetic acid. Nonspecific binding was blocked by incubating the membranes in 5% nonfat milk in TBST buffer (50 mm Tris-HCl, pH 7.5, 150 mm NaCl, and 0.1% Tween 20). Membranes were incubated in the primary antibody for 2 h, washed three times for 15 min in TBST, and incubated for 1 h in 1:10,000 diluted horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG antibody (Promega). Blots were developed using the enhanced chemiluminescence system from Amersham Biosciences.

**Immunolocalization of Surface β3 Integrin**—Cells were washed two times with ice-cold PBS, cooled on ice, and incubated in the presence of 0.5 mg/ml sulfo-N-hydroxysuccinimide-biotin (Pierce) at 4 °C for 30 min to biotinylate proteins on the cell surface. Excess biotin was quenched by incubating in 50 mm Tris-HCl for 10 min at 4 °C. Cells were washed twice and harvested in mRIPA buffer.

**Analysis of Differences in Transfected CTRs**—RT-PCR, immunoblotting, and Western blotting of different transfected CTRs, biotinylated proteins were bound to 25 μl of resin of immobilized streptavidin (Pierce) by incubating for 2 h at 4 °C in the presence of 0.1% bovine serum albumin (w/v). After the incubation, the resin was pelleted by centrifugation and washed four times with immunoprecipitation-washing buffer contain-
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Overexpression and Purification of GST Fusion Proteins—Exch. richia coli, strain BL-21, harboring the empty GST vector or GST-C-tail over the time course, using the same channels as described above. Membrane areas, and the GFP and RFP fluorescence was measured caused by absorption of light energy. The region of interest was set to be the RFP with the 568-nm krypton laser. An increase of the donor fluorescence (GFP) was interpreted as evidence of FRET from GFP to RFP. An unbleached area of membrane in the same cell served as control. To perform FRET analysis, we used the C-terminal GFP- and RFP-tagged receptor constructs as described in Ref. 31. HEK 293 cells were seeded in 35-mm glass bottom dishes (MatTek Corp., Ashland, OR) and 24 h later transfected with the GFP- and RFP-tagged CTR constructs as described above. Images were taken by placing the dish in a heated stage (DH-35; Warner Instruments, Hamden, CT) in a Zeiss LSM 510 confocal microscope (Zeiss, Jena, Germany) to maintain a temperature of 37 °C. The culture medium was replaced by 37 °C warm HEPES buffer containing glucose. A 63 × 1.25 numerical aperture water immersion objective was used with the pinhole set to 2.5 Airy disc units. GFP was excited at 488 nm by an argon laser, and RFP was excited at 568 nm using a krypton laser. Emission was measured in the green channel from 505 to 530 nm and in the red channel from 610 to 670 nm. FRET was measured by imaging GFP before and after photobleaching the RFP with the 568-nm krypton laser. An increase of the donor fluorescence (GFP) was interpreted as evidence of FRET from GFP to RFP. An unbleached area of membrane in the same cell served as control. All experiments analyzed at least 10 cells, the mean change of GFP fluorescence was calculated, and statistical analysis of the results was performed by using Student’s t test. For time lapse studies, cells were prepared as described above. For the imaging, the excitation energy was lowered to minimize photobleaching and cell damage caused by absorption of light energy. The region of interest was set to membrane areas, and the GFP and RFP fluorescence was measured over the time course, using the same channels as described above.

Fluorescence Resonance Energy Transfer (FRET) Experiments—FRET analysis was performed using the C-terminal GFP- and RFP-tagged receptor constructs as described in Ref. 31. HEK 293 cells were transfected with either the C1a-GFP construct (left panel) or the Δe13-GFP construct (right panel). 36 h after transfection, cells were examined by confocal microscopy. Both the C1a and the Δe13 construct accumulate intracellularly, but the membrane staining of the cells transfected with the Δe13-GFP construct was much less than that of the C1a construct. The white bar represents 5 μm. B; FACS analysis confirmed that the surface expression of the Δe13-GFP (upper right panel) was much lower than that of the C1a-GFP construct (upper left panel). The N-terminally HA-tagged receptors on the surface were labeled using anti-HA and a PE-conjugated secondary antibody, as described under “Experimental Procedures.” Cells with high fluorescence from both GFP and PE (i.e. cells with GFP-tagged CTR on the cell surface) are located in the upper right quadrant of the dot plots. The plot represents a typical result of six independent experiments. The lower panel shows mean values of cell-associated PE of six independent samples. HEK 293 cells not transfected with a CTR construct were used as a negative control.

Results

The Cell Surface Expression Levels of the C1a and the Δe13 Isoform of the Rabbit CTR Differ.—The decrease of the cAMP response previously reported for the Δe13 isoform of the CTR could reflect, at least in part, different levels of cell surface expression of the two isoforms. To examine the subcellular localization of the two isoforms, we first generated C-terminal GFP constructs for the C1a and the Δe13 isoforms and expressed both in HEK 293 cells. Both isoforms showed marked accumulation within the cell (Fig. 1A). Similar intracellular
accumulations were found when the CTR isoforms were tagged with the FLAG epitope (data not shown). We previously reported that these accumulations are a recycling compartment for the C1a isoform (30). Moreover, we found that much less of the Δe13-GFP chimera (Fig. 1A, right panel) was expressed on the cell surface relative to the C1a-GFP chimera (Fig. 1A, left panel). To better quantify the difference in the cell surface expression, we used a flow cytometric method to measure the cell surface expression of the two constructs. An HA tag near the N terminus of the C1a- and Δe13-GFP constructs was used to label the receptors on the cell surface with fluorescent phycoerythrin (PE). FACS analysis showed that whereas the expressions of the two GFP-tagged CTR isoforms were similar (Fig. 1B, upper panels), there was much less PE labeling of the cells expressing the GFP-Δe13 chimera (Fig. 1B, lower panel), indicating that the C1a isoform is much more highly expressed on the cell surface of HEK 293 cells than the Δe13 isoform. The difference was statistically significant (p < 0.001).

The C1a and the Δe13 Isoform of the Rabbit CTR Form Dimers—To address the question of whether or not the two isoforms of the CTR form hom- or heterodimers, we coexpressed FLAG- and/or GFP-tagged C1a and Δe13 receptors in HEK 293 cells as described in Fig. 2. After immunoprecipitation of the FLAG-tagged CTR, we blotted the immune complexes with the anti-GFP antibody. In all cases, we found that the immune complexes contained both the CTR-FLAG chimera and the CTR-GFP chimera, suggesting that the CTR forms dimers or oligomers (Fig. 2A, upper panel). We found C1a/Δe13 heterodimers (lane 6) as well as C1a/C1a (lane 5) and Δe13/Δe13 (lane 7) homodimers.

We next asked whether ligand binding affects the formation of CTR dimers. HEK 293 cells transfected with the C1a-GFP construct alone or in combination with the C1a-FLAG construct were stimulated with 10 nM sCT for different time periods, the cells were lysed, and the FLAG-tagged CTR was immunoprecipitated. Blotting of the immune complexes with the anti-GFP antibody showed no significant change in the amount of co-immunoprecipitating GFP-tagged C1a-CTR after ligand stimulation (Fig. 2B, upper panel), suggesting that the dimerization of the CTR is ligand-independent. Since large amounts of the CTR-GFP chimera, especially the Δe13 variant, were located intracellularly, the dimers that we isolated might be present only in the intracellular compartments. To determine whether dimerized receptors are present at the cell surface, the surface proteins of cells that had been co-transfected with GFP-tagged and FLAG-tagged CTR were labeled with biotin. This allowed the specific isolation of the receptors that were present at the cell surface using immobilized streptavidin, as described under “Experimental Procedures.” Once the biotinylated proteins were eluted from the streptavidin, the FLAG-tagged C1a was immunoprecipitated, and the immune complexes were blotted with anti-GFP antibody (Fig. 2C, upper panel). When both the FLAG-tagged and the GFP-tagged CTR were the C1a isoform, C1a-GFP was found in the immune complex, indicating that C1a homodimers are present at the cell surface. In contrast, GFP-tagged Δe13 was not detected in the biotinylated complexes, despite the fact that we had shown that Δe13/C1a heterodimers do form. This is consistent with the observed absence of Δe13 at the cell surface and indicates that the dimers are stable under the conditions used here to isolate the receptor dimers.

In order to demonstrate the presence of CTR dimers in living cells, we did a FRET experiment using RFP- and GFP-tagged CTR constructs. In this technique, a GFP in close proximity to an RFP, as in a dimer, will transfer light energy nonfluorescently to the RFP, which will then emit the energy as fluorescence at a longer wavelength, resulting in decreased fluorescence by the GFP (31). HEK 293 cells were co-transfected with GFP-C1a and C1a-RFP constructs, and small regions of the surface membrane were photobleached. Increased green fluo-
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Fluorescence resonance energy transfer analysis confirms a constitutive dimerization of the CTR. A. HEK 293 cells co-transfected with the GFP-tagged and RFP-tagged C1a-constructs were placed in a heated stage in a confocal microscope. GFP was photobleached by repeated scanning of the membrane region in the rectangular areas with the 568-nm line of the laser. The GFP and RFP fluorescence intensities were recorded before and after the photobleaching. An example of a cell expressing the C1a-GFP and C1a-RFP constructs before (left panel) and after (right panel) photobleaching is shown. Note the loss of GFP fluorescence after photobleaching. B, the mean change in GFP fluorescence energy after photobleaching of RFP for 10 independent experiments for each pairing is shown. The increase in GFP fluorescence after photobleaching the RFP was statistically significant for all CTR pairings compared with all controls used (*, p < 0.05). C, the RFP (■) and GFP (▲) fluorescence at the plasma membrane of HEK 293 cells co-expressing C1a-GFP and C1a-RFP was measured every 3 s during perfusion of the chamber with 10 nM sCT for 5 min. The figure shows the change of fluorescent intensity compared with the beginning of the experiment for each dye over time. The figure shows a typical example of five independent experiments.

Fluorescence was detected in all fluorescent cells, regardless of the intensity of fluorescence (level of expression) of the fluorescent protein-tagged CTRs. Fig. 3A shows an example of a cell before (left panel) and after photobleaching RFP (right panel). The mean changes of GFP fluorescence after photobleaching RFP in 10 different experiments is shown in Fig. 3B. The GFP fluorescence increased by about 7%, suggesting that there had been energy transfer between the GFP- and RFP-tagged C1a constructs. Similar results were obtained if the photobleaching was performed in an area of the intracellular accumulation of the C1a receptors (data not shown). The same experiment was done using C1a-GFP/Δe13-RFP and Δe13-GFP/Δe13-RFP pairs. For these pairings, colocalization was only observed in areas of the intracellular accumulations, and therefore the RFP-photobleaching was done in this area. In both instances, photobleaching of RFP was accompanied by a significant increase of GFP fluorescence, providing further support for the presence of C1a-GFP/Δe13-RFP and Δe13-GFP/Δe13-RFP dimers. In contrast, photobleaching of the C-terminally RFP-tagged vasopressin (V1a) receptor in the plasma membrane of cells co-transfected with the GFP-tagged C1a construct did not result in an increased GFP fluorescence (Fig. 3B). Moreover, photobleaching of RFP using GFP/RFP pairs where one fluorescent protein was untagged RFP or GFP, respectively, did not result in an increased GFP fluorescence, suggesting that the effect observed for the CTR RFP/GFP chimera was specific (Fig. 3B).

FRET was also used to monitor ligand-induced changes in the CTR dimerization status in living cells. HEK 293 cells co-transfected with the C1a-GFP and C1a-RFP construct were placed in a heated stage of a confocal microscope, and the RFP and GFP fluorescence were monitored during perfusion of the chamber with 10 nM sCT. As shown in Fig. 3C, the fluorescence intensities of neither RFP nor GFP changed during sCT perfusion, suggesting that binding of the ligand is not increasing or decreasing the dimerization of the CTR, consistent with our earlier findings (Fig. 2B).

The Dimerization of the CTR Involves Multiple Domains of the Receptor—GPCR dimerization has been reported to involve various domains of the receptors, depending on the specific receptor being studied. The extracellular domain of some GPCRs was found to be involved (32), whereas the transmembrane domains or the C-terminal tail of other receptors mediate dimer formation (20, 21). We first examined the role of the C-terminal tail of the CTR in the dimerization. A construct encoding the complete C-terminal domain of the CTR with a C-terminal FLAG tag was expressed in HEK 293 cells in the absence or presence of C1a-RFP and as a control in the presence of the (V1a) vasopressin RFP chimera. Immunoprecipitation of the receptors with the anti-RFP antibody co-immunoprecipitated the small C-terminal tail construct when the C1a construct was co-expressed but not when the vasopressin receptor was present (Fig. 4A, upper panel), suggesting that the C-terminal tail binds to the full-length CTR. To explore the role of the cytoplasmic tail of the CTR in the receptor dimerization in more detail, we established an in vitro binding assay. Constructs of the complete C-terminal tail fused to glutathione S-transferase (GST) were expressed in bacteria and purified with glutathione-conjugated agarose beads. Samples of the beads were subjected to SDS-PAGE, and the bound proteins had the expected molecular weight (Fig. 4B, upper panel). The purified fusion proteins were incubated with total cell lysates from HEK 293 cells expressing either full-length C1a-GFP, a GFP chimera lacking the C-terminal tail (CTRΔ397), or the V1a vasopressin-RFP chimera. As predicted by the initial results, the GST-C-terminal tail fusion protein bound to the full-length C1a-GFP protein (Fig. 4B, second panel). It did not bind to the CTRΔ397 (Fig. 4B, third panel), suggesting that the C-terminal tail of the CTR binds only the cytoplasmic tail and no other domains of the receptor. Moreover, no binding of the vasopressin-RFP chimera to the GST C-terminal tail GST fusion protein could be observed (Fig. 4B, bottom panel).
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To determine whether the interaction between the C-terminal domain of the two receptors is required for dimerization, we co-transfected cells with the full-length FLAG-tagged C1a construct or the C1a-FLAG construct without the C-terminal tail and either the full-length GFP-tagged C1a construct or the C1a-GFP construct without the C-terminal tail. As shown before, immunoprecipitation of the FLAG-tagged full-length C1a construct co-immunoprecipitated both the GFP-tagged C1a and the C-terminally truncated GFP-tagged CTRA397 (Fig. 4C, upper panel, lane 2 and 3, respectively). The truncated GFP-tagged CTRA397 also co-immunoprecipitated with the truncated CTRA397 FLAG construct (Fig. 4C, upper panel, lane 4). This result, together with the failure of the GST-C-terminal fusion protein to bind to the truncated receptor, indicates that the C-terminal tail of the CTR is dispensable for CTR dimerization. Thus, the CTR dimerization appears to involve at least two domains of the receptor, one of which is the C-terminal tail of the receptor.

Co-expression of the C1a and the Δe13 Isoforms of the Rabbit CTR Decreases the Surface Expression of the C1a Isoform—The significant difference in the cell surface expression of the C1a and the Δe13 isoforms and the fact that the isoforms form heterodimers raised the question of whether the heterodimerization of the two isoforms would either increase the surface expression of the Δe13 isoform or decrease the surface expression of the C1a isoform. To address this question, we co-transfected HEK 293 cells with the GFP-tagged Δe13 construct and the RFP-tagged C1a construct. When co-expressed with the Δe13 isoform in HEK 293 cells, the C1a-RFP was clearly present at the cell surface as well as intracellularly (Fig. 5A, left panel), as we had observed when the C1a-GFP chimera was expressed alone in HEK 293 cells (Fig. 1A). Also, the pattern of the Δe13-GFP localization in the cell was not changed when co-expressed with the C1a isoform (Fig. 5A, middle panel). Consequently, the overlay of both micrographs (Fig. 5A, right panel) shows a clear colocalization in the region of the intracellular accumulations, whereas no colocalization could be observed at the cell surface.

This result, together with our finding that the Δe13 isoform was not detectable at the cell surface when co-expressed with the C1a isoform (Fig. 2C), indicated that the C1a isoform does not promote the cell surface expression of the Δe13 isoform. On the other hand, an inhibition of the cell surface expression of the C1a isoform by the Δe13 isoform cannot be excluded by confocal microscopy. We therefore quantified the effect of the Δe13 isoform on the C1a surface expression by FACS analysis using a N-terminal HA-tagged C1a isoform and N-terminal Myc-tagged Δe13-GFP construct. As shown in Fig. 5B, the PE staining of the HA-tagged C1a-CTR was reduced by about 50% when the Δe13 isoform was co-expressed. In contrast, surface staining of the Myc-tagged Δe13 isoform was unchanged in the presence of the C1a isoform (Fig. 5B, last bar), confirming the result of the confocal microscopy.

To confirm the finding of an inhibitory effect of the Δe13 isoform on cell surface expression of the C1a isoform with a different method, we again used the biotinylating compound to label the CTRs at the surface of cells expressing either the C1a-FLAG construct or the Δe13-FLAG construct alone or both the C1a-FLAG construct and the Δe13-Myc construct. The FLAG-tagged CTRs were immunoprecipitated and assayed for biotin with horseradish peroxidase-conjugated avidin (Fig. 5C, upper panel). As expected, we detected little or no biotinylation of the Δe13 isoform when it was expressed alone, confirming the very low degree of surface expression of this isoform (Fig. 5C, upper panel). In contrast and as expected, the C1a-FLAG construct was strongly biotinylated when expressed alone, re-
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**FIG. 5.** Co-expression of the CTRΔe13 isoform with the C1a isoform reduces the surface expression of the C1a isoform. A, HEK 293 cells co-expressing the GFP-tagged Δe13 construct and the RFP-tagged C1a construct were examined using a confocal microscope. The C1a-RFP was clearly localized at the cell membrane and in strongly fluorescent perinuclear vesicles (left panel), whereas the Δe13-GFP construct preferentially localized in the perinuclear vesicles (middle panel). Merging the two images showed colocalization primarily in the perinuclear region (right panel). The white bar represents 5 μm. B, HEK 293 cells were transfected with HA-tagged C1a-GFP alone, Myc-tagged Δe13-GFP alone, or HA-tagged C1a-GFP and Myc-tagged Δe13 together. The cell surface expression of the CTR-GFP chimeras was measured by FACS as described under "Experimental Procedures." Surface expression of the C1a isoform was measured using the anti-HA antibody as primary antibody; surface expression of the Δe13 isoform was measured using the anti-Myc antibody as primary antibody. The mean values of cell-associated PE from four independent samples are shown. The Student’s t test was used to analyze the statistical significance of the difference between PE-values for the C1a isoform in the presence or absence of the Δe13 variant. C, HEK-293 cells were mock-transfected (pDNA 3.1 alone), transfected with C1a-FLAG or Δe13-FLAG alone, or co-transfected with the C1a-FLAG and Δe13-Myc constructs. After 48 h, cells were surface-biotinylated at 4 °C and lysed. The FLAG-tagged CTR was immunoprecipitated (IP) using the monoclonal anti-FLAG antibody. The immune complexes were blotted with an avidin-horseradish peroxidase conjugate to visualize the FLAG-tagged C1a receptor that was exposed at the cell surface (upper panel, arrows). The membrane was stripped and rebotted with the anti-FLAG antibody to ensure that the same amounts of CTR-FLAG were immunoprecipitated (lower panel). IB, immunoblot.

Reflecting the high degree of surface expression of this isoform, however, when the C1a-FLAG was co-expressed with the Δe13 isoform, we found a significant decrease of the biotin signal, again indicating that the presence of the Δe13 isoform markedly decreased the surface expression of the C1a isoform. Importantly, the amount of C1a-FLAG protein was unchanged in the presence of the Δe13 isoform (Fig. 5C, lower panel), indicating that the transport of the C1a isoform to the cell surface is inhibited and suggesting that the protein synthesis and degradation are not affected.

A reduction in the level of surface expression of the C1a isoform of the CTR, when co-expressed with the Δe13 isoform, should result in a reduced response to ligand stimulation. In order to address this question, we stimulated HEK 293 cells expressing the C1a isoform alone, the Δe13 isoform alone, or a combination of both isoforms with increasing concentrations of sCT and measured the generation of cAMP. As previously reported (8), the C1a-transfected cells showed a clear dose-dependent increase of cAMP, and the Δe13 isoform, although only poorly expressed on the cell surface, also showed a dose-dependent increase in cAMP after sCT stimulation, although the amplitude of the response was decreased to about half of the C1a isoform (Fig. 6A). When the Δe13 isoform was co-expressed with the C1a isoform, the increase in cAMP levels after sCT stimulation was clearly reduced relative to that seen with the C1a isoform alone (Fig. 6A), supporting the conclusion that the Δe13 isoform reduced the surface expression of C1a isoform.

The CAMP response in the Δe13-transfected cells suggests that at least some cells are expressing a small number of Δe13 at the cell surface when overexpressed, which is consistent with the result of the FACS analysis in Fig. 1B. To further test the hypothesis of a dominant-negative effect of the Δe13 isoform on surface expression of the C1a isoform, we examined CT-in-
Reduced Erk phosphorylation in HEK 293 cells that expressed the C1a and Δe13 isoforms alone or in combination (Fig. 6B). Treatment with sCT for 5 min induced significantly more Erk phosphorylation when cells expressed only the C1a isoform than when both the C1a and the Δe13 isoforms were expressed together (Fig. 6B), despite indistinguishable levels of the C1a receptor (Fig. 6B, third panel). In contrast, no CT-induced Erk phosphorylation was detected in cells expressing only the Δe13 isoform, even at very high sCT concentrations up to 100 nM, consistent with our published findings (8, 10, 17).

If the inhibition of cell surface expression of the C1a isoform by the Δe13 isoform has a physiological role, then both splice variants must be present together in a cell that naturally expresses the CTR. To determine whether this is the case in osteoclasts, we generated rabbit OCLs, separated single OCLs in a 96-well PCR plate by FACS, and performed a reverse transcription of the RNA. PCR was performed using a primer amplifying a region that contained exon 13, generating a PCR product of 356 bp when derived from the C1a cDNA or 308 bp when derived from the Δe13 cDNA. The PCR products were separated on a 2.5% agarose gel and stained with ethidium bromide.

The present study adds the CTR to the group of GPCRs that are expressed together in OCLs. Rabbit OCLs were generated as described under “Experimental Procedures,” and single OCLs were distributed in single wells of a 96-well PCR plate by FACS. After lysis resulting in PCR products with different sizes when derived from the C1a and Δe13 isoforms alone or in combination (Fig. 6B), despite indistinguishable levels of the C1a receptor (Fig. 6B, third panel). In contrast, no CT-induced Erk phosphorylation was detected in cells expressing only the Δe13 isoform, even at very high sCT concentrations up to 100 nM, consistent with our published findings (8, 10, 17).

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The present study adds the CTR to the group of GPCRs that form dimers or oligomers. Ligand binding to the receptor can increase (33) or decrease dimerization or have no effect on receptor dimerization, depending on the receptor studied (20, 21). Using both co-immunoprecipitation and the more sensitive FRET technique, we found that the CTR dimerization is ligand-independent. FRET also allowed us to examine dimerization of the CTR in different compartments of the cell. The fact that FRET occurred both at the cell surface and in intracellular vesicles supports the finding that the CTR dimerization is ligand-independent.

Our results suggest that the C-terminal tail might be involved in the receptor dimerization. The result of the co-immunoprecipitation experiment, confirmed by the in vitro binding assay, showed that the C-terminal tail bound to the full-length CTR but not to the receptor construct without the C-terminal domain (CTRΔ397), suggesting that interactions between C-terminal domains contribute to dimer formation. However, the truncated CTRΔ397 co-immunoprecipitated both with the full-length receptor and with another CTRΔ397 molecule, indicating that at least one other domain, in addition to the C-terminal tail, is involved in the dimer formation. Studies of the structure of GPCR dimers have revealed the involvement of various domains in receptor dimerization. The only available crystal structure of a GPCR dimer demonstrated that a disulfide bridge in the extracellular part of the metabotropic glutamate receptor 1 might be involved in the formation of the receptor dimers (32). Dimerization of the calcium sensing receptor (34), the metabotropic glutamate receptor 5 (35), and the M3 muscarinic receptor (36) were also suggested to involve disulfide bridges between extracellular domains of the receptors. In most of these cases, however, the disulfide bridges were not the only point of contact, and noncovalent interactions involving other receptor domains were also proposed. For other receptors, hydrophobic interactions involving transmembrane domains (37) or the C-terminal tail (23) contribute to receptor dimerization. Interestingly, whereas the binding of the C-terminal tail of GRB2 to GRB1 is required for cell surface transport of GRB1, deletion of the C-terminal tail did not prevent receptor dimerization (26).

The reason for the inefficient expression of the Δe13 splice variant on the cell surface and dimerization-dependent retention of the C1a isoform is not clear. We considered the possibility that surface expression of Δe13 might, in contrast to the C1a isoform, require binding to one or another member of the receptor activity-modifying protein family to be efficiently transported to the cell surface, much like the closely related calcitonin receptor-like receptor (38). However, the surface expression of the Δe13 variant was unaffected by the overexpression of any of the three receptor activity-modifying proteins (data not shown). The difference between the C1a and Δe13 splice variants is that the Δe13 isoform lacks the C-terminal 14 amino acids of the seventh transmembrane domain. It may be that splicing out of this region of the Δe13 isoform generates a motif that inhibits the transit of the Δe13 isoform to the cell surface, much like the retention signal that has been described for the GRB1 isoform of the GABAB receptor (26). The GRB1 motif is however not present in the Δe13 isoform.

The findings of this study establish the CTR as another example of heterodimerization of GPCR isoforms or mutants changing the cell surface expression. Heterodimerization of the rabbit CTR isoforms prevents transport of the receptor to the cell surface, the converse of the effect reported for the GABAB receptor, where dimerization of the GRB1 with the GRB2 isoform is required for the transport of the GRB1 isoform to the cell surface. On the other hand, there are reports describing negative effects of mutant receptors on the cell surface transport of other GPCRs, including the V2 vasopressin receptor (V2R) (20, 39, 40), the human chemokine receptor 5 (CCR5).
The Δe13 Variant of the CTR Inhibits CTR Surface Expression

(41–43), and the dopamine D3 receptor (44). In contrast to these reports, the Δe13 splice variant of the CTR is a naturally occurring variant of the CTR that is co-expressed with the C1a isoform in osteoclasts, kidney, brain, heart, muscle, and lung (8). Interestingly, splice variants arising from similar deletions of the highly conserved small exon that encodes 14 amino acids in the putative seventh transmembrane domain have been described for two other members of the receptor family, the CRH-R1 (18) and the parathyroid hormone/parathyroid hormone-related protein receptors (19). It is not known if these splice variants also interact with the predominant isoform in a dominant-negative manner. A preliminary report indicated that the insert-positive isoform of the human CTR also acts in a dominant-negative fashion, but only with regard to the induction of calcium signaling by the C1a isoform (45), suggesting that the mechanism of this dominant-negative effect does not involve an inhibition of the cell surface expression of one isoform. The results shown here suggest that the CTRΔe13 splice variant plays a role as a negative regulator of the CTR. Interestingly, the ratio of C1a/Δe13 transcripts in single osteoclast-like cells was highly variable, suggesting that this ratio is regulated. The questions of how the splicing of the C1a and the Δe13 variant is regulated and whether physiological circumstances lead to a change in the ratio of C1a and Δe13 transcripts remain to be addressed. The difference in the relative levels of expression of the two isoforms in individual osteoclasts might explain our past observation that not all osteoclasts respond to CT(4), although this remains to be shown.

In summary, we found that both the rabbit CTR C1a and Δe13 splice variants constitutively homodimerize and heterodimerize. The functional relevance of the splice variant heterodimers appears to involve the retention of the Δe13/C1a heterodimer within the cell, which leads to a marked reduction of the C1a isoform at the cell surface and reduction in the C1a-mediated signaling. Thus, one splice variant of the CTR acts in a dominant-negative manner to reduce the surface expression of another, providing an additional and novel mechanism for regulating signaling by the CTR and possibly other GPCRs.

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