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ACCELERATED COMMUNICATION

Centaurin-α_1, an ADP-Ribosylation Factor 6 GTPase Activating Protein, Inhibits β_2-Adrenoceptor Internalization

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ABSTRACT

The small GTP-binding protein ADP ribosylation factor 6 (ARF6) has recently been implicated in the internalization of G protein-coupled receptors (GPCRs), although its precise molecular mechanism in this process remains unclear. We have recently identified centaurin α_1 as a GTPase activating protein (GAP) for ARF6. In the current study, we characterized the effects of centaurin α_1 on the agonist-induced internalization of the β_2-adrenoceptor transiently expressed in human embryonic kidney (HEK) 293 cells. Using an enzyme-linked immunosorbent assay as well as confocal imaging of cells, we found that expression of centaurin α_1 strongly inhibited the isoproterenol-induced internalization of β_2-adrenoceptor. On the other hand, expression of functionally inactive versions of centaurin α_1, including an R49C mutant, which has no catalytic activity, and a double pleckstrin homology (PH) mutant (DM; R148C/R273C), which has mutations in both the PH domains of centaurin α_1, rendering it unable to translocate to the cell membrane, were unable to inhibit β_2-adrenoceptor internalization. In addition, a constitutively active version of ARF6, ARF6Q67L, reversed the ability of centaurin α_1 to inhibit β_2-adrenoceptor internalization. Finally, expression of centaurin α_1 also inhibited the agonist-induced internalization of β_2-adrenoceptor endogenously expressed in HEK 293 cells, whereas the R49C and DM mutant versions of centaurin α_1 had no effect. Together, these data indicate that by acting as an ARF6 GAP, centaurin α_1 is able to switch off ARF6 and so inhibit its ability to mediate β_2-adrenoceptor internalization. Thus, ARF6 GAPs, such as centaurin α_1, are likely to play a crucial role in GPCR trafficking by modulating the activity of ARF6.

The internalization of G protein-coupled receptors (GPCRs) occurs in response to agonist stimulation, often after receptor desensitization (Ferguson, 2001). In many cases, GPCR internalization is important for the recycling or down-regulation (Marchese et al., 2003) of receptors and has also been implicated in GPCR signaling (Ostrom and Insel, 2004). For some well characterized GPCRs, such as the β_2-adrenoceptor, phosphorylation by GPCR kinases (GRKs) leads to binding of nonvisual arrestins to the receptor and subsequent targeting of receptor to clathrin-coated pits for internalization (Krupnick and Benovic, 1998). However, the molecular details of the trafficking of GPCRs inside the cell remain to be fully characterized.

The ADP-ribosylation factor (ARF) family of small GTPases has been implicated in the regulation of vesicle trafficking (Moss and Vaughan, 1998). These proteins regulate trafficking by shuttling between an active GTP-bound form and an inactive GDP-bound form. Of the six mammalian ARF isoforms (ARFs 1–6), ARF6 is of particular interest with regard to GPCR trafficking because it is localized to both

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ABBREVIATIONS: GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; ARF, ADP-ribosylation factor; GEF, guanine nucleotide exchange factor; ARNO, ARF nucleotide-binding site opener; GAP, GTPase-activating protein; GIT1, ARF6-GAP-GRK interacting protein 1; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; EGF, epidermal growth factor; DMEM, Dulbecco's modified Eagle's medium; [^{3}H]CGP 12177, (−)-4(3-*t*-butylamino-2-hydroxypropoxy)benzimidazol-2-one [5,7-^{3}H]; HA, hemagglutinin; PH, pleckstrin homology; DM, double PH mutant; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; Ro 201724, 4-(3-butoxy-4-methoxybenzyl) imidazolidin-2-one; WT, wild-type; β_2-AR, β_2-adrenoceptor.

plasma membrane and endosomes. Although it was thought that ARF6 existed at the plasma membrane in a GTP-bound form, and in endosomes in the GDP-bound form, recent findings indicate that GTP/GDP exchange for ARF6 occurs at the plasma membrane (Macia et al., 2004). ARF6 regulates cargo transport between plasma membrane and endosomes and is also implicated in cortical actin rearrangement at the plasma membrane, which is important for such processes as internalization, chemotaxis, and focal adhesion (Donaldson, 2003). Other proteins in turn regulate the activity of ARFs. ARF guanine nucleotide exchange factors (GEFs), such as the ARF6 GEF cytohesin 2 [also known as ARF nucleotide-binding site opener (ARNO)], activate ARFs by promoting the replacement of GDP by GTP on the ARF. On the other hand, ARF GTPase-activating proteins (GAPs), such as the ARF6 GAP GRK interacting protein 1 (GIT1), enhance the intrinsic GTPase activity of ARF6, resulting in the hydrolysis of ARF6-bound GTP to GDP (Vitale et al., 2000; Hawadle et al., 2002). Another ARF6 GAP that has recently been identified is centaurin α_1 (Hammonds-Odie et al., 1996; Venkateswarlu and Cullen, 1999). This protein, which is enriched in brain, has an N-terminal zinc finger motif as with other ARF GAPs, which is necessary for ARF GAP activity; centaurin α_1 also contains two PH domains required for the binding of the second-messenger phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which is an important determinant of the recruitment of ARF6 to the plasma membrane (Venkateswarlu et al., 1999; Venkateswarlu and Cullen, 2000). We have shown recently that centaurin α_1 exerts GAP activity against ARF6 in vivo and can functionally switch off ARF6-activated processes, such as EGF-stimulated cortical actin rearrangement in HeLa cells (Venkateswarlu et al., 2004).

Because ARF6 has been implicated in the trafficking of GPCRs (Hunzicker-Dunn et al., 2002), it is thus possible that centaurin α_1 can also modify receptor internalization. Therefore, in the present study, we investigated the effect of centaurin α_1 on the widely studied and well characterized internalization of the β_2-adrenoceptor. We found that centaurin α_1 inhibits the agonist-induced internalization of the β_2-adrenoceptor and that this activity depends upon the ability of centaurin α_1 to act as a functional ARF6 GAP. Thus, the functional manipulation of ARF6 can modify GPCR trafficking in vivo, further supporting the importance of ARF6 as a regulator of receptor trafficking.

Materials and Methods

Materials. Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, LipofectAMINE 2000, and penicillin-streptomycin were obtained from Invitrogen (Paisley, Scotland, UK). [^{3}H]CGP 12177 (specific activity, 1110–2220 GBq/mmol) was from PerkinElmer (Beaconsfield, Buckinghamshire, UK). The mouse monoclonal anti-HA antibody was from Cambridge Biosciences (Cambridge, UK), the colorimetric alkaline phosphatase substrate kit was from Bio-Rad (Hemel Hempstead, Hertfordshire, UK), and the enhanced chemiluminescence (ECL) kit, the anti-mouse horseradish peroxidase-conjugated antibody, and [^{3}H]cyclic AMP (specific activity, 925 GBq/mmol) were from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). The anti-mouse IgG alkaline phosphatase conjugate antibody, mouse monoclonal anti-FLAG M2 antibody, and mouse monoclonal anti-FLAG M2-Cy3 antibody, were obtained from Sigma (Poole, Dorset, UK). The QuikChange II site-directed mutagenesis kit was purchased from Stratagene (Amesterdam, The Netherlands).

Plasmids. FLAG-β_2-adrenoceptor/pcDNA3, HA-ARF6/pXS, HA-ARF6(T27N)/pXS, HA-ARF6(Q67L)/pXS and HA-ARF1(Q71L)/pXS were described previously (Radhakrishna and Donaldson, 1997). HA-ARF6(T44N)/pXS mutant was generated using the sequence specific mutagenic primers (sense, 5′-GTGACCACCATTCCCAATGT-GGGTTTCAACGTG-3′; antisense, 5′-CACGTTGAAACCCACATT-GGGAATGGTGGTCAC-3′), with HA-ARF6/pXS as template and with the use of the QuikChange II site-directed mutagenesis kit and by following the manufacturer's instructions. Centaurin α_1 WT, centaurin α_1 R49C and centaurin α_1 DM (double PH mutant; R149C/R273C) were excised from the pEGFP vector (Venkateswarlu et al., 1999, 2004) by EcoRI-SalI digestion and subcloned into the EcoRI-XhoI sites of pCMV3-Myc.

Cell Culture and Transient Transfection. HEK 293 cells were maintained in culture using DMEM supplemented with 10% (v/v) fetal calf serum, penicillin (100 units/ml), streptomycin (0.1 mg/ml), and 2 mM glutamine. Cells were transfected using LipofectAMINE 2000 according to the manufacturers' instructions using a total of 4 μg of plasmid DNA for ELISA and immunofluorescence (60-mm plates) and 6 μg of plasmid DNA for cyclic AMP assays (100-mm plates). After transfection, cells were split onto 24-well plates and used for experimentation 48 h after transfection.

Agonist-Induced Receptor Internalization. For transiently expressed β_2-adrenoceptor, the loss of surface receptor in response to agonist treatment was quantified by ELISA exactly as described previously using mouse monoclonal anti-FLAG M2 antibody and anti-mouse IgG alkaline phosphatase conjugate secondary antibody (Mundell et al., 2001). For endogenous β_2-adrenoceptor, cell surface loss of receptor was assessed using the radioligand [^{3}H]CGP 12177. In brief, cells grown on 60-mm dishes (transfected with constructs 48 h previously) were incubated in the presence or absence of 10 μM isoproterenol for various lengths of time. Cells were then detached and washed three times with ice-cold phosphate-buffered saline (PBS), and the cells were resuspended in ice-cold binding buffer (20 mM HEPES, 1 mM $MgCl_2$, pH 7.4). Cells were then incubated with 10 nM [^{3}H]CGP 12177 $\pm$ 10 μM propranolol (the latter was included to define specific binding, which was $\sim$70% of total binding) for 90 min at 4°C. Cells were then collected on filter paper using a Brandel cell harvester, and the filter disks were added to scintillant for scintillation counting.

Immunofluorescence and Confocal Imaging. Transiently transfected HEK 293 cells were seeded onto 0.1 mg/ml poly-L-lysine-coated 13-mm coverslips, using normal growth media. The next day, the medium was removed and cells incubated with monoclonal anti-FLAG M2-Cy3, diluted 1:200 in 1% (w/v) bovine serum albumin/DMEM for 1 h at 4°C. The cells were washed twice in PBS and stimulated with 0.5 μM isoproterenol at 37°C for the required length of time, then cells were fixed using 4% (w/v) paraformaldehyde in PBS for 30 min at room temperature. The cells were washed twice with PBS. The coverslips were mounted onto glass slides using Mowiol/DABCO as described previously (Venkateswarlu, 2003). Immunofluorescence staining of the cells was examined using a Leica TCS-NT confocal microscope (Leica, Wetzlar, Germany).

Gel Electrophoresis and Immunoblotting. Whole-cell lysates were prepared from transiently transfected HEK 293 cells. Proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred onto polyvinylidene fluoride membrane, and immunoblotted using a mouse monoclonal anti-HA antibody as described previously (Venkateswarlu et al., 2004).

Cyclic AMP Assay. Medium was removed, and 0.5 ml of pre-warmed fresh medium containing a 250 μM concentration of the phosphodiesterase inhibitor Ro 201724 was added to each well, followed immediately by isoproterenol (0.5 μM) or water (control). This was incubated for 0 to 30 min at 37°C, and the reaction was terminated by the addition of 20 μl of 100% (w/v) ice-cold trichloroacetic acid. The cyclic AMP content of the samples was analyzed with the use of a protein binding assay using [^{3}H]cyclic AMP as described previously (Mundell et al., 1998).

Statistical Analysis. Unless indicated otherwise, data were analyzed by one-way ANOVA with Bonferroni post test. Differences were taken as statistically significant when $p < 0.05$.

Results

Agonist-Induced Internalization of Transiently Expressed β_2-Adrenoceptor Is Dependent on Functional ARF6. In initial experiments, both the concentration and time dependence of agonist-induced β_2-adrenoceptor internalization in HEK 293 cells transiently transfected with receptor was assessed by ELISA. When added to the cells for 30 min, the β_2-adrenoceptor agonist isoproterenol induced receptor internalization with an EC_{50} of ~0.3 μM, with a maximum ~35% internalization being observed at 1 to 10 μM isoproterenol. Addition of 0.5 μM isoproterenol to the cells led to time-dependent internalization of β_2-adrenoceptor (percentage surface receptor loss was 23.5 ± 4.1, 29.3 ± 3.3, and 27.5 ± 2.6 at 15, 30, and 60 min after addition of agonist, respectively; values are means ± S.E.M., $n = 3$–4 in each case) with maximum β_2-adrenoceptor internalization observed 10 to 20 min after agonist addition. Confocal imaging of cells confirmed the agonist-dependent internalization of β_2-adrenoceptor (see Fig. 1B, left). Unless otherwise indicated, a standard agonist treatment protocol of 0.5 μM isoproterenol for 30 min was used in further experiments. We next sought to determine the effects of ARF6 on agonist-induced β_2-adrenoceptor internalization. Cells were transiently transfected with β_2-adrenoceptor and either pcDNA3 as control, wild-type ARF6 (ARF6 WT), or a constitutively inactive mutant form of ARF6 (ARF6 T27N). Whereas ARF6 WT had no effect on agonist-stimulated internalization, β_2-adrenoceptor internalization was inhibited by ARF6 T27N (Fig. 1A). Confocal imaging of β_2-adrenoceptor localization (Fig. 1B) showed that whereas in control cells or those transfected with ARF6 WT, agonist treatment led to the marked accumulation of receptor in endosome-like structures in the cell cytoplasm, this accumulation was greatly reduced when ARF6 T27N was cotransfected into the cells. Western blotting confirmed that both ARF6 WT and ARF6 T27N are expressed after transient transfection (Fig. 1C). Another constitutively inactive mutant form of ARF6 (ARF6 T44N; Macia et al., 2004) was also able to inhibit β_2-adrenoceptor internalization (internalization after 0.5 μM isoproterenol for 30 min was 47 ± 6% in pcDNA3-transfected cells, and 18 ± 4% in ARF6 T44N-transfected cells, $p < 0.05$; values are means ± S.E.M., $n = 6$).

Agonist-Induced Internalization of Transiently Expressed β_2-Adrenoceptor Is Inhibited by Centaurin α_1. Centaurin α_1 is thought to be a GAP for ARF6 (Venkateswarlu et al., 2004), so we investigated whether cotransfection of this GAP into the cells would affect the internalization of β_2-adrenoceptor. Centaurin α_1 greatly reduced the agonist-induced internalization of β_2-adrenoceptor; after 15 min of isoproterenol treatment, the internalization of β_2-adrenoceptor in centaurin α_1-transfected cells was only approximately one third of that in plasmid-transfected controls (Fig. 2A). Certain mutations of centaurin α_1 have been shown to inhibit the ability of this protein to function as an ARF6 GAP. Thus, both the catalytically inactive R49C form of centaurin α_1 and the form of the protein with both PH domains mutated (DM; R149C/R273C) are unable to function as ARF6 GAPs (Venkateswarlu et al., 2004). Therefore, when either the R49C or the DM form of centaurin α_1 was transfected into HEK 293 cells, there was no inhibition of agonist-induced β_2-adrenoceptor internalization as observed with centaurin α_1 WT (Fig. 2, B and C).

To determine the specificity of the interaction of centaurin

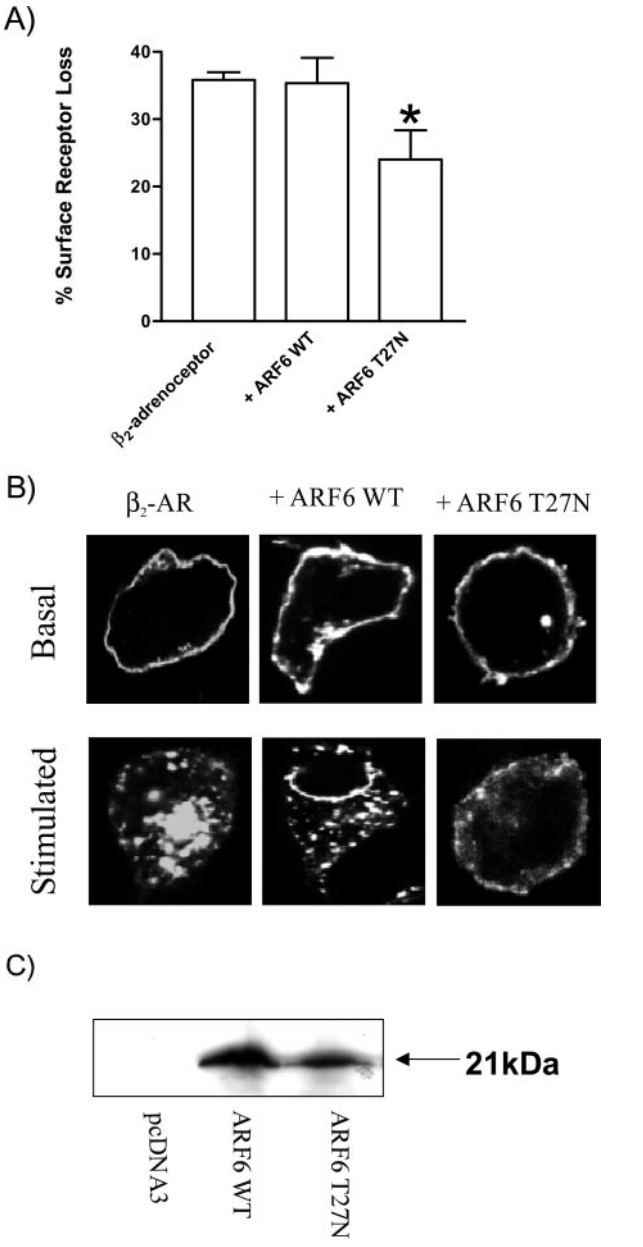

Fig. 1. ARF6 is required for the agonist-induced internalization of transiently expressed β_2-adrenoceptor. HEK 293 cells were transiently transfected with FLAG-tagged β_2-adrenoceptor, as well as either pcDNA3, wild-type ARF6 (ARF6 WT), or a dominant-negative mutant form of ARF6 (ARF6 T27N), and used for internalization assays 48 h later. A, cell surface receptor loss in response to 0.5 μM isoproterenol for 30 min assayed by ELISA. Coexpression of ARF6 T27N significantly reduced agonist-induced internalization of β_2-adrenoceptor (*, $p < 0.01$; values are means ± S.E.M., $n = 4$–12). B, representative confocal images showing agonist-induced β_2-adrenoceptor (β_2-AR) redistribution in the presence of pcDNA3 (left), ARF6 WT (middle), or ARF6 T27N (right) after addition of 0.5 μM isoproterenol to cells for 30 min. Receptor immunofluorescence was undertaken with a monoclonal anti-FLAG M2-Cy3 antibody as described under *Materials and Methods*. C, Western blot with anti-HA antibody to illustrate the expression of the ARF6 constructs in HEK 293 cells after transient expression.

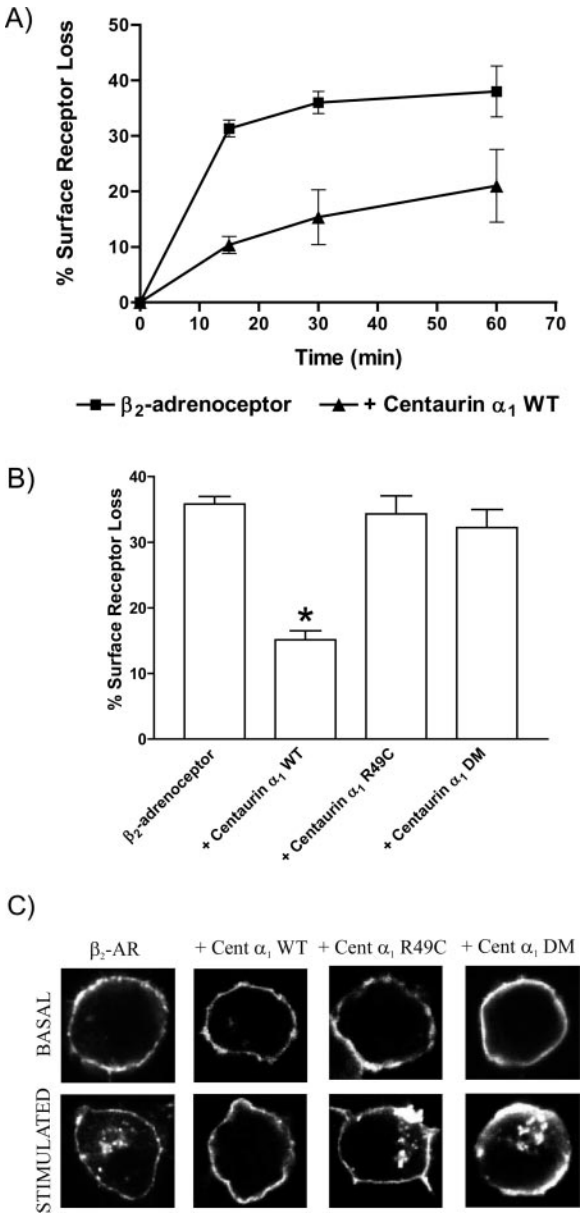

Fig. 2. Centaurin α_1 inhibits the agonist-induced internalization of transiently expressed β_2-adrenoceptor. HEK 293 cells were transiently transfected with FLAG-tagged β_2-adrenoceptor and Myc-tagged wild-type centaurin α_1 (centaurin α_1 WT), or in some cases with centaurin α_1 R49C or centaurin α_1 DM, and used for internalization assays 48 h later. A, time course of effect of centaurin α_1 on β_2-adrenoceptor internalization. Cell surface receptor was assayed by ELISA after stimulation with 0.5 μM isoproterenol for the time points indicated. The internalization of β_2-adrenoceptor was markedly reduced in the presence of centaurin α_1 ($p < 0.0001$ by 2-way ANOVA; values are means ± S.E.M., $n = 3$). B, effect of centaurin α_1 mutants on β_2-adrenoceptor internalization. Cells transfected with the indicated constructs were treated with 0.5 μM isoproterenol for 30 min and cell surface loss of receptor assayed by ELISA. Neither centaurin α_1 R49C nor centaurin α_1 DM had any effect on agonist-induced β_2-adrenoceptor internalization. On the other hand, centaurin α_1 WT inhibited cell surface loss (*, $p < 0.05$; values are means ± S.E.M., $n = 5$–10). C, representative confocal images showing agonist-induced β_2-adrenoceptor (β_2-AR) redistribution in the presence of pcDNA3, centaurin α_1 WT, centaurin α_1 R49C, or centaurin α_1 DM after addition of 0.5 μM isoproterenol to cells for 30 min. Receptor immunofluorescence was undertaken with a monoclonal anti-FLAG M2-Cy3 antibody as described under *Materials and Methods*. Note the lack of agonist-induced β_2-adrenoceptor internalization in centaurin α_1 WT-transfected cells.

α_1 with ARF6, we transfected cells with β_2-adrenoceptor and centaurin α_1 WT, and either pcDNA3, ARF6 Q67L, or ARF1 Q71L. The latter two constructs are constitutively active forms of ARF6 and ARF1, respectively (Radhakrishna and Donaldson, 1997). Whereas ARF6 Q67L completely reversed the inhibitory effect of centaurin α_1 on β_2-adrenoceptor, ARF1 Q71L had no effect on the ability of centaurin α_1 to inhibit β_2-adrenoceptor internalization (Fig. 3A). Confocal imaging of the β_2-adrenoceptor confirmed these findings; in cells expressing centaurin α_1 and ARF6 Q67L, isoproterenol treatment promoted the intracellular accumulation of receptor in endosome-like structures throughout the cytoplasm, whereas in cells expressing centaurin α_1 and ARF1 Q71L, there was much less internalization of receptor, and any endosome-like structures containing receptor were near the plasma membrane (Fig. 3B). Expression of ARF1 Q71L alone did not affect β_2-adrenoceptor internalization, whereas expression of ARF6 Q67L alone moderately reduced internalization (Fig. 3A).

Centaurin α_1 Increases β_2-Adrenoceptor Mediated Cyclic AMP Production in HEK 293 Cells. We also assessed the ability of centaurin α_1 to modulate the functional coupling of β_2-adrenoceptor (Fig. 4). In cells transiently expressing β_2-adrenoceptor, basal cyclic AMP accumulation was not affected by expression of centaurin α_1 (basal values were 0.72 ± 0.05, 0.65 ± 0.06, 0.71 ± 0.07, and 0.69 ± 0.06 pmol of cyclic AMP/well for pcDNA3, centaurin α_1 WT, centaurin α_1 R49C, and centaurin α_1 DM transfected cells, respectively; values are means ± S.E.M., $n = 4$). In the absence of centaurin α_1, isoproterenol induced a time-dependent increase in cyclic AMP accumulation, which leveled off after 5 to 10 min (Fig. 4A). When centaurin α_1 was coexpressed, agonist-stimulated cyclic AMP accumulation was greater than in pcDNA3-transfected controls. The effect of centaurin α_1 was dependent upon the functional activity of the protein, because expression of neither the R49C nor the DM form of centaurin α_1 was able to enhance isoproterenol-stimulated cyclic AMP accumulation (Fig. 4B).

Centaurin α_1 Inhibits the Agonist-Induced Internalization of Endogenous β_2-Adrenoceptor in HEK 293 Cells. Using an intact cell binding assay with the hydrophilic ligand [^{3}H]CGP12177, we determined the effects of centaurin α_1 on the agonist-induced internalization of endogenous β_2-adrenoceptor. Isoproterenol treatment produced a rapid and extensive internalization of cell surface endogenous β_2-adrenoceptor with >60% cell surface loss of receptor after 30 min (Fig. 5A). The agonist-induced internalization was strongly inhibited in cells expressing centaurin α_1 (Fig. 5, A and B), whereas expression of centaurin α_1 R49C or centaurin α_1 DM did not affect isoproterenol-stimulated internalization. Finally, as with cells transiently expressing β_2-adrenoceptor (see Fig. 4A), in centaurin α_1-transfected cells, cyclic AMP accumulation caused by activation of endogenous β_2-adrenoceptor was greater than in plasmid-transfected control cells (Fig. 5C).

Discussion

The present results extend our earlier findings (Venkateswarlu et al., 2004) with regard to the functional effects of centaurin α_1 as an ARF6 GAP. We showed previously that centaurin α_1 acts as an ARF6 GAP in vivo and consequently

that centaurin α_1 inhibits the ability of ARF6 to mediate the rearrangement of cortical actin in cells in response to the agonist EGF. In the present study, we show that centaurin α_1 inhibits the ability of ARF6 to support the agonist-induced internalization of a well characterized GPCR, the β_2-adrenoceptor. We also show for the first time that centaurin α_1 can regulate the function of endogenously expressed β_2-adrenoceptor.

Using constitutively inactive mutant forms of ARF6, including the ARF6 T27N mutant used in the current study, an important role for ARF6 in the agonist-induced internalization of the β_2-adrenoceptor has previously been demonstrated (Claing et al., 2001). Furthermore, these authors showed that the ARF6 GEF, ARNO, was able to enhance agonist-stimulated β_2-adrenoceptor internalization. In the present study, we also found that inhibition of endogenous ARF6 activity with the GTP binding-defective ARF6 T27N mutant could significantly inhibit agonist-induced internalization of β_2-adrenoceptor. A recent study has questioned the effectiveness of ARF6 T27N as a dominant-negative mutant of ARF6 (Macia et al., 2004), suggesting that whereas ARF6 T27N could act as a dominant-negative mutant, its effectiveness was compromised by a tendency for the mutant protein to denature in vivo. Therefore, we found that the GTP binding defective mutant designed by these authors (ARF6 T44N) is a very effective inhibitor of β_2-adrenoceptor internalization. Together, our findings are in agreement with those of Claing et al. (2001) and Macia et al. (2004) and are further

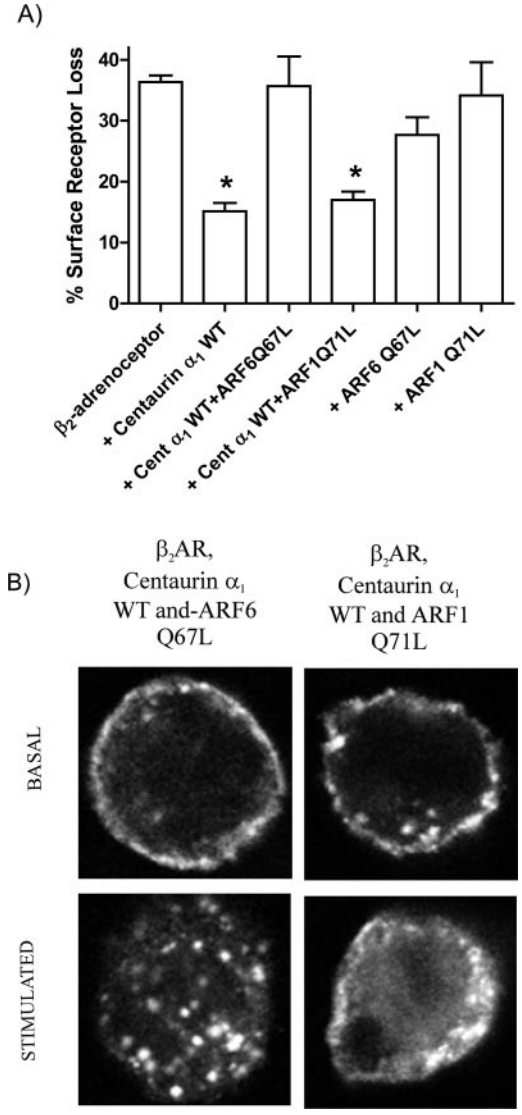

Fig. 3. The inhibition of β_2-adrenoceptor internalization by centaurin α_1 is reversed by a constitutively active form of ARF6. HEK 293 cells were transiently transfected with FLAG-tagged β_2-adrenoceptor and either pcDNA3 or wild-type centaurin α_1 (centaurin α_1 WT), and either constitutively active ARF6 (ARF6 Q67L) or constitutively active ARF1 (ARF1 Q71L) and used for internalization assays 48 h later. A, cell surface receptor loss in response to 0.5 μM isoproterenol for 30 min assayed by ELISA. Coexpression of ARF6 Q67L but not ARF1 Q71L reversed the centaurin α_1-mediated inhibition of β_2-adrenoceptor internalization (internalization in the presence of centaurin α_1 WT or centaurin α_1 WT plus ARF1Q71L was significantly less than control (*, $p < 0.005$; values are means ± S.E.M., $n = 5$–10). B, representative confocal images showing agonist-induced β_2-adrenoceptor (β_2-AR) redistribution in the presence of centaurin α_1 WT, and either ARF6 Q67L or ARF1 Q71L. For stimulated cells, 0.5 μM isoproterenol was added to the cells for 30 min. Receptor immunofluorescence was undertaken with a monoclonal anti-FLAG M2-Cy3 antibody as described under *Materials and Methods*. After agonist stimulation, β_2-adrenoceptor undergoes redistribution into intracellular compartments when expressed with centaurin α_1 and ARF6 Q67L but largely remains at the cell membrane when expressed with ARF1 Q71L.

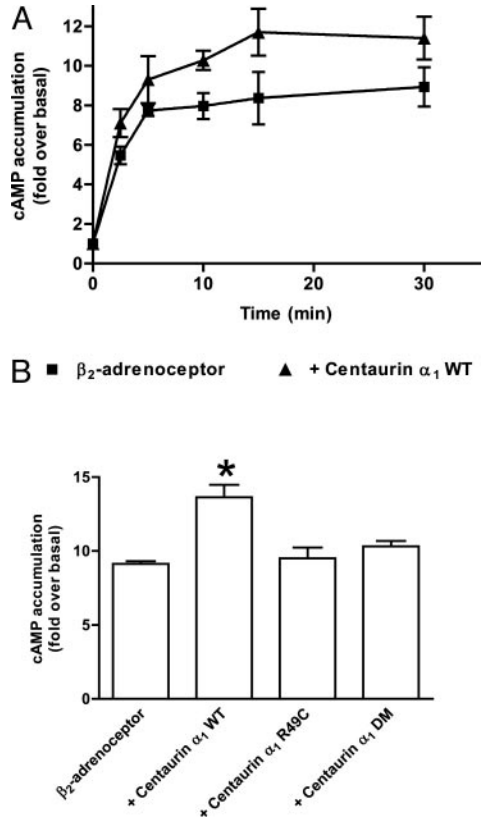

Fig. 4. Centaurin α_1 enhances isoproterenol-stimulated cyclic AMP accumulation in cells transiently expressing β_2-adrenoceptor. HEK 293 cells were transiently transfected with FLAG-tagged β_2-adrenoceptor and either pcDNA3, centaurin α_1 WT, centaurin α_1 R49C, or centaurin α_1 DM and used for cyclic AMP assays 48 h later. A, time course of 0.5 μM isoproterenol-stimulated cyclic AMP (cAMP) accumulation in cells transfected with either pcDNA3 (control) or wild-type centaurin α_1 (centaurin α_1 WT). Cyclic AMP accumulation was greater in the presence of centaurin α_1 WT ($p < 0.001$, 2-way ANOVA; values are means ± S.E.M., $n = 3$–6). B, the mutant forms of centaurin α_1, centaurin α_1 R49C and centaurin α_1 DM, have no effect on isoproterenol-stimulated cyclic AMP (cAMP) accumulation. Agonist-induced cyclic AMP accumulation in the presence of centaurin α_1 WT was greater than control (*, $p < 0.05$; values are means ± S.E.M., $n = 4$).

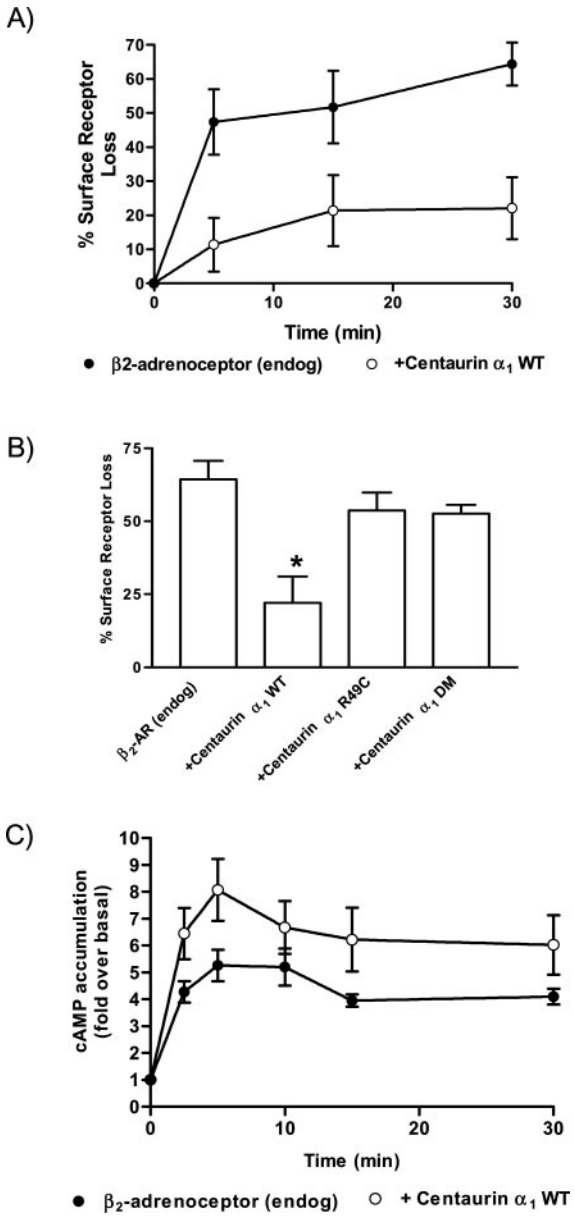

Fig. 5. Centaurin α_1 inhibits the agonist-induced internalization of endogenous β_2-adrenoceptor. HEK 293 cells were transiently transfected with Myc-tagged wild type centaurin α_1 (centaurin α_1 WT), or in some cases with centaurin α_1 R49C or centaurin α_1 DM, and used for internalization assays 48 h later. A, time course of effect of centaurin α_1 on endogenous β_2-adrenoceptor internalization. Cell surface receptor was assayed using [^{3}H]CGP 12177 binding to intact cells in suspension, after stimulation with 0.5 μM isoproterenol for the time points indicated. The internalization of endogenous β_2-adrenoceptor was markedly reduced in the presence of centaurin α_1 ($p < 0.0001$ by 2-way ANOVA; values are means ± S.E.M., $n = 3$). B, effect of centaurin α_1 mutants on endogenous β_2-adrenoceptor (β_2-AR) internalization. Cells transfected with the indicated constructs were treated with 0.5 μM isoproterenol for 30 min and cell surface loss of receptor assayed with [^{3}H]CGP 12177. Neither centaurin α_1 R49C nor centaurin α_1 DM had any effect on agonist-induced internalization of endogenous β_2-adrenoceptor, whereas centaurin α_1 WT inhibited cell surface loss (*, $p < 0.05$; values are means ± S.E.M., $n = 3$). C, centaurin α_1 enhances cyclic AMP accumulation caused by endogenous β_2-adrenoceptor activation in HEK 293 cells. Shown is the time course of 0.5 μM isoproterenol-stimulated cyclic AMP (cAMP) accumulation in cells transfected with either pcDNA3 (control) or wild-type centaurin α_1 (centaurin α_1 WT). Cyclic AMP accumulation was greater in the presence of centaurin α_1 WT ($p < 0.001$, 2-way ANOVA; values are means ± S.E.M., $n = 3$).

supported by a very recent study showing that the reduction of cellular ARF6 by small interfering RNA markedly reduces the internalization of transiently expressed β_2-adrenoceptor (Houndolo et al., 2005).

We found that expression of centaurin α_1 in HEK 293 cells strongly inhibited the agonist-induced internalization of β_2-adrenoceptor, whether endogenously expressed or transiently introduced into the cells. To determine whether this was caused by the ARF6 GAP activity of centaurin α_1, we employed two mutant forms of the protein. Centaurin α_1 R49C is mutated in the zinc finger domain, which is critical for the ARF6 GAP activity. Upon EGF stimulation of HeLa cells, this mutant translocates to the plasma membrane in a manner indistinguishable from that of centaurin α_1 WT, but it is unable to inhibit the ability of ARF6 to mediate the rearrangement of cortical actin in the cells. On the other hand, centaurin α_1 DM has the two PH domains mutated (R149C/R273C; Venkateswarlu et al., 1999), which are necessary for the PIP_3-mediated translocation of centaurin α_1 to the membrane in cells stimulated by PIP_3-generating agonists such as EGF. Although this mutant is unable to translocate, it is still effective as an ARF6 GAP if it is constitutively associated with the plasma membrane by the addition of a CAAX motif to the protein (Venkateswarlu et al., 2004). Like the R49C mutant, the DM mutant is unable to inhibit the EGF-stimulated rearrangement of cortical actin in cells. In the present experiments and in contrast to centaurin α_1 WT, neither the centaurin α_1 R49C nor the centaurin α_1 DM mutant was able to inhibit agonist-induced internalization of β_2-adrenoceptor. This provides very strong evidence that the ARF6 GAP activity and correct localization are essential for the ability of centaurin α_1 to regulate ARF6 and hence GPCR trafficking.

We have previously shown that the constitutively active form of ARF6, ARF6 Q67L (Venkateswarlu et al., 2004), is able to reverse the functional effects of centaurin α_1 by acting as a dominant-negative mutant for centaurin α_1. Thus, ARF6 Q67L prevented the inhibition of cortical actin formation by centaurin α_1 in EGF-stimulated cells. In the present experiments, we also found that ARF6 Q67L could fully reverse the effects of centaurin α_1 on β_2-adrenoceptor internalization. It is noteworthy that this effect was highly selective because a constitutively active form of ARF1, ARF1 Q71L, was unable to reverse the effects of centaurin α_1, just as this constitutively active mutant was unable to reverse the effects of centaurin α_1 on cortical actin rearrangement (Venkateswarlu et al., 2004). On its own, ARF6 Q67L moderately inhibited β_2-adrenoceptor internalization, which might be unexpected for a constitutively active ARF6 mutant. However, this effect on β_2-adrenoceptor internalization is the same as that reported previously for ARF6 Q67L (Claing et al., 2001) and may reflect the necessity of constant ARF6 GTP/GDP cycling for normal function.

Finally, we assessed the effects of centaurin α_1 on β_2-adrenoceptor signaling. Expression of centaurin α_1 enhanced the agonist-stimulated accumulation of cyclic AMP, an effect that would be consistent with the ability of centaurin α_1 to inhibit β_2-adrenoceptor internalization and thus prolong signaling. It is noteworthy that neither of the centaurin α_1 mutants was able to increase β_2-adrenoceptor-stimulated cyclic AMP accumulation, indicating that the effect of centaurin α_1 on β_2-adrenoceptor signaling is related to the ARF6

GAP activity of the protein. It is interesting that another ARF6 GAP, GIT1, a GRK2-interacting protein, also inhibits the agonist-induced internalization of β_2-adrenoceptor (Premont et al., 1998; Claing et al., 2001) as well as some other GPCRs (Claing et al., 2000). However, unlike centaurin α_1 in the present study, GIT1 has been reported to inhibit β_2-adrenoceptor-stimulated cyclic AMP accumulation. The reason for this difference is not clear at present, but it should be noted that GIT1 does interact with other cellular proteins (such as GRK2) that potentially could modulate β_2-adrenoceptor-stimulated cyclic AMP accumulation apart from ARF6 GAP activity.

In summary, the present results suggest an important role for centaurin α_1 as a regulator of ARF6 in the trafficking of endogenous GPCRs. In the future, it will be crucial to assess the role of endogenous centaurin α_1 in the regulation of ARF6 and GPCR trafficking using a small interfering RNA or related approach. It will also be important to determine exactly where in the internalization pathway ARF6 exerts its regulatory activity, although our results and others suggest that ARF6 is important in the very early steps of GPCR internalization.

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