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Interaction Between the μ Opioid Receptor and Filamin A Is Involved in Receptor Regulation and Trafficking

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ABSTRACT

The carboxyl tail of the human μ opioid receptor was shown to bind the carboxyl terminal region of human filamin A, a protein known to couple membrane proteins to actin. Results from yeast two-hybrid screening were confirmed by direct protein-protein binding and by coimmunoprecipitation of filamin and μ opioid receptor from cell lysates. To investigate the role of filamin A in opioid receptor function and regulation, we used the melanoma cell line M2, which does not express filamin A, and its subclone A7, transfected with human filamin A cDNA. Both cell lines were stably transfected with cDNA encoding myc-tagged human μ opioid receptor. Fluorescent studies, using confocal microscopy, provided evidence that filamin and μ opioid receptors were extensively colocalized on the membranes of filamin-expressing melanoma cells. The immunostaining of μ opioid receptors indicated that the lack of filamin had no detectable effect on membrane localization of the receptors. Moreover, μ opioid receptors function normally in the absence of filamin A, as evidenced by studies of opioid binding and DAMGO inhibition of forskolin-stimulated adenylyl cyclase. However, agonist-induced receptor down-regulation and functional desensitization were virtually abolished in cells lacking filamin A. The level of internalized μ-opioid receptors, after 30-min exposure to agonist, was greatly reduced, suggesting a role for filamin in μ opioid receptor trafficking. During these studies, we observed that forskolin activation of adenylyl cyclase was greatly reduced in filamin-lacking cells. An even more unexpected finding was the ability of long-term treatment with [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin of M2 cells, containing μ opioid receptors, to restore normal forskolin activation. The mechanism of this effect is currently unknown. It is postulated that the observed effects on μ opioid receptor regulation by filamin A and, by implication, of the actin cytoskeleton may be the result of its role in μ opioid receptor trafficking.

Opioid receptors belong to the superfamily of G protein coupled receptors (GPCRs). They are involved in numerous physiological functions, including analgesia, respiration, and cardiovascular functions. These receptors are also involved in the development of the physical and psychological dependence that are important aspects of drug abuse.

Current work in many laboratories, including our own, is directed toward understanding opioid receptor signaling, regulation, and trafficking. Agonist binding induces a change in the conformation of GPCRs because of interaction of the receptors with different kinases, including second messenger-dependent protein kinases and G-protein coupled receptor kinases (GRK). Phosphorylation of the receptors promotes binding of β-arrestins and uncouples the activated GPCRs from G-proteins. Uncoupling of the receptor from heterotrimeric G-proteins after short-term agonist exposure leads to receptor desensitization, which results in a decrease in agonist affinity and receptor function. In addition to receptor desensitization, GRKs and β-arrestins are required to mediate receptor internalization into intracellular endosomes via clathrin-coated vesicles. Endosome-associated receptors can either be resensitized after dephosphorylation and recycled back to the plasma membrane or directed to the lysosomes for degradation. The loss of binding sites because of receptor degradation is termed down-regulation and is commonly observed after long-term exposure to agonist.

Studies on GPCRs, including opioid receptors, have shown that the third cytoplasmic loop and the carboxyl terminal tail

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ABBREVIATIONS: GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; PDZ, postsynaptic density 95/disc-large/ZO-1; hMOP, human μ opioid receptor; aa, amino acids; TM, transmembrane domain; ABP, actin binding protein; PCR, polymerase chain reaction; hMOP-ΔC, human μ opioid receptor, shorter carboxyl tail; hMOP-C, human μ opioid receptor, carboxyl tail; GST, glutathione *S*-transferase; HEK, human embryonic kidney; DAMGO, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin; MAP, mitogen-activated protein kinase; SAPK, stress-activated protein kinase; CaR, calcium sensing receptor; DOP, δ-opioid receptor; His-fil, hexahistidine-tagged fusion protein of filamin fragment; PBS, phosphate-buffered saline; GASP, G protein-coupled receptor-associated sorting protein.

are very important for signal transduction (Law et al., 2000), regulation (Law and Loh, 1999), and internalization of GPCRs (Trapaidze et al., 1996; Keith et al., 1998) and are frequently involved in the association of the receptors with other proteins. Examples of proteins that can interact with GPCRs are GRKs (Pitcher et al., 1998), β-arrestins (Lefkowitz, 1998), PDZ domain-containing proteins (Cao et al., 1999; Li et al., 2002a) and SH3 domain-containing adaptor molecules (Milligan and White, 2001).

To discover proteins that can interact with the μ opioid receptor and may play a role in its function, regulation, and/or trafficking, we performed a yeast two-hybrid screen on a human cDNA library, in which the carboxyl terminal tail (C-tail) of the human μ opioid receptor (hMOP) served as bait. We found a number of proteins that seem to associate with the hMOP-C-tail. Here we report evidence for a direct association between the hMOP-C-tail and filamin A, a protein known to couple membrane proteins to actin, as well as some of the effects produced by this association.

Materials and Methods

Yeast Two-Hybrid Screen. The Matchmaker GAL4 yeast two-hybrid system 3 (BD Biosciences Clontech, Palo Alto, CA) was used to detect proteins interacting with the C-tail of the hMOP. The cDNA fragment encoding the last 68 amino acids (aa 333–400, including so called "fourth" loop and 9 aa of TM7) of hMOP (Fig. 1A), hereafter termed C-tail or hMOP-C, was amplified by PCR and subcloned into vector pGBKT7, containing the Gal4 DNA binding domain. This was used as a bait to screen a human brain cDNA library, constructed in the vector pACT2 containing the Gal4 DNA activation domain. The bait and library plasmids were sequentially transformed into yeast strain AH109, using the lithium acetate method. To retest positive clones in the yeast two-hybrid system, we used the long C-tail described above as well as the DNA fragment encoding a shorter C-tail (ΔC) (aa 354 to 400, including the last 47 aa down-stream from cysteine residue 353, which is usually palmitoylated) of the hMOP (hMOP-ΔC) (Fig. 1A). The transformed yeast was grown on high-stringency medium lacking leucine, tryptophan, histidine, and adenine. The resulting clones were grown on the same medium, containing X-α-Gal, and assayed for α-galactosidase expression. The DNA from positive yeast clones was isolated, amplified in *Escherichia coli*, and sequenced at the DNA sequencing facility of the Skirball Research Center at NYU School of Medicine.

Column Overlay Assays. DNA fragments encoding the hMOP-C and hMOP-ΔC were subcloned into bacterial expression vector pGEX-5-X-1 (Amersham Biosciences, Piscataway, NJ) to generate the glutathione *S*-transferase (GST) fusion proteins, GST-C (aa 333–400) and GST-ΔC (aa 354–400). BL21 bacterial lysates containing GST and GST fusion proteins were applied to glutathione agarose beads to produce GST, GST-C, and GST-ΔC affinity columns.

The DNA fragment identified from the yeast two-hybrid screen, encoding the carboxyl terminal region of human filamin A (aa 2355–2647), was subcloned into the pTrcHisC vector (Invitrogen, Carlsbad, CA), to generate a hexahistidine-tagged fusion protein of the filamin fragment (His-fil). His-fil was expressed in *E. coli* and affinity-purified on ProBond Nickel-chelating resin (Invitrogen). Resins with immobilized GST, GST-C, or GST-ΔC fusion proteins were incubated with purified His-fil at 4°C for 16 h. Washed resin-bound proteins were eluted with 10 mM glutathione in 50 mM Tris buffer, pH 8.0. The fractions of eluted proteins were run on SDS/polyacrylamide gel electrophoresis, and immunoblots were visualized by the enhanced chemiluminescence method (Pierce, Rockford, LI), using monoclonal anti-polyhistidine and polyclonal anti-GST antibodies purchased from Sigma (St. Louis, MO) and Amersham Biosciences, respectively.

Coimmunoprecipitation. HEK cells stably transfected with myc-tagged hMOP cDNA were lysed in a buffer containing 50 mM Tris, pH 7.4, 1% Triton X-100, 10% glycerol, 300 mM NaCl, 1.5 mM $MgCl_2$, 1 mM $CaCl_2$, and protease inhibitors. Cell lysates were incubated overnight at 4°C with polyclonal anti-myc antibody A-14, obtained from Santa Cruz Biotechnology (Santa Cruz, CA). After addition of 50 μl (50% slurry) of protein A/G agarose, the samples were incubated for 2 h at 4°C. Resin-bound proteins were washed three times in lysis buffer and then eluted with Laemmli sample buffer at 65°C for 15 min. Eluted samples were separated on SDS/polyacrylamide gel electrophoresis, and Western blot analysis was performed using polyclonal anti-myc and monoclonal anti-filamin antibodies (BD Transduction Laboratories, Lexington, KY).

Mapping the Interacting Sites on Filamin A. The cDNA fragment of human filamin A, identified from the yeast two-hybrid screen (His-fil22C-24, aa 2355–2647) was used as a template in PCR to amplify different deletion mutants of the carboxyl terminal portion of human filamin A. The PCR products were subcloned to generate fusion proteins of His-filamin deletions (Fig. 1B). All His-filamin fusion proteins were affinity purified on ProBond Nickel-chelating resin and used in column overlay assays as described above.

Cell Culture and Transfection. Human melanoma cell line M2, which does not express endogenous filamin, and M2 subclone A7,

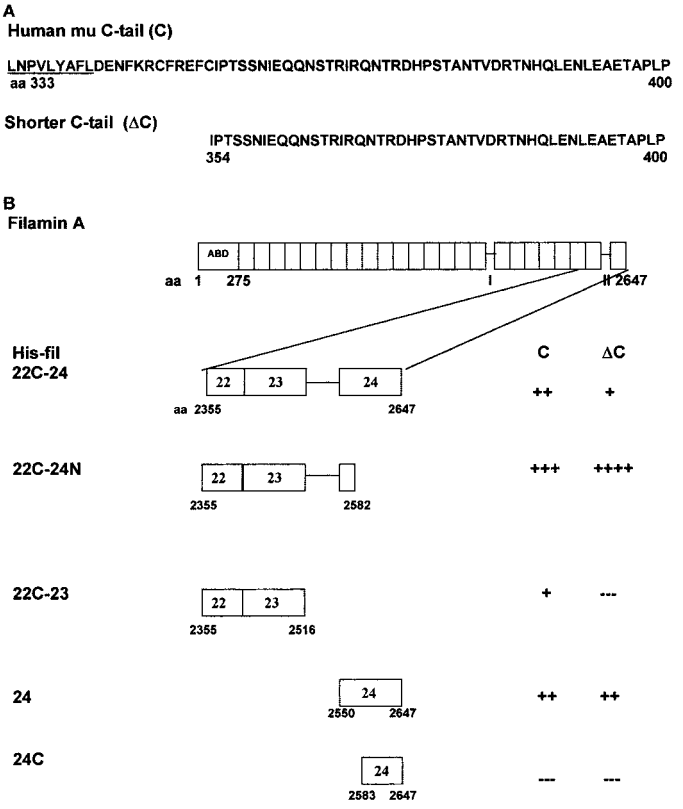

Fig. 1. Sequence of hMOP C-tail and schematic structures of human filamin A, full length and deletions. A, sequences of hMOP C-tail (C), and shorter C-tail (ΔC). The underlined amino acids in C are part of TM7. B, schematic of full-length filamin A (280kDa) containing 24 repeats (boxes ~96 aa each) and two hinge regions (I and II) between repeats 15 and 16 and 23 and 24, respectively. The N-terminal actin-binding domain is labeled ABD. The carboxyl terminal fragment of filamin A, identified by yeast two-hybrid screen, is enlarged and shown as His-fil (22C-24). Different fusion proteins of deletion mutants of His-fil are shown on the left side of the illustration and identified by the repeats they include. On the right side, C and ΔC indicate GST-C and GST- ΔC fusion proteins, respectively, used in overlay assays with different His-fil fusion proteins. +, weak interaction; ++++, strong interaction; ---, no interaction, based on the intensity of the bands (see Fig. 2).

stably transfected with filamin cDNA (Cunningham et al., 1992), were kindly provided to us by Drs. Thomas Stossel and Yasutaka Ohta at Harvard. They were grown in minimal essential medium supplemented with 8% newborn calf serum and 2% fetal calf serum. HEK, M2, and A7 cells were transfected with myc-tagged hMOP cDNA, using LipofectAMINE (Invitrogen). Stable clones were selected and tested for hMOP expression. Cell clones with similar levels of expression of hMOP were used for further studies.

Immunocytochemistry. A7 and M2 melanoma cells stably transfected with myc-tagged hMOP cDNA were grown on poly-L-lysine–coated glass coverslips in complete medium. Attached cells were washed in 1× PBS and then fixed in 2% freshly prepared paraformaldehyde for 10 min. After washing in 1× PBS, the cells were blocked in 1× PBS containing 1% bovine serum albumin and 3% normal donkey serum for 1 h and incubated with 5 μg/ml polyclonal anti-myc and/or 1:40 dilution of monoclonal anti-filamin antibodies purchased from Chemicon (Temecula, CA). After washing with 1× PBS, the cells were incubated with 1:250 dilution of rhodamine-coupled donkey anti-rabbit IgG and fluorescein isothiocyanate-coupled donkey anti-mouse IgG, respectively. These antibodies were obtained from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Permanently mounted cells were examined on Nikon PCM 2000 Laser Scanning confocal microscope using a Simple32 C-Imaging 1280 image analysis system.

Receptor Radioligand Binding. Cell membranes from stably transfected M2 and A7 cells were prepared as described previously (Onoprishvili et al., 1999). For saturation experiments, cell membranes were incubated with increasing concentration of [^{3}H]diprenorphine (50 Ci/mmol; PerkinElmer Life Sciences, Boston, MA) or [^{3}H]DAMGO (45–50 Ci/mmol) obtained from Multiple Peptide Systems (San Diego, CA), a gift from the National Institute on Drug Abuse. Nonspecific binding was determined in the presence of 1 μM naloxone. All binding experiments (in triplicate) were incubated at room temperature for 1 h. Retained radioactivity was measured in a Beckman Coulter scintillation counter in EcoScint A purchased from National Diagnostics (Atlanta, GA).

hMOP Down-Regulation. Confluent monolayers of M2 and A7 cells, stably transfected with hMOP cDNA, were incubated in serum-free medium for 24 h at 37°C in the presence or absence of increasing concentrations of DAMGO (10^{-10}–10^{-4}M). Control cells were incubated in serum-free medium for 24 h, and DAMGO (5 μM) was added for 1 min before harvesting to test the completeness of washing. After this, cells were washed three to four times in 1× PBS to remove excess agonist. Radioligand binding was performed on whole cells with [^{3}H]diprenorphine as described above.

Internalization of hMOP. Cultures of M2 and A7 cells at 80% confluence were harvested in Versene and pelleted at 800*g*. The pellet was resuspended in sodium-free choline buffer (Strahlendorf et al., 2001) and incubated at 37°C for 30 min in the presence or absence of 5 μM DAMGO. Sodium-free choline buffer was used in these experiments because of the recognized sodium-sensitivity of agonist binding by the MOP. After incubation, the cells were washed three times and resuspended in choline buffer for separate determinations of [^{3}H]DAMGO (2.3 nM) and [^{3}H]diprenorphine (1.6 nM) binding at room temperature. A decrease in the binding of [^{3}H]DAMGO, which interacts with surface receptors only, reflects receptor internalization. [^{3}H]Diprenorphine binds to both surface and internal receptors and was therefore used as a control for total binding, which should remain constant.

cAMP Assay. Transfected melanoma cells were grown to confluence in 24-well plates. The cells were treated with 5 μM DAMGO in serum-free medium for different time periods at 37°C. After removing the agonist, the cells were washed with 1× PBS and incubated for 15 min at 37°C in serum-free medium containing 0.25 mM 1-methyl-3-isobutylxanthine and 25 μM forskolin in the presence or absence of 1 μM DAMGO. After incubation, the cells were washed and extracted with 5% trichloroacetic acid. Levels of cAMP were determined using a radioimmunoassay kit from Biomedical Technologies (Stoughton,MA).

Results

The C Terminus of Filamin A Binds to Human μ Opioid Receptor C-Tail in Yeast-Two Hybrid Assay. To isolate proteins capable of binding to the μ opioid receptor, the cDNA fragment encoding the carboxyl tail plus a few amino acids of the 7th TM domain (aa 333–400) of the hMOP (hMOP-C) was used as bait in the yeast two-hybrid system to screen a human brain cDNA library. One positive clone was found to encode a carboxyl terminal fragment (aa 2355–2647) of the actin-binding protein, human filamin A.

Filamin A (see schematic in Fig. 1B; also known as ABP280 (actin binding protein 280), filamin 1, or nonmuscle filamin) is a large 280-kDa actin–cross-linking protein localized in peripheral cytoplasm. It stabilizes three-dimensional branching of actin filaments and links membrane proteins to the actin cytoskeleton (Gorlin et al., 1990). The N-terminal region of filamin is an actin-binding domain that encompasses the first 275 amino acids. It is followed by a rod-shaped domain composed of 24 repeats, each containing approximately 96 amino acids. The hinge regions I and II are located between repeats 15 and 16 and 23 and 24, respectively, and contain calpain cleavage sites. The last portion of the carboxyl-terminal region of filamin (65 aa), located in repeat 24, is the dimerization domain (Gorlin et al., 1990). The fragment isolated in the yeast two-hybrid screen (Fig. 1B) comprised the region including about two thirds of repeat 22 to the C terminus (22C-24).

To determine that the filamin sequence obtained from the yeast two-hybrid screen was not a false positive, the filamin clone was transformed back into the AH109 yeast strain with hMOP-C or hMOP-ΔC, which is a shorter C-tail (aa 354–400), as bait (see Fig. 1A) or with empty vectors pGBKT7 and pACT2 respectively. Neither the bait vectors nor the isolated filamin A clone was able to activate transcription of the reporter gene when the other vector was empty, whereas transformation of the filamin clone with hMOP-C and hMOP-ΔC bait vectors into the yeast produced positive blue colonies, even when grown on a high-stringency medium. This finding provides support for an interaction of filamin with the hMOP C-tail.

Filamin A Interaction with HMOP-C Confirmed by Direct Binding Assays. The interaction between the hMOP C-tail and the carboxyl terminal region of filamin A was further confirmed by in vitro protein-protein binding assays. GST fusion proteins of hMOP-C (GST-C, ~33 kDa) and hMOP-ΔC (GST- ΔC, ~30 kDa) and the hexahistidine fusion protein of the C-terminal filamin A fragment (His-fil, ~32 kDa) were generated and purified as described under *Materials and Methods*. Glutathione-agarose beads, bound to GST fusion proteins or GST, were incubated with purified His-fil. The resin-bound proteins were eluted with glutathione elution buffer and analyzed by Western blotting with anti-polyhistidine antibodies. To ensure that approximately equal amounts of the eluted GST and GST fusion proteins were loaded on the gel, nitrocellulose membranes were probed with anti-GST antibodies (not shown). As shown in Fig. 2A, Western blots of fractions eluted from GST-C and GST-ΔC columns contained the bands corresponding to His-fil,

whereas no band was detected in the fractions eluted from the control GST column. When we tested interaction between the GST fusion protein of hMOP third intracellular loop (GST-IC3, aa 257–283) and His-fil in the column overlay assay, GST-IC3 did not bind to His-fil (Fig. 2A). Similar results were obtained when anti-filamin instead of anti-polyhistidine antibodies were used (not shown). These results indicate that the carboxyl terminal portion of filamin A interacts specifically with the carboxyl tail of hMOP.

Coimmunoprecipitation. We performed coimmunoprecipitation experiments to determine whether hMOP interacts with endogenously expressed filamin A. Myc-tagged hMOP was immunoprecipitated from a lysate of HEK cells, stably transfected with myc-hMOP, using polyclonal anti-myc antibodies (Fig. 3A). The immunoprecipitated receptor was tested for coprecipitation of filamin A with monoclonal human anti-filamin (anti-ABP280) antibodies. As shown on Fig. 3B, the band corresponding to 265 to 270 kDa indicates that endogenously expressed filamin was able to coimmunoprecipitate with hMOP, whereas no band was detected in a similarly treated sample of nontransfected HEK cells. It should be noted that we also obtained coprecipitation of filamin when we used HEK cells transfected with bovine FLAG-tagged MOP (not shown). The C-tail of bovine MOP differs from the human by only two amino acids. These results indicate that the association between MOP and filamin is not dependent on species.

Deletion Studies of the C terminus of Filamin A. To obtain a more detailed definition of the binding domain of filamin A, which interacts with the hMOP-C, we generated a series of His-fil fusion protein deletion mutants, depicted in Fig. 1B. When the carboxyl terminal portion of repeat 24 was deleted, the resulting fusion protein (His-fil 22C-24N) bound to GST-C (Fig. 2B) with higher affinity (as reflected by heavier bands at the same concentration of proteins) than the undeleted His-fil 22C-24 itself. The binding affinity was even higher when the fusion protein of the shorter C-tail, GST-ΔC (Fig. 2C) was used. His-filamin fusion protein lacking repeat 24 and hinge region II (His-fil 22C-23) gave a very weak band with fusion protein GST-C and no detectable band with the shorter C-tail, GST-ΔC. The importance of the N-terminal portion of repeat 24 was confirmed by the finding that repeat 24 alone gave strong bands with both GST-C and GST- ΔC, whereas the carboxyl terminal region of repeat 24 gave no perceptible binding. The evidence from the overlay experiments (Fig. 2, B and C) suggests that the N-terminal part of repeat 24, containing 33 aa (2550–2582), is responsible for the major binding between filamin A and hMOP. However, the existence of more than one binding motif in the filamin A carboxyl terminal for the hMOP C-tail remains a possibility.

To investigate possible roles for the interaction between filamin A and hMOP, a melanoma cell line M2 that does not express filamin, and A7, an M2 subclone stably transfected with filamin A cDNA, were used. These cells were transfected with myc-tagged hMOP cDNA.

Immunofluoresence Studies. Immunofluorescent double-staining experiments using confocal microscopy revealed extensive colocalization of filamin and hMOP on the membranes of filamin-expressing A7 cells, resulting in orange fluorescent staining from the combined red (for myc) and green (for filamin) (Fig. 4A). The staining of N-terminally tagged hMOP in A7 and M2 melanoma cells indicated that the lack of filamin had no detectable effect on the membrane localization of μ opioid receptors (Fig. 4, A and B).

Evidence that Filamin A Has no Role in MOP Binding or Function. Saturation binding studies showed that the affinity of the opioid receptor antagonist, [^{3}H]diprenorphine, and of the μ opioid receptor agonist, [^{3}H]DAMGO, for hMOP were very similar in the M2 and A7 cell lines, expressing comparable numbers of hMOP, as depicted in Table 1. These results show that the lack of filamin does not affect either the affinity of the antagonist, diprenorphine, or that of the μ agonist, DAMGO, for hMOP.

To determine whether the absence of filamin A has an effect on the function of MOP, we studied the inhibition of forskolin-stimulated cAMP accumulation by DAMGO in M2 and A7 human melanoma cells stably transfected with hMOP. No significant differences were observed in the maximum inhibition of cAMP production by DAMGO between A7 and M2 melanoma cells. Thus, in naive cells, 1 μM DAMGO inhibited forskolin-stimulated cAMP production by 54% and 49% in A7 and M2 cells, respectively. These results are similar to results we obtained with HEK cells expressing hMOP (66% inhibition; data not shown) and are in agreement with previously published results from different laboratories (Keith et al., 1998; Koch et al., 2001). We have also

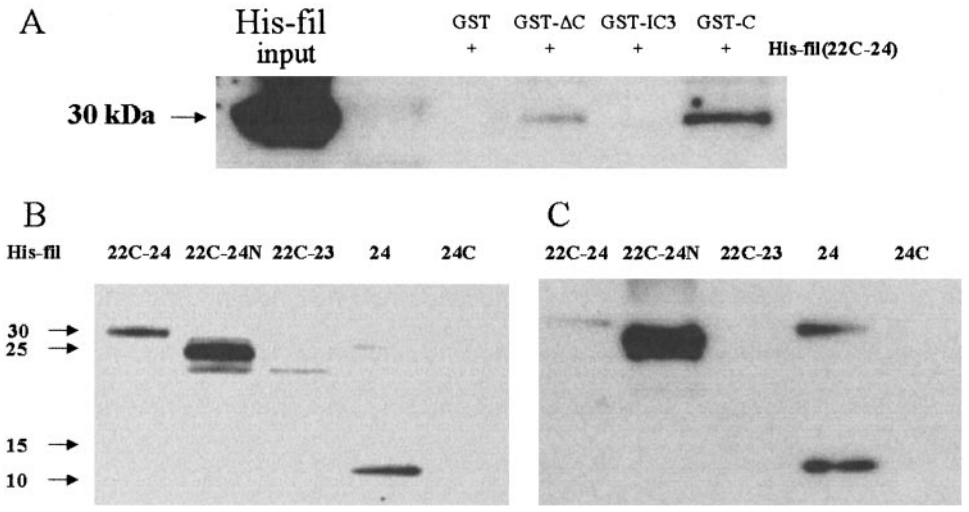

Fig. 2. In vitro binding between hMOP C and the carboxyl terminal region of filamin A. A, Western blot analysis of GST, and GST-IC3, GST-ΔC, and GST-C fusion proteins, derived from hMOP, eluted from glutathione-agarose column after incubation with His-fil fusion protein (fragment of filamin A identified from the yeast two hybrid screen). B and C, in vitro binding of GST-C and GST-ΔC, respectively, with different deletion fragments of His-fil fusion protein (see *Materials and Methods*). All blots were probed with monoclonal anti-polyhistidine antibodies.

obtained data suggesting that the activation of MAP kinase by DAMGO is unaffected by the absence of filamin (data not shown). These results indicate that in melanoma cells lacking filamin A, both binding and function of hMOP were comparable with those found in filamin-containing cells.

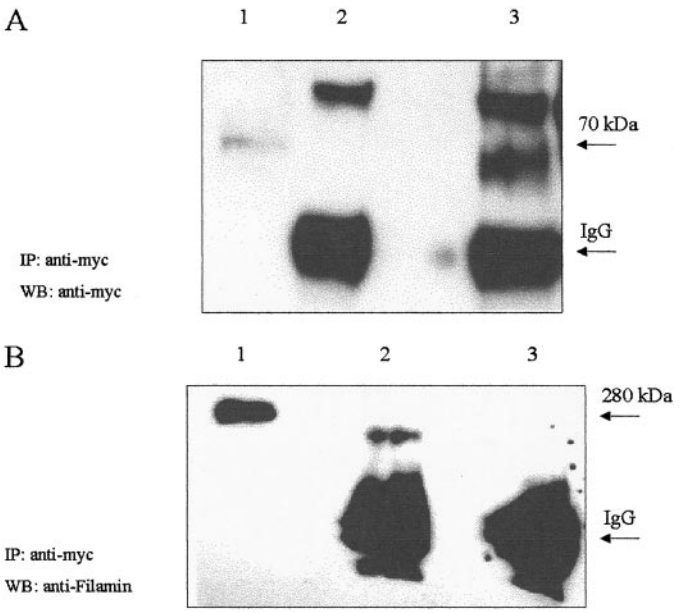

Fig. 3. CoImmunoprecipitation of filamin-A with myc-tagged hMOP. Lysates of untransfected HEK cells and HEK cells expressing myc-tagged hMOP were immunoprecipitated with anti-myc antibodies as described under *Materials and Methods*. The blots were probed with anti-myc (A) and anti-filamin (B) antibodies. A, lane 1, myc-tagged hMOP from HEK lysates (input). Lanes 2 and 3 show the blots of extracts of immunoprecipitated pellets from untransfected HEK and myc hMOP-transfected HEK cells, respectively. The band at ~65 kDa corresponds to myc-hMOP. B, lane 1, filamin from lysates of myc hMOP-transfected HEK cells (input); lanes 2 and 3 are samples from immunoprecipitated pellets of myc hMOP transfected HEK and untransfected HEK cells, respectively. A band of molecular mass somewhat below than 280 kDa (indicated by arrow) is seen only with hMOP-containing HEK lysate (lane 2) and seems to be coimmunoprecipitated filamin.

Evidence for a Role of Filamin A in MOP Down-Regulation. Our next approach toward determining a possible role of filamin A in μ opioid receptor regulation, was to perform down-regulation studies. The results are illustrated in Fig. 5, where down-regulation is shown as a function of DAMGO concentration. The EC_{50} for DAMGO-induced down-regulation showed dramatic differences. In A7 cells, the EC_{50} for DAMGO was 0.7 μM, whereas in M2 cells, lacking filamin, this value was more than 1000-higher (>1 mM).

The possibility was considered that the observed differences might be influenced by clonal variations, by different expression levels of MOP, or by species differences. Different clones expressing the myc-tagged human MOP at different levels (two clones isolated from A7 and 3 clones from M2) were examined. In all of these clones, the differences in down-regulation of MOP between filamin-containing and filamin-lacking cells were the same. Neither clonal variation nor significant differences in expression levels of MOP had any effect on the results. We carried out similar studies in M2 and A7 cells transfected with the bovine MOP and found that the species from which the receptor was derived, human or bovine, was also without effect (results not shown).

Filamin and Functional Desensitization. To determine the possible role of filamin A in agonist-induced functional desensitization of hMOP, human melanoma cells were pretreated with DAMGO for different time periods (0, 2, 4, and 24 h). Receptor desensitization was measured as the decreased ability of the agonist, DAMGO, to inhibit forskolin-stimulated cAMP accumulation after pretreatment with the agonist. As depicted in Fig. 6A, in A7 cells expressing hMOP, DAMGO exhibited reduced ability to inhibit forskolin-stimulated cAMP production after agonist pretreatment. Inhibition decreased from 54% in naive (zero time exposure) cells to

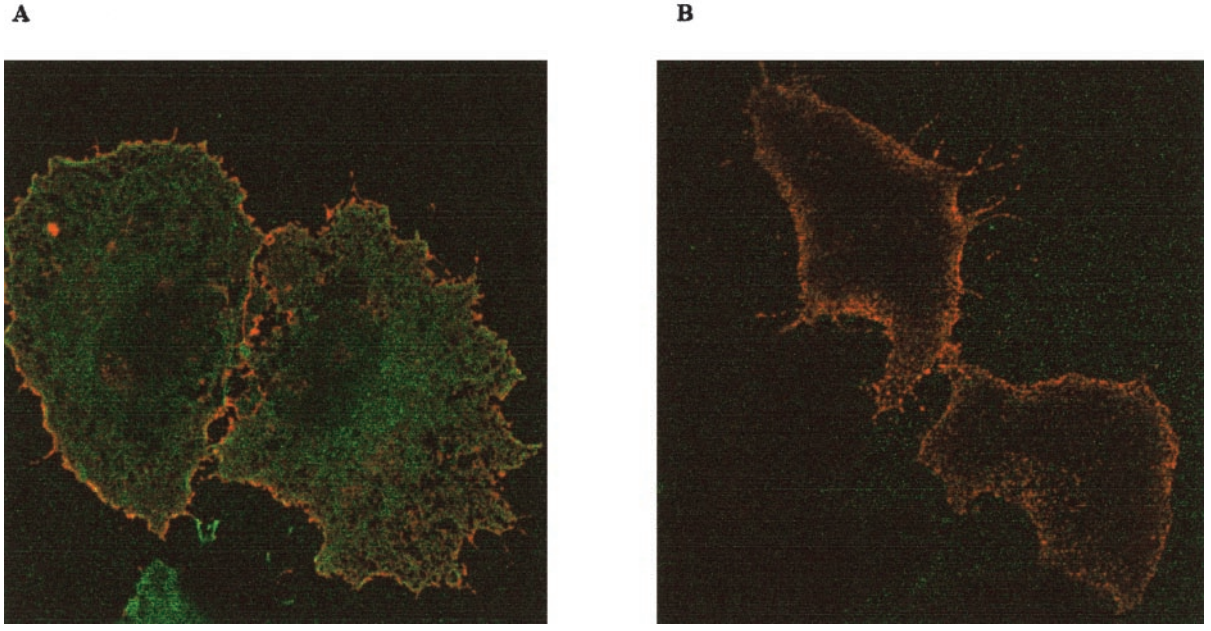

Fig. 4. Confocal microscope images of the localization of myc-tagged hMOP and filamin in human melanoma cells. A7 (A) and M2 (B) cells were double-labeled with anti-myc (A-14) and anti-filamin (MAB 1680) antibodies, followed by rhodamine (red)-coupled donkey anti-rabbit IgG and fluorescein isothiocyanate (green)-coupled donkey anti-mouse IgG, respectively. The orange areas represent colocalization of hMOP and filamin. These confocal microscopic images illustrate representative results from three independent experiments.

14% inhibition at 4 h. After 24 h of agonist exposure, the inhibition was almost back to untreated levels (~46%). In contrast, in cells lacking filamin, there was no functional desensitization. Moreover, as shown in Fig. 6A, the potency of DAMGO to inhibit forskolin-stimulated cAMP accumulation seemed to be enhanced by prolonged agonist pretreatment. Although naive M2 cells exhibited 49% inhibition by DAMGO, there was a gradual increase in inhibition with time, reaching 86% after 24 h of DAMGO treatment. The reason for this apparent increase in the inhibitory potency of DAMGO is explained below.

Preliminary results indicate that the desensitization of MAP kinase activation by opioids is also virtually abolished in cells lacking filamin A, although, as stated earlier, the stimulation itself was unaffected by the absence of filamin (data not shown).

Internalization Studies. We ascertained whether filamin A has a role in the internalization of hMOP using the two-ligand method described under *Materials and Methods*. A loss in surface receptors while total receptor number remains constant represents internalized receptors. After a 30-min exposure to DAMGO (5 μM), the total number of receptors in both melanoma cell lines remained unchanged (Fig. 7A). The decrease in surface hMOP in A7 cells showed that 42 ± 11% of the receptors were internalized, whereas in M2 cells, there was no detectable change in surface receptors (105 ± 17), suggesting that internalization is abolished in the absence of filamin (Fig. 7B).

Superactivation of Adenylyl Cyclase in Melanoma Cells. While doing these studies, we made the following interesting observation. A7 melanoma cells expressing high levels of filamin A exhibited 13-fold stimulation of cAMP formation by forskolin (25 μM) in agonist-naive cells (Fig. 6B). By contrast, in agonist-naive M2 cells, stimulation by forskolin was only about 3-fold. After 24 h in the presence of DAMGO, A7 cells showed forskolin stimulation of ~15-fold.

TABLE 1
Binding characteristics of hMOP expressed in A7 and M2 human melanoma cells

K_d and B_{max} values were determined by saturation binding experiments on melanoma cell membranes and analyzed using GraphPad Prism software. Data shown are means ± S.E. of four independent experiments performed in triplicate.

Cell Line	[^{3}H]Diprenorphine		[^{3}H]DAMGO	
	K_D	B_{max}	K_D	B_{max}
	nM	*fmol/mg*	*nM*	*fmol/mg*
A7	0.22 ± 0.05	1915 ± 114	2.3 ± 0.36	977 ± 69
M2	0.31 ± 0.08	2309 ± 151	2.8 ± 0.45	1200 ± 65

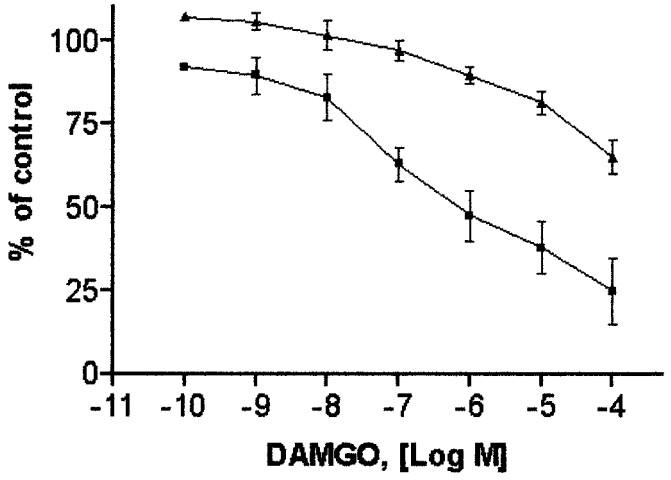

Fig. 5. DAMGO concentration dependence of down-regulation of hMOP in M2 and A7 melanoma cells. Melanoma cells expressing hMOP were incubated with increasing concentrations of DAMGO (10^{-10}–10^{-4} M) for 24 h at 37°C. [^{3}H]diprenorphine binding was performed on intact cells. In A7 cells (■), the IC_{50} for down-regulation was 0.7 μM; in M2 cells (▲), it was >1 mM. Data were analyzed with Prism software (GraphPad, Software, San Diego, CA).

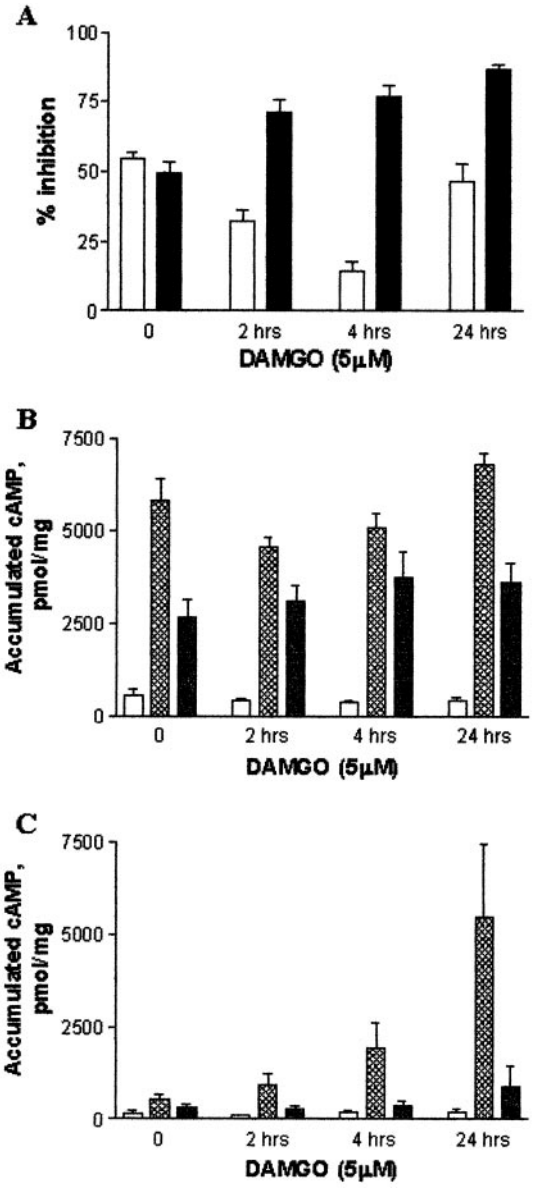

Fig. 6. Desensitization of DAMGO inhibition of forskolin-stimulated adenylyl cyclase in melanoma cells. A7 and M2 cells expressing hMOP were exposed to 5 μM DAMGO for the indicated times. Inhibition of forskolin-stimulated cAMP accumulation was measured after incubation of cells with 25 μM forskolin alone or in the presence of 1 μM DAMGO. A, percentage inhibition of forskolin-stimulated cAMP accumulation by DAMGO in A7 (□) and M2 (■) cells. B and C, the actual cAMP accumulation expressed as picomoles per milligram of protein in A7 and M2 cells, respectively. □, basal cAMP accumulation; ▨, forskolin-stimulated cAMP accumulation; ■, forskolin-stimulated cAMP accumulation in the presence of DAMGO. The data represent the means ± S.E. of four experiments.

This modest increase in forskolin-stimulated adenylyl cyclase may reflect superactivation, which in other cell lines (HEK or Chinese hamster ovary) was shown to be ~2- to 3-fold (Avidor-Reiss et al., 1995). As shown in Fig. 6C, in M2 cells, whereas the base levels remain constant, forskolin-stimulated levels of cAMP accumulation increase with time of exposure to DAMGO from 3- to 32-fold. The actual level of cAMP accumulation becomes comparable with that in A7 cells after 24-h exposure to DAMGO. This striking superactivation is the reason for the apparent increase in inhibition of adenylyl cyclase by DAMGO after long-term exposure to the agonist.

We carried out similar studies in M2 and A7 cells that had not been transfected with opioid receptor cDNA. These cells exhibited the same differences in adenylyl cyclase stimulation by forskolin as the cells containing MOP. As expected, DAMGO had no effect on forskolin stimulation of adenylyl cyclase in the cells lacking MOP.

Discussion

In the present study, we identified Filamin A, a protein of the actin cytoskeleton, as a direct binding partner for the human μ opioid receptor. Binding between these proteins was detected using the yeast two-hybrid system, with hMOP C-tail as bait, and revealed interaction of the hMOP C-tail with a carboxyl terminal fragment of human filamin A (aa 2355–2647), comprising repeats 22C-24. Direct binding between these proteins was confirmed by overlay experiments, using a GST fusion protein of the hMOP C-tail and a hexahistidine (His) fusion protein of the carboxyl terminal fragment of filamin A. The interaction was specific for hMOP C-tail, because the GST fusion protein of the third intracellular loop of hMOP failed to bind the His-filamin fusion protein. Further verification was obtained by coimmunoprecipitation experiments of filamin and hMOP from HEK cell lysates. It has consistently been observed that the immunoprecipitated filamin is slightly smaller than the filamin in cell lysates (265–270 instead of 280 kDa). We do not know the reason, but we suggest that this may be the result of splitting at one of the calpain cleavage sites present in filamin, which would remove a fragment of ~10 to 12 kDa. Fluorescence studies using confocal microscopy provided evidence for colocalization of hMOP and filamin on the membrane of melanoma cells.

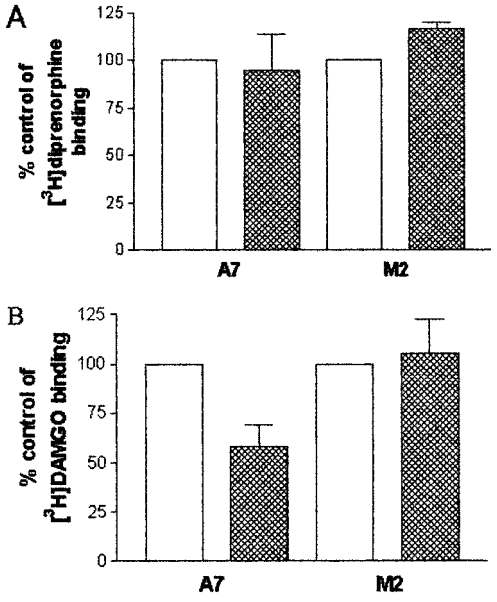

Fig. 7. Internalization of hMOP in A7 and M2 melanoma cells by exposure to DAMGO. Melanoma cells expressing hMOP were incubated without (□) and with (▨) 5 μM DAMGO for 30 min and whole cells were used to assess total receptor ([^{3}H]diprenorphine) (A) and surface receptor ([^{3}H]DAMGO) (B) binding, as described under *Materials and Methods*. The bars represent binding as percentage of that observed in untreated cells. The mean ± S.E. is derived from triplicate samples of two experiments.

Filamin A is a cytoskeleton protein that crosslinks actin filaments into an orthogonal network and maintains the integrity of the cell cytoskeleton. It has a rod-like structure containing 24 repeat domains, each approximately 96 amino acids long, and two hinge regions. It binds to actin at its N terminus (aa 1–275) and homodimerizes with another filamin molecule at its C terminus (repeat 24, aa 2583–2647). Filamin A is known to bind to different membrane and signaling molecules (Stossel et al., 2001; van der Flier and Sonnenberg, 2001). Several examples will be cited.

Filamin interacts with transmembrane molecules, such as β-integrins (Loo et al., 1998), platelet glycoprotein Ibα (Xu et al., 1998), presenilins (Zhang et al., 1998), and the voltage-gated potassium channel Kv4.2 (Petrecca et al., 2000). In addition, filamin binds to a number of signaling molecules. For example, SEK-1 (also known as MKK-4 or c-Jun NH_2-terminal kinase kinase), an activator for stress-activated protein kinase (SAPK), interacts with the carboxyl terminal fragment of filamin. Absence of this interaction causes a defect in the regulation of SAPK (Marti et al., 1997). Bellanger et al. (2000) determined that Trio, a member of the guanine nucleotide-exchange factor family, which activates Rho GTPases, binds to filamin with its Pleckstrin-homology domain. Thus, filamin may be part of a scaffold for the spatial organization of Rho-GTPase-mediated signaling pathways. Filamin is also known to be involved in other cellular processes, such as endocytosis of the proprotein-processing proteinase, furin (Liu et al., 1997), possible regulation of caveolae internalization (Stahlhut and van Deurs, 2000), and receptor-mediated phosphorylation of Smad, proteins that are involved in intracellular signal transduction of the transforming growth factor-β protein family (Sasaki et al., 2001).

Although filamin binds to a wide variety of proteins, each interaction involves different repeat domains of filamin and there is relatively little homology between repeats, which explains why there is no sequence motif for protein binding to filamin (van der Flier and Sonnenberg, 2001). While this project was in progress, other laboratories demonstrated the association of filamin with various GPCRs, including the D2 and D3 dopamine receptors (Li et al., 2000, 2002b; Lin et al., 2001), the calcium-sensing receptor (CaR) (Awata et al., 2001; Hjalm et al., 2001), the metabotropic glutamate receptor type 7 (Enz, 2002) and, most recently, the calcitonin receptor (Seck et al., 2003).

Both sites and the functional effects of the interaction with filamin differ significantly among the various GPCRs. The reports on the D2 and D3 DA receptors agree that the third intracellular loop, not the C-terminal tail, of dopamine receptors binds to repeat domains 16 to 19 of filamin, whereas in the case of the CaR, its carboxyl terminal tail interacts with repeats 14 to 15 of filamin. Studies on the metabotropic

glutamate receptor type 7 (Enz, 2002) indicated that its C-tail interacts with repeats 21 and 22 of filamin. A tyrosine residue in the glutamate receptor C-tail was found to be essential for filamin binding. The C-tail of the calcitonin receptor was found to bind filamin at repeats 20 and 21 (Seck et al., 2003).

In our results, filamin A binds to the C-tail of hMOP, but the major binding site on filamin is the N-terminal portion of repeat 24 (aa 2550–2582). Contributions to the binding from residues within repeats 22 to 23 are possible.

The involvement of repeat 24 was rather surprising because it is so close to the homodimerization site of filamin A. There is one similar example in the literature. The guanine nucleotide exchange factor Trio, which activates small Rho GTPases, binds to filamin somewhere in its C-terminal 112 amino acids (Bellanger et al., 2000). This sequence contains repeat 24 and a portion (15 or 16 amino acid) of the hinge domain II, suggesting that Trio binds close to the filamin homodimerization domain.

The reported roles of the association with filamin differ between the various GPCRs. For the dopamine receptors, it was found that the association with filamin was necessary for function. Thus, in cells lacking filamin, the ability of D2 and D3 receptors to inhibit forskolin-stimulated cAMP accumulation was greatly reduced (Li et al., 2000, 2002b). In the case of CaR, filamin was also found to be involved in function, as illustrated by a requirement for filamin for CaR-mediated activation of MAP kinase (Awata et al., 2001; Hjalm et al., 2001). In addition, the experiments on dopamine receptors suggest a function for filamin in the proper localization of receptors. The distribution of D2 receptors on the plasma membrane in filamin-deficient cells revealed uniform distribution, whereas in filamin-expressing cells, membrane-bound receptors had a clustered appearance (Li et al., 2000). Another report (Lin et al., 2001) indicated that filamin is required for the localization of D2 receptors on the plasma membrane, because they found most of the receptors in the cytoplasm of filamin-deficient melanoma cells.

In contrast, we found that the absence of filamin had no effect on any hMOP function examined, including membrane localization, ligand binding, agonist-induced adenylyl cyclase inhibition, and MAP kinase activation. The effects we observed were on receptor regulation and trafficking. Thus, in melanoma cells lacking filamin, agonist-induced down-regulation and functional desensitization of hMOP were virtually abolished. We also report here that filamin is necessary for normal receptor trafficking, as demonstrated by a decreased intracellular level of hMOP in filamin-deficient cells after a 30-min treatment with agonist.

It should be noted, especially in the light of results by Whistler et al. with the δ opioid receptor (DOP), discussed below, that our results do not distinguish between decreased rate of internalization and increased rate of receptor recycling to the membranes. We postulate that the observed changes in down-regulation and desensitization may result from alterations in receptor trafficking. The exact opposite effects were reported for the calcitonin receptor (Seck et al., 2003); i.e., the absence of filamin resulted in an increase in receptor degradation and a decrease in its recycling from endosomes to the cell membrane.

Trafficking of GPCRs involves a specific endocytic sorting mechanism, which separates receptors to be resensitized (recycling to plasma membrane after dephosphorylation) from those destined to be degraded (Tsao et al., 2001). Studies addressing receptor internalization and down-regulation indicate that residues located in the carboxyl-terminal tail of several GPCRs, including opioid receptors, specify the mechanism of receptor trafficking pathways.

Whistler et al. (2002) recently identified a novel protein, G protein-coupled receptor-associated sorting protein (GASP), that binds preferentially to the cytoplasmic tail of DOP. The authors showed that this interaction modulates endosomal sorting between receptors destined for lysosomal degradation and those to be recycled to the cell membrane and resensitized. The interaction with GASP is involved in directing DOP to the lysosomes, because disruption of this interaction, by competition from a dominant-negative fragment of GASP, significantly decreased down-regulation of DOP. GASP was found to interact only weakly with MOP.

We seem to be observing the mirror image of these findings. Filamin A interacts strongly with the C-tail of MOP but neither exhibits detectable interaction with the C-tail of DOP nor affects DOP trafficking (J. Nyberg, I. Onoprishvili, M. L. Andria, N. Ancevska-Taneva, J. M. Hiller, and E. J. Simon, unpublished results). The trafficking of μ opioid receptors seems to occur largely via the pathway involving clathrin-coated vesicles. We suggest that filamin may be involved in the sorting of receptors between molecules to be recycled and those to be degraded, a role suggested by Whistler et al. (2002) for GASP in DOP trafficking. Further research is planned to determine whether this hypothesis is correct.

One additional question is the importance of phosphorylation in MOP-filamin interactions. We know that the unphosphorylated C-tail binds filamin, because it was used in all of our binding and immunoprecipitation experiments. However, phosphorylation of one or more residue in the C-tail may be of importance in the functions of the filamin-receptor association or for the removal of filamin from the receptor. Studies to explore this are planned.

The κ opioid receptor may interact with yet another protein that modulates its trafficking, because a PDZ domain-containing phosphoprotein, EBP50/NHERF, has been found to associate with the C-tail of the human κ opioid receptor (Li et al., 2002a). However, the effects seem to be opposite those seen with filamin and GASP; i.e., the interaction leads to increased recycling and decreased breakdown.

We observed effects of filamin on forskolin activation of adenylyl cyclase as well as on the superactivation of the enzyme by long-term exposure to opioid agonist. Melanoma cells lacking filamin have only a modest level of stimulation of adenylyl cyclase by forskolin. In contrast, forskolin stimulation is significantly higher in A7 cells, in which a high level of filamin is expressed. After long-term treatment with DAMGO, only a modest level of superactivation is observed. This may be caused by the overexpression of filamin in A7 cells, which results in a high level of forskolin stimulation even in agonist-naive cells. The most striking result is seen in M2 cells, where long-term exposure (24 h) to the μ agonist DAMGO increases forskolin stimulation of cAMP accumulation dramatically (from 3-fold to 32-fold). The actual level of cAMP accumulation after DAMGO treatment is comparable with that seen in DAMGO-treated A7 cells. We hypothesize that filamin is an important factor in adenylyl cyclase activation by forskolin and that long-term exposure to DAMGO

compensates, in some as-yet-unknown manner, for the absence of filamin. Whether this effect is also a result of the modulation of receptor trafficking by filamin remains to be explored. Such a hypothesis, however, is supported by the results of Finn and Whistler (2001). These authors showed that long-term morphine treatment resulted in considerable superactivation of adenylyl cyclase in cells containing wild-type μ opioid receptor, where endocytosis induced by morphine is very slow. However, in a mutant receptor (a μ receptor containing the cytoplasmic tail of the δ receptor), in which rapid endocytosis was induced by morphine, there was significantly less adenylyl cyclase superactivation after long-term morphine exposure.

In conclusion, we have shown that the carboxyl tail of the μ opioid receptor interacts with filamin. Our data strongly indicate that association with filamin, and by inference with the actin cytoskeleton, is required for proper trafficking and regulation of the μ opioid receptor.

Acknowledgments

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