Membrane Glycoprotein M6a Interacts with the µ-Opioid Receptor and Facilitates Receptor Endocytosis and Recycling*

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Using a yeast two-hybrid screen, the neuronal membrane glycoprotein M6a, a member of the proteolipid protein family, was identified to be associated with the µ-opioid receptor (MOPr). Bioluminescence resonance energy transfer and co-immunoprecipitation experiments confirmed that M6a interacts agonist-independently with MOPr in human embryonic kidney 293 cells co-expressing MOPr and M6a. Co-expression of MOPr with M6a, but not with M6b or DM20, exists in many brain regions, further supporting a specific interaction between MOPr and M6a. After opioid treatment M6a co-localizes with MOPr to cell surface in transfected human embryonic kidney 293 cells. Moreover, the interaction of M6a and MOPr augments constitutive and agonist-dependent internalization as well as the recycling rate of µ-opioid receptors. On the other hand, overexpression of a M6a-negative mutant prevents µ-opioid receptor endocytosis, demonstrating an essential role of M6a in receptor internalization. In addition, we demonstrated the interaction of M6a with a number of other G protein-coupled receptors (GPCRs) such as the β-opioid receptor, cannabinoid receptor CB1, and somatostatin receptor sst2A, suggesting that M6a might play a general role in the regulation of certain GPCRs. Taken together, these data provide evidence that M6a may act as a scaffolding molecule in the regulation of GPCR endocytosis and intracellular trafficking.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screen—The MATCHMAKER GAL4 Yeast Two-hybrid System 3 (Clontech) was used to investigate interacting proteins of rat µ-opioid receptor (MOPr). The cDNA fragments encoding the full-length, a truncation (258 – 398 aa), and the carboxyl tail (340 – 398 aa) of MOPr (NCBI GenBank accession number GI: 6981309) were amplified by PCR and subcloned into pGBKT7, containing the Gal4 DNA binding domain. These fusion genes were used as baits to screen a rat brain cDNA library (Clontech) constructed in the pACT2 containing the Gal4 DNA activation domain. As negative control, Gal4-BD-MOPr with empty Gal4-AD and Gal4-AD-M6a-(108 – 278) with empty Gal4-AD, as well as a fusion of Gal4-AD with human lamin C, which neither forms complexes nor interacts with most other proteins, were used. The murine p53 fused with the Gal4-AD and SV40 large T-antigen fused with Gal4-AD served as positive controls. The screen was carried out according to the manufacturer’s protocol. From clones positive on plates lacking leucine, tryptophan, histidine, and adenine, six were confirmed by yeast mating and filter lift assays for β-galactosidase, with three clones encoding for known proteins of opioid receptors are regulated by direct interaction with membranal and/or cytosolic proteins (7).

In the course of identifying new µ-opioid receptor-interacting proteins using a yeast two-hybrid method, we isolated a cDNA encoding for the membrane glycoprotein M6a. M6a is a member of the proteolipid protein (PLP) family of tetraspan membrane proteins and mainly expressed in neurons (8 – 10). M6a shares 40% homology with DM20, the smaller splice isoform of the major central nervous system myelin proteolipid PLP, which is mainly expressed in myelinating glial cells (11). M6a is 55% homologous to proteolipid M6b, which is expressed in neurons and oligodendrocytes (8, 9, 12). M6a is suggested to play a role as a modulator for neurite outgrowth (13) and spine formation (14) and as nerve growth factor-gated Ca²⁺ channel in neuronal differentiation (15). Here, we found that M6a binds to the µ-opioid receptor and markedly affects receptor endocytosis and recycling.

* This work was supported by the SFB 426/A2 and the Graduate College 1167 of the Deutsche Forschungsgemeinschaft. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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teins including M6a-(108–287) and three clones encoding for novel proteins (16). MOPr and M6a showed the strongest interaction in all tests, including the bioluminescence resonance energy transfer (BRET) assay (data not shown).

**Immunoprecipitation and Western Blot Analysis**—The coding region of M6a gene (GI: 31981997) was subcloned from pCMV-SPORT6-M6a (RZPD) into pCMV-Myc (Clontech) by PCR, which generates amino-terminal Myc-tagged M6a (Myc-M6a). Similarly, the coding region of DM20 gene (GI: 200408) was subcloned from pCMV-DM20 plasmid (kindly provided by Prof. Klaus-Armin Nave (Göttingen, Germany) into pCMV-Myc expression vector. HEK293 cells with and without expressing HA-MOPr were transfected by pCMV-Myc-M6a or pCMV-Myc-DM20 using Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen). 48 h after transfection, cells were lysed and the resulting extract was subjected to either Western blot analysis or immunoprecipitation as described (17, 18).

**BRET Assay**—The coding sequences without a stop codon of MOPr or its truncations, or DOPr, sst2A, CB1R, mGluR1a, and mGlR5a, were amplified by PCR and were ligated into humanized pBlc-N3 (BioSignal Packard). Similarly, the coding sequences of M6a and its truncation, or DM20, were subcloned into humanized pGFP-C4. For BRET, HEK293 cells were cotransfected with Rluc and GFP fusion plasmids using Lipofectamine 2000. 45 h post-transfection, cells were detached and BRET signal was determined as described (17, 18).

**GST Affinity Chromatography**—GST pulldown experiments were carried out as described (18). Simply, to generate the glutathione S-transferase (GST) fusion proteins, cDNA fragments encoding the fourth, fifth, and sixth transmembrane domains of MOPr (amino acids 185–303, μ-(185–303)) and the carboxyl tail of MOPr (amino acids 340–398, μ(C-tail)) were subcloned in-frame into the bacterial expression vector pGEX-2KT encoding GST (Amersham Biosciences). Myc-tagged M6a proteins from stably transfected HEK293 cells were purified and incubated with GST fusion protein or GST beads at 4 °C for 3 h. Following complete wash, bound proteins were eluted and run on SDS-PAGE, and immunoblots were visualized.

**In Situ Hybridization**—A previously described probe (19) was used to detect mRNAs for MOPr (GI: 6981309, nucleotides 1249–2077). The coding sequences of M6a, M6b (GI: 9502118), and DM20 were amplified by PCR and then subcloned into the pGEM-T easy vector (Promega) for producing relative riboprobes. Generation of 35S-labeled and digoxigenin-labeled riboprobes, hybridization, washing procedures, and detection of hybridization signals were performed as described (20). Hybridization signal densities of the sense probes were defined as background levels. Expression patterns detected with the probes for MOPr, M6a, M6b, and PLP/DM20 mRNAs were in excellent agreement with those detected by others (9, 21). Because a riboprobe directed against the entire DM20 coding region was used, which does not distinguish the mRNA expression of DM20 and PLP, both DM20 and PLP mRNAs should be detected. The visualization of two different mRNA transcripts in the same section was performed by combining radioactive and nonradioactive in situ hybridization as described (19).

**Generation of Stable Cell Lines**—MOPr was amino-terminal tagged with the HA epitope tag using PCR and subcloned into pEAK10 (Edge Bio Systems). M6a was amino-terminal tagged with the Myc epitope tag using PCR and subcloned into pcDNA3.1 (Invitrogen). HEK293 cells were first transfected with pEAK10-HA-MOPr using Lipofectamine 2000. Stable transfectants were selected in the presence of 1.25 μg/ml puromycin (Sigma). To generate double stable cell lines, stable HA-MOPr cells were subjected to a second round of transfection with pcDNA3.1-Myc-M6a and selected in the presence of 1.25 μg/ml puromycin and 400 μg/ml hygromycin B (Invitrogen). Multiclones in similar receptor density were used for further study.

**Radioligand Binding Assay**—Binding studies were performed on membranes prepared from stably transfected cells. The dissociation constant ($K_d$) and number of $[^{3}H]DAMGO$ binding sites ($B_{max}$) were calculated by Scatchard analysis using at least seven concentrations of $[^{3}H]DAMGO$ in a range from 0.25 to 10 nM as described (22).

**Immunocytochemistry**—Cells were grown onto poly-L-lysine-coated coverslips overnight. For the observation of surface receptor and/or M6a internalization, HA-MOPr and/or M6a were surface-labeled with anti-HA and/or anti-M6a antibodies (Medical & Biological Laboratories Co., Ltd.) at a concentration of 1 μg/ml at 4 °C for 1.5 h. Subsequently, cells were treated as indicated in this paper prior to fixation and permeabilization as described (23). Bound primary antibody was detected with cyanine 2.18- and/or cyamine 3.18-conjugated secondary antibodies (Jackson Immunoresearch). Cells were permanently mounted in DPX (Fluka) and examined using a Leica TCS-NT confocal microscope.

**Quantitative Analysis of Receptor Trafficking**—Cells were seeded at a density of 5.0 × 10⁴/well in poly-L-lysine-treated 96-well plates overnight. To estimate endocytosis, cells were surface-labeled with anti-HA antibody (1 μg/ml) at 4 °C for 1.5 h and then incubated with and without agonist at 37 °C for 30 min. Subsequently, cells were fixed and incubated with peroxidase-conjugated antibody (1:1000; Amersham Biosciences) at room temperature for 2 h. The plate was developed with 50 μl of 2,2′-azino-di-[3-ethylbenzthiazolinesulfonate (6)] solution (ABTS) (Roche Applied Science). The reaction was analyzed at 405 nm using an Expert Plus Microplate Reader (ASYS) during 15–25 min in real time. The endocytosis was evaluated as the loss of labeled surface receptors. To measure recycling, cells were first exposed to 10 μM DAMGO for 2 h to drive agonist-induced endocytosis to a steady-state level. After wash, cells were subsequently incubated at 37 °C for 30 and 60 min in the presence of 10 μM naloxone to block residual agonist-stimulated internalization of MOPr. Cells were chilled with 4 °C phosphate-buffered saline to stop receptor trafficking. Afterward, receptors were surface-labeled at 4 °C for 2 h. After wash of unbound antibody, cells were fixed and surface receptors were detected as described above. The recycling rate was estimated as a percentage of recovered surface receptors to endocytosed receptors.
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Bonferroni test.

negative control analyzed by one-way analysis of variance followed by
versus
combination with GFP alone (similar results).

markers are indicated on the

INPUT

Ab:

HA

Myc

HA

Myc

HA

Myc

HA

Myc

Anti-HA

Anti-Myc

C

GFP-DM20

GFP-M6a

Negative

Positive

BRET Signal

FIGURE 1. MOPr interacts with M6a. A. yeast mating and β-galactosidase assays. The interaction of M6a-(108–278) (fused with the Gal4-activating domain) with the full-length (1–398 aa) but not the truncation (258–398 aa) including the third intracellular loop or the C-tail (340–398 aa) of MOPr (fused with Gal4 binding domain) was verified in a yeast mating and β-galactosidase assay. Results from penta-duplicate are shown. B, co-immunoprecipitation. Cell lysate proteins from different transfected cells as indicated were extracted and either immunoblotted directly (INPUT) or immunoprecipitated (IP) with anti-HA or -Myc antibody. The resulting immunoprecipitates were electrophoretically separated, transferred to nitrocellulose membrane, and detected with anti-Myc or anti-HA antibody. The positions of molecular mass markers are indicated on the left (in kDa). Two additional experiments gave similar results. C, BRET assay. Cells were cotransfected with MOPr-Rluc in combination with GFP alone (negative control), GFP-M6a, or GFP-DM20. A treatment of GFP-Rluc fusion protein was used as positive control (Positive). Cells were harvested 45 h post-transfection and were incubated in the BRET buffer for 30 min. The energy transfer was initiated by addition of 5 µl Deep Blue C, and BRET signals were measured immediately. Histograms show mean ± S.E. of three to five independent experiments performed in duplicate. *p < 0.0001 versus negative control analyzed by one-way analysis of variance followed by Bonferroni test.

RESULTS

Protein Interaction Studies of the µ-Opioid Receptor with M6a—A yeast two-hybrid screen of a rat cDNA library using full-length MOPr as bait led to the identification of a cDNA encoding amino acids 108–278 of the membrane glycoprotein M6a. A strong interaction between this M6a fragment and full-length MOPr was verified in yeast mating and β-galactosidase assays (Fig. 1A).

To confirm whether an interaction of MOPr with full-length M6a also occurs in mammalian cells, we carried out co-immunoprecipitation studies in HEK293 cells. Expression of epitope-tagged proteins (HA-MOPr, Myc-M6a, and Myc-DM20) was examined by directly immunoblotting extracts from these cells with specific antibodies against HA tag or Myc tag (Fig. 1B, INPUT). For co-immunoprecipitation, HA-MOPr was immunoprecipitated from different extracts using anti-HA antibody (IP, Ab). The resulting precipitates were immunoblotted with anti-Myc antibody. As shown in Fig. 1B, lane 2, Myc-tagged M6a migrating at ~35 kDa was detected in immunoprecipitates from co-expressing cells. In contrast, no M6a was detectable in immunoprecipitates prepared under identical conditions from cells expressing only HA-MOPr or Myc-M6a or from a mixture of cells expressing HA-MOPr or Myc-M6a individually (Fig. 1B, lanes 4, 5, and 7, IP Ab, respectively), indicating that the MOPr-M6a complex preexisted in cells prior to cell lysis and was not artificially formed during sample preparation. In the reciprocal co-immunoprecipitation with anti-Myc antibody and blot with anti-HA antibody, HA-MOPr was precipitated from co-expressing cells (Fig. 1B, lane 3), but not from cells expressing only HA-MOPr or Myc-M6a (data not shown). Though M6a and DM20 share high homology, DM20 was not co-immunoprecipitated by MOPr (Fig. 1B, lane 9). In addition, the interaction was confirmed by a biophysical method, BRET. A high BRET signal was obtained in cells co-expressing MOPr-Rluc with GFP-M6a, but not with GFP-DM20 (Fig. 1C). This finding indicates a specific constitutive interaction of MOPr with M6a also in living cells.

To estimate the binding domains of M6a and MOPr, a series of truncation mutants of M6a and MOPr were generated. The interactions between these truncations and the full length of M6a or MOPr were analyzed by BRET. As depicted in Fig. 2B, BRET signals for the M6a truncation (108–278 aa, yeast two-hybrid hit) indicated a very strong interaction with full-length MOPr. When this M6a mutant (108–278 aa) was truncated into two parts (108–163 and 164–278 aa), both of them failed to interact with the receptor, suggesting that the second extracellular large loop of M6a may play a major role in the MOPr-M6a interaction. However, the second extracellular loop (152–222 aa) itself showed no interaction with the receptor, indicating that both the third and fourth transmembrane domains (TMDs 3 and 4) are required for the direct interaction of MOPr-M6a. This is in agreement with the finding that there is an interaction between the M6a truncation (129–255 aa, including TMDs 3 and 4) and full-length MOPr. The MOPr truncation without its C-tail is an interaction between the M6a truncation (108–278 aa, yeast two-hybrid hit) indicated a very strong interaction with full-length MOPr. This finding indicates a specific constitutive interaction of MOPr-M6a interaction. Furthermore, we found that the MOPr truncation (1–303 aa) interacted with full-length M6a (Fig. 2C), whereas the MOPr truncations (1–258 and 258–303 aa) showed no positive BRET signal with full-length M6a. In addition, the truncation (186–303 aa, including the fourth, fifth, and sixth transmembrane domains) exhibited interaction with full-length M6a (Fig. 2C). In summary, these findings suggest that the protein stretch/domain including TMDs 3 and 4 of M6a are important regions for the MOPr-M6a interaction.
To confirm the direct interaction of MOPr-M6a, GST affinity chromatography was carried out. The MOPr truncation (185–303 aa, including the TMDs 4, 5, and 6) was expressed as a glutathione S-transferase fusion protein (GST-μ185–303 aa) and tested in GST pulldown experiments performed on purified Myc-M6a proteins. Following complete wash, precipitated protein was analyzed by Western blotting with anti-Myc antibody. Note that M6a directly binds to the protein stretch/domain including TMDs 4, 5, and 6 of MOPr. Two additional experiments gave similar results.

Consistent with the yeast mating and /-galactosidase assays, GST pulldown experiment also confirmed that the C-tail of M6a was not the binding region for M6a (Fig. 3).

M6a Interaction with a Subset of GPCRs—Because the fourth, fifth, and sixth transmembrane domains of the μ-opioid receptor, which are involved in the interaction with M6a, show high sequence homology with the transmembrane domains of a number of GPCRs, we next investigated whether M6a also interacts with other G protein-coupled receptors. As depicted in Fig. 4, M6a also interacted with another opioid receptor, the δ-opioid receptor (DOPr), with the cannabinoid type 1 receptor (CB1R), and with the somatostatin receptor sst2A, but not with the metabotropic glutamate receptors (mGluR1a and 5a). This finding suggested that M6a might play a role in the regulation of certain GPCRs.

Co-expression of MOPr and M6a in Rat Brain—Previous researchers reported that there is a good correlation between the distribution of mRNA and protein of MOPr in the central nervous system (19, 24). In addition, the distribution of the M6a antigen has been demonstrated to fit well with the pattern of mRNA and protein expression of MOPr.
M6a mRNA expression (9, 10, 25). Because an anti-M6a antibody for Western blot and immunohistochemical studies is currently unavailable, we evaluated whether there is a co-expression of MOPr and M6a in native cells using double in situ hybridization. In rat brain, both MOPr and M6a mRNAs were expressed in large proportions of thalamic cells (Fig. 5, A and B, respectively), suggesting the co-expression of both MOPr and M6a in the same cells within the thalamus, a major area of pain control in brain (26). As predicted, virtually all MOPr-positive cells expressed M6a mRNA and most M6a-expressing cells were MOPr-positive in many thalamic nuclei. As a representative example from thalamic region, the co-expression of MOPr and M6a (Fig. 5, D) was shown as a representative example in thalamus, providing further support for the specific interaction of MOPr and M6a.

**M6a Does Not Interfere with MOPr Binding**—To elucidate the effect of MOPr-M6a interaction on μ-opioid receptor binding and signal transduction, we stably expressed MOPr and/or M6a in HEK293 cells (Fig. 6, A–C). Expression of MOPr and/or M6a was monitored by confocal microscopy and Western blot.

For MOPr or MOPr-M6a stable cell line, multiclones in similar expression level of μ-opioid receptors were used for further study because MOPr density affects receptor trafficking and signaling (16, 18, 22, 23, 27, 28). Saturation binding experiments revealed no substantial differences between MOPr- and MOPr-M6a-expressing cells with respect to their affinities ($K_D$) to [3H]DAMGO (2.25 ± 0.10 and 2.17 ± 0.22 nM for MOPr and MOPr-M6a, respectively, $n = 3$) and number of binding sites ($B_{max}$) (2.68 ± 0.26 and 2.10 ± 0.15 pmol/mg protein for MOPr and MOPr-M6a, respectively, $n = 3$).

**M6a Augments MOPr Endocytosis and Recycling**—Consistent with previous reports (16, 29), the μ-opioid receptor was localized mainly at the plasma membrane in MOPr-transfected HEK293 cells (Fig. 6A). However, after co-expression of M6a, MOPr was localized both at the plasma membrane and in endosome-like compartments (Fig. 6C). In addition, the treatment of monensin (a blocker of vesicle recycling) induced a remarkable co-localization of MOPr and M6a in intracellular vesicles (Fig. 6D).

This finding prompted us to investigate whether MOPr-M6a interaction increases the constitutive endocytosis of μ-opioid receptors. The anti-HA antibody recognizes the HA epitope tag located in the extracellular N-terminal tail of HA-MOPr, and the anti-M6a monoclonal antibody recognizes the extracellular loop of the M6a antigen. These properties of the antibodies make it possible to specifically label MOPr and/or M6a at the
surface of living cells and only monitor the trafficking of labeled surface MOPr and/or M6a. As shown in Fig. 7, A and B, in MOPr- and MOPr-M6a-expressing cells, M6a and/or MOPr were exclusively confined to the plasma membrane at low temperature (4 °C), which prevents internalization. Incubation of only MOPr-expressing cells at 37 °C for 30 min did not induce substantial constitutive receptor internalization (Fig. 7C). In marked contrast, in co-expressing cells, both MOPr and M6a were found in the cytoplasm (Fig. 7D), indicating M6a enhances the constitutive internalization of μ-opioid receptors. In addition to the M6a-facilitated agonist-independent μ-receptor internalization, the treatment of MOPr-M6a-co-expressing cells with μ-agonist resulted in an enhanced receptor internalization (Fig. 7F) compared with cells expressing the receptor alone (Fig. 7E). Quantification of receptor internalization by enzyme-linked immunosorbent assay confirmed an enhanced constitutive internalization in cells co-expressing M6a (9.4 ± 0.5% versus 26.3 ± 1.4% for MOPr and MOPr-M6a cells, respectively, Fig. 7G, vehicle) but also an augmented DAMGO-induced receptor internalization by co-expression of M6a 32.4 ± 2.7% versus 47.8 ± 1.0% for MOPr and MOPr-M6a cells, respectively, Fig. 7G, DAMGO). In addition, we found that co-expression of M6a also facilitates etorphine-induced endocytosis of MOPr, whereas M6a does not increase morphine-induced MOPr internalization (data not shown).

Because M6a strictly co-internalizes with MOPr (Fig. 7, D3 and F3), we investigated the potential effect of M6a on the μ-receptor recycling. Therefore, after 2 h of agonist treatment agonists were washed out and cells were exposed to naloxone in agonist-free medium up to 60 min to allow receptor recycling. As shown in Fig. 8, ~32% of endocytosed receptors were recycled after 30 min of agonist withdrawal in cells co-expressing M6a. By contrast, in cells expressing the μ-receptor alone, only ~9% of internalized receptors was recycled after 30 min. After 60 min of agonist withdrawal, from 30% up to 40% recycling of internalized receptors was detected in MOPr- and MOPr-M6a-expressing cell lines. This finding indicates that M6a accelerates the post-endocytic sorting of internalized receptors into recycling pathway.

**Truncated Mutant of M6a Prevents Agonist-induced MOPr Internalization**—From the yeast two-hybrid assay, we know that fragment 108–278 of M6a is important for M6a interaction with MOPr (Fig. 1A). Furthermore, BRET analysis revealed that the shorter truncation mutant (129–255 aa) of M6a also shows interaction with the μ-opioid receptor (Fig. 2B). This finding led us to test whether this truncation mutant influences receptor internalization. In cells co-expressing GFP-tagged wild type M6a, a remarkable agonist-induced intracellular...
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Localization of μ-opioid receptors was seen (Fig. 9A). This observation was also confirmed by quantitative enzyme-linked immunosorbent assay (Fig. 9C). However, after overexpression of GFP-tagged M6a truncation mutant, agonist-induced receptor internalization was almost completely blocked whereas receptors in adjacent cells that did not express this mutant internalized efficiently in response to μ-agonist (Fig. 9B). In line with this observation, quantitative assay revealed a significantly decreased receptor internalization in MOPr cells transiently transfected with the M6a truncation mutant (Fig. 9C). Reverse transcription PCR studies and quantitative PCR revealed that there was no significant difference in the mRNA expression of endogenous M6a in MOPr-HEK293 cells with and without co-expression of GFP-M6a-(129–255) (data not shown). Therefore, a possible explanation for the observed effect is that the truncation mutant is not functional but could compete for binding of endogenously expressed M6a to μ-opioid receptors, and thus impair receptor internalization. The expression of endogenous M6a protein in HEK293 cells is further supported by the detection of M6a antigen in mouse kidney (13). Thus, our findings indicate an essential role of endogenous M6a in μ-opioid receptor internalization.

DISCUSSION

Most of the identified opioid receptor-interacting proteins have been found to bind to the carboxyl tail (C-tail) of opioid receptors (16, 30–34). However, experiments with C-terminal chimeras or truncations of the μ- and δ-opioid receptors revealed that the C-tail cannot be the only region involved in receptor regulation (35–38). It is further questionable whether a MOPr-derived endocytic recycling sequence (MRS) recently identified in the C terminus of μ-opioid receptors (39) is in fact essential for inducing receptor recycling because the C-terminal MOPr splice variants (MOPr-B and MOPr-D), which lack the MRS motif, undergo faster agonist-induced endocytosis and recycling than MOPr (40). These findings suggest that other motifs in addition to the C-tail could participate in opioid receptor regulation and prompted us to investigate other interacting proteins.

Using the μ-opioid receptor as bait, a cDNA encoding amino acids 108–278 of the membrane glycoprotein M6a was screened out and identified. The present studies provide several lines of evidence for a direct interaction between the μ-opioid receptor and M6a. First, the interaction was observed in the yeast two-hybrid system and confirmed by yeast mating and β-galactosidase assay. Second, in co-immunoprecipitation assay, M6a was specifically pulled down by the μ-opioid receptor only under physiological condition. Third, M6a shows a strong BRET signal with the μ-opioid receptor in living cells. The protein stretch/domain including the fourth, fifth, and sixth TMDs of MOPr and the protein stretch/domain including TMDs 3 and 4 of M6a are important for the interaction between
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MOPr and M6a. Moreover, the direct binding of TMDs 4, 5, and 6 of MOPr with M6a was revealed by GST pulldown experiment. Further sequence comparison revealed that TMDs 5 and 6 of various GPCRs such as μ, δ, and κ opioid receptors, the cannabinoid receptor CB1, and the somatostatin receptor sst2A share great homology. Therefore, it was reasonable to suggest that M6a might interact with certain GPCRs. In fact, we found that in BRET analysis M6a interacts not only with the μ-opioid receptor but also with the δ-opioid receptor, the cannabinoid receptor CB1, and the somatostatin receptor sst2A, but not with the metabotropic glutamate receptors (mGluR1a and 5a). This result can be explained by the fact that the TMDs 5 and 6 of the mGluRs differ markedly from those of the μ-opioid receptor.

Next we asked whether interaction between M6a and MOPr might also take place under physiological conditions. M6a, M6b, and PLP/DM20 belong to the same PLP family and are structurally related (8–10). In rat brain, there was a high level of co-expression between MOPr and M6a, whereas a co-expression of MOPr with M6b or PLP/DM20 was not detected in all tested brain regions, indicating the specificity of MOPr-M6a interaction. Furthermore, both co-immunoprecipitation and BRET assays demonstrated that the μ-opioid receptor cannot interact with DM20, further supporting the specific interaction of MOPr with M6a.

Our results clearly demonstrate that M6a does not affect the expression or agonist binding of the MOPr but facilitates constitutive and agonist-dependent MOPr internalization. In addition, M6a accelerates the recycling and reactivation of internalized MOPr. Due to the fact that M6a interacts with various GPCRs, our results suggest that M6a might play a general role in the internalization and recycling of certain GPCRs. Endocytosed GPCRs are either recycled to the plasma membrane or degraded in the lysosomes (41–44). The mechanism of post-endocytic sorting for opioid receptors is still incompletely understood. A protein termed GASP (GPCR-associated sorting protein) was identified that binds preferentially to the carboxyl tail of DOPr (30). The interaction of DOPr with GASP directs the receptor into lysosomal pathway destined for proteolytic degradation. MOPr-M6a interaction enhances the receptor recycling. Consistent with these findings is that GASP binds much less strongly to MOPr than to DOPr (30), and we observed a lower interaction of M6a with DOPr in comparison to MOPr (Fig. 4). It is reasonable to assume that the different post-endocytic sorting might be caused by the selective binding of M6a and GASP to the receptors.

In summary, we identified and characterized the membrane glycoprotein M6a as a scaffolding molecule of μ-opioid receptor endocytosis. We found that M6a enhances constitutive and agonist-dependent endocytosis of μ-opioid receptors. Because it has been well demonstrated that μ-opioid receptor endocytosis reduces the development of opioid tolerance by promoting fast receptor recycling and resensitization (27–29, 45–49), it is reasonable to suggest that M6a-enhanced endocytosis/recycling of μ-opioid receptors attenuates the development of opiate tolerance.

Acknowledgments—We thank Anke Reichenauer, Michaela Böx, and Evelyn Kahl for excellent technical assistance.

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