The Adaptor Complex 2 Directly Interacts with the α\textsubscript{1b}-Adrenergic Receptor and Plays a Role in Receptor Endocytosis*

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Desensitization to the effects of hormones and neurotransmitters is a fundamental regulatory mechanism of G protein-coupled receptor (GPCR)\textsuperscript{d} function defined by a specific loss of responsiveness for those receptors that have been repeatedly stimulated by the agonist. Receptor desensitization results from the combination of multiple biochemical events occurring at different time frames: receptor-G protein uncoupling in response to receptor phosphorylation (seconds to minutes), internalization or endocytosis of cell surface receptors to intracellular compartments (minutes), and down-regulation of the total pool of receptors due to their decreased synthesis and/or increased degradation (hours) (1). A prominent role in homologous desensitization of GPCRs is played by G protein-coupled receptor kinases. Once the receptor is occupied by the agonist, it is recognized by the G protein-coupled receptor kinases and becomes phosphorylated (2). The subsequent uncoupling of the receptor from the G protein is then mediated by arrestin proteins, which preferentially bind to the agonist-occupied phosphorylated receptor (3). However, during the last decade, β-arrestins have emerged as key regulatory molecules controlling various steps of receptor desensitization. Beyond their role in physical uncoupling of GPCRs from the G proteins (3), it has been demonstrated that β-arrestins target GPCRs to the endocytic machinery (4). In fact, it is believed that for those GPCRs that internalize in a clathrin-dependent manner, like the β\textsubscript{2}-adrenergic receptor (AR), targeting of the receptor-β-arrestin complexes to clathrin-coated vesicles is mediated by a dual interaction of β-arrestin with both clathrin heavy chain and the β\textsubscript{2} subunit of the heterotetrameric clathrin adaptor complex 2 (AP2) (5–7).

The AP2 complex directly links the clathrin coat with cargo transmembrane proteins that are sorted into coated pits and vesicles (8) and is composed of two large subunits, α and β\textsubscript{2}, of about 100 kDa and two smaller subunits, µ\textsubscript{2} and σ\textsubscript{2}, of 50 and 17 kDa, respectively (9). The AP2 adaptor can initiate endocytosis of membrane receptors by either associating directly with their cytoplasmic tail or by interacting with additional molecules, such as β-arrestins, as described for the β\textsubscript{2}-AR (5, 7). Direct interactions between AP2 and transmembrane proteins have been demonstrated, for example, for the transferrin receptor (10), the epidermal growth factor receptor (11, 12), and the cystic fibrosis transmembrane conductance regulator (13) but not for GPCRs. They are principally mediated by the µ\textsubscript{2} subunit, which specifically associates with endocytosis signals including YXXΦ (where Φ represents a bulky hydrophobic residue) (14) and dileucine motifs (15) on the cytoplasmic portion of the transmembrane proteins (8). Interestingly, recent evidence suggests that the β\textsubscript{2} subunit also participates in the recognition of dileucine motifs on the proteins (16).

Previous reports have shown that the α\textsubscript{1b}-AR undergoes rapid endocytosis upon exposure to the agonist. This was shown both in DDT1-FM2 smooth muscle cells expressing endogenous receptors as well as in cells expressing the recombinant receptor (17, 18). The molecular determinants involved in agonist-induced endocytosis of the α\textsubscript{1b}-AR reside within the C-tail of the receptor as demonstrated by the fact that truncation of this region almost completely abolishes receptor desensitization and internalization (19). A previous report suggested that the α\textsubscript{1b}-AR can internalize in clathrin-coated vesicles as shown by that fact that α\textsubscript{1b}-AR endocytosis can be blocked by hypertonic sucrose and that internalized receptors colocalize with transferrin receptors (18). Moreover, we previously reported that agonist-induced internalization of the α\textsubscript{1b}-AR is, at least in part, mediated by β-arrestins (20). Despite this experimental evidence, our knowledge on the biochemical mechanisms and the molecular mediators controlling the clathrin-
mediated endocytosis of the α1b-AR is still at an early stage. To identify new proteins interacting with the α1b-AR that could potentially be involved in regulating receptor function, we have used the yeast two-hybrid system and identified the µ2 subunit of the AP2 complex. In this study, we demonstrate that the α1b-AR and µ2 subunit can directly interact through a polyarginine motif located on the C-tail of the receptor and that this interaction plays a role in agonist-induced internalization of the receptor. Our findings highlight a previously unappreciated mechanism that might also be involved in the endocytosis of other GPCRs.

EXPERIMENTAL PROCEDURES

Expression Constructs—A cDNA fragment encoding the last 165 amino acids of the hamster α1b-AR (amino acids 351-515) (Fig. 2) was PCR-amplified and subcloned at EcoRI/SalI in pGEX4T1, pET30a, and pLexA plasmids to construct fusion proteins with GST, His6, and LexA at the N terminus of the C-tail of the receptor, respectively. For constructing GST fusion proteins with different fragments of the C-tail, cDNA fragments encoding amino acids 351–380, 351–395, 351–425, 351–449, and 351–477 of the α1b-AR were PCR-amplified and subcloned at EcoRI/SalI in pGEX4T1. The Y386A, Y447A, L450A/L451A, and L473A/L474A mutations were introduced into the C-tail-pGEX4T1 and DNA fragments encoding glycine residues 351–477 of the receptor. Our findings highlight a previously unappreciated mechanism that might also be involved in the endocytosis of other GPCRs.
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The radish labeled protein was separated from free \([\gamma^\text{32P}]\text{ATP} \) on an Excelgel GF-5 cellulose column (Pierce) equilibrated in TBS-Tween.

Conofocal Microscopy—HEK-293 cells grown on glass coverslips were transfectected with the cDNAs encoding different GFP-tagged receptors. 48 h after transfection, cells were incubated in serum-free DMEM for 1 h and treated for various times with \( 10^{-4} \) M epinephrine (Sigma) at 37 °C. After the treatment, cells were placed on ice, washed twice with ice-cold PBS, fixed for 10 min in PBS plus 3.7% formaldehyde, and mounted using Prolong (Molecular Probes, Inc., Eugene, OR). In the experiments measuring the effect of K44A dynamin mutant on receptor endocytosis, HEK-293 cells were cotransfected with the cDNAs encoding the GFP-tagged \( \alpha_{1b} \)-AR and the HA-tagged dynamin K44A mutant. 48 h after transfection, cells were incubated in serum-free DMEM for 1 h and treated with \( 10^{-4} \) M epinephrine at 37 °C. After two washes with ice-cold PBS, cells were fixed for 10 min in PBS plus 3.7% formaldehyde and permeabilized for 5 min with 0.2% (v/v) Triton X-100 in PBS. Cells were incubated in PBS plus 1% bovine serum albumin for 1 h and with 1:250 dilution of anti-HA polyclonal antibody (Santa Cruz Biotechnology) for 1 h, followed by another incubation with Texas Red-conjugated donkey anti-rabbit secondary antibody (Jackson Immunoresearch) for 1 h. The cells were then mounted using Prolong (Molecular Probes). GFP fluorescence or immunofluorescent staining were visualized on a laser-scanning confocal microscope (Zeiss).

Cell Surface Biotinylation Experiments—HEK-293 cells grown in 100-mm dishes were transfectected with the cDNAs encoding the HA-tagged \( \alpha_{1b} \)-AR or its T368 and A371–378 mutants. 48 h after transfection, cells were incubated in serum-free DMEM for 1 h and treated for various times with \( 10^{-4} \) M epinephrine (Sigma) at 37 °C. After the incubation, cells were placed on ice and washed twice with ice-cold PBS. Surface proteins were biotinylated by incubating cells with 500 μg/ml of the membrane-impermeable biotin analogue sulfo-NHS-S-s-biotin (Pierce) in PBS for 30 min at 4 °C. After two washes with ice-cold PBS, cells were fixed for 10 min in PBS plus 3.7% formaldehyde and permeabilized for 5 min with 0.2% (v/v) Triton X-100 in PBS. Cells were incubated in PBS plus 1% bovine serum albumin for 1 h and with 1:250 dilution of anti-HA polyclonal antibody (Santa Cruz Biotechnology) for 1 h, followed by another incubation with Texas Red-conjugated donkey anti-rabbit secondary antibody (Jackson Immunoresearch) for 1 h. The cells were then mounted using Prolong (Molecular Probes). GFP fluorescence or immunofluorescent staining were visualized on a laser-scanning confocal microscope (Zeiss).

RESULTS

Identification of the \( \mu_2 \) Subunit of the AP2 Complex as a Protein Interacting with the \( \alpha_{1b} \)-AR—It was previously reported that the C-tail of the \( \alpha_{1b} \)-AR plays an important role in the regulation of receptor desensitization and internalization (19, 22, 23). However, the molecular players involved in these processes are not fully characterized. To identify novel regulatory proteins interacting with the C-tail of the \( \alpha_{1b} \)-AR, the last 165 amino acids of the receptor were used as bait to screen a human brain cDNA library using the yeast two-hybrid system. Three independent positive clones encoding the \( \mu_2 \) subunit of the AP2 complex were identified from \( 13 \times 10^6 \) cotransformants, as assessed by their ability to grow in the absence of histidine and to produce \( \beta \)-galactosidase. To confirm the interaction between the C-tail of the receptor and \( \mu_2 \), the L40 yeast strain was transformed with the bait plasmid expressing the C-tail of the \( \alpha_{1b} \)-AR (\( \alpha_{1b} \)-AR C-tail-pLexA) in combination with either a pACT2 vector containing the \( \mu_2 \) cDNA (\( \mu_2 \)-pACT2) or with empty pACT2 vector. As shown in Fig. 1A, yeast transformed with \( \mu_2 \)-pACT2 were able to grow in the absence of histidine (upper panel) and to produce \( \beta \)-galactosidase (lower panel), whereas those transformed with the empty pACT2 vector did not.

To provide biochemical evidence that the \( \alpha_{1b} \)-AR can associate with the \( \mu_2 \) subunit of the AP2 complex, pull-down experiments were performed by incubating the GST-tagged C-tail of the receptor with cell extracts of HEK-293 cells. We found that the \( \mu_2 \) subunit was specifically associated with the purified His\(\alpha\)-tagged C-tail using a solid phase overlay assay. The autoradiography shown in Fig. 1C (left panel) indicates that the C-tail of the \( 32\text{P} \)-labeled His\(\alpha\)-tagged C-tail of the \( \alpha_{1b} \)-AR could specifically interact with GST-\( \mu_2 \) but not with GST alone. Control experiments showed that the GST-\( \mu_2 \) did not interact with 100 μl of an unrelated radish labeled protein (regulatory subunit of protein kinase A) (results not shown). Altogether, these results strongly suggest that the \( \mu_2 \) subunit of the AP2 complex can directly interact with the C-tail of the \( \alpha_{1b} \)-AR.

Identification of the Binding Site for \( \mu_2 \) on the \( \alpha_{1b} \)-AR—The \( \mu_2 \) subunit of the AP2 complex has been shown to recognize endocytosis signals including tyrosine-based motifs (YXX\(\Phi \)) and dileucine motifs on the cytoplasmic portion of membrane receptors (9). Analysis of the primary sequence of the C-tail of the \( \alpha_{1b} \)-AR revealed the presence of two potential tyrosine-based motifs at positions 386 and 442 and of two potential dileucine motifs at positions 450 and 473 (Fig. 2). Moreover, two additional YXX\(\Phi \) sequences can be found at positions 144 and 153 in the second intracellular loop of the receptor (Fig. 2). To assess whether these motifs can mediate the interaction between the \( \alpha_{1b} \)-AR and \( \mu_2 \), we generated GST-C-tail fusion proteins in which tyrosines 386 and 442 as well as the leucine doublets 450–451 and 473–474 were individually substituted by alanines and assessed their ability to interact with \( \mu_2 \) endogenously expressed in HEK-293 cells. Surprisingly, none of these mutations was able to disrupt the interaction between the C-tail of the receptor and \( \mu_2 \) (Fig. 3A). Similar results were obtained using the yeast two-hybrid system as an interaction assay (Fig. 3B). To investigate whether the two YXX\(\Phi \) sequences located at positions 144 and 153 of the \( \alpha_{1b} \)-AR were involved in the interaction with \( \mu_2 \), we also constructed a GST fusion protein, including the second intracellular loop of the receptor. This fusion protein was not able to interact with \( \mu_2 \) from HEK-293 cell extracts (results not shown). Altogether, these results suggest that the structural determinants of the \( \alpha_{1b} \)-AR mediating its interaction with \( \mu_2 \) are different from the canonical tyrosine-based or dileucine motifs.

To further investigate the binding site for \( \mu_2 \) on the C-tail of the \( \alpha_{1b} \)-AR, we fused to GST a series of fragments of the C-tail carrying progressive truncations (Fig. 2) and assessed their ability to interact with \( \mu_2 \) endogenously expressed in HEK-293 cells. As shown in Fig. 3C, whereas different fusion constructs truncated up to residue 380 could interact with \( \mu_2 \), the GST-T368 fusion construct did not, thus suggesting that the region included between residues 380 and 368 is crucial for this interaction. Interestingly, the deletion of eight arginines at positions 371–378 completely abolished the binding of the C-tail to \( \mu_2 \), suggesting that the \( \alpha_{1b} \)-AR interacts with the \( \mu_2 \) subunit of the AP2 complex through a novel arginine-based binding domain (Fig. 2).

Identification of the Binding Site for \( \alpha_{1b} \)-AR on \( \mu_2 \) —Recently, the crystal structure of the \( \mu_2 \) subunit of the AP2
complex has been solved (12, 24). The first 157 residues of the protein are organized in a predominantly \( \alpha \)-helical structure, whereas the C-terminal fragment of \( \mu_2 \) is largely composed of \( \beta \)-sheet structures that are folded into two subdomains (Fig. 4A) (12). The first subdomain, comprising residues 158–282, contains the binding site for tyrosine-based endocytic motifs, whereas the second subdomain, comprising residues 283–435, contains an interaction site for synaptotagmin, a neuronal AP2-binding protein involved in synaptic vesicles exocytosis (25).

To identify the region within \( \mu_2 \) that interacts with the C-tail of the \( \alpha_{1b} \)-AR, we tagged with GFP the three fragments of the \( \mu_2 \), \( 1 \)–157, 158–282, and 283–435, and expressed them in HEK-293 cells. Pull-down experiments were performed by incubating the GST-C-tail construct with cell extracts overexpressing different GFP-tagged \( \mu_2 \) fragments (Fig. 4A). We

Fig. 1. Identification of the \( \mu_2 \) subunit of the AP2 complex as a protein interacting with the C-tail of the \( \alpha_{1b} \)-AR. A, yeast two-hybrid screening. The C-tail-pLexA plasmid was used to transform the L40 yeast strain in combination with either empty pACT2 vector or with a pACT2 containing the \( \mu_2 \) cDNA. Double transformants were plated on Trp, Leu, His plates to select for histidine prototrophy (upper panel). The His\(^+ \) positive clones encoding \( \mu_2 \) were restreaked on selective medium and assayed for \( \beta \)-galactosidase activity by a filter assay (lower panel). Quantitative analysis of the interaction between the C-tail and \( \mu_2 \) was performed using the liquid \( \beta \)-galactosidase assay. B, GST pull-down assay. Extracts from HEK-293 cells expressing endogenous \( \mu_2 \) were incubated with glutathione-Sepharose beads coupled to GST alone or to the GST-C-tail. The \( \mu_2 \) eluted from the beads was detected by Western blotting using anti-\( \mu_2 \) monoclonal antibodies (left panel). The amount of extract loaded on the gel was 10% of that used for the pull-down. A control protein staining indicating the expression level of the GST and GST-tagged C-tail used in the pull-down assay is shown (right panel). The results are representative of three independent experiments. C, solid phase overlay assay. GST or GST-\( \mu_2 \) proteins were separated on SDS-PAGE and electroblotted onto nitrocellulose membranes. The membrane was incubated with 100 nM of \(^{32} \)P-labeled His\(_6\)-tagged C-tail (left panel) and revealed by autoradiography. A protein staining showing the relative amount of GST and GST-\( \mu_2 \) used in the assay is shown (right panel). The results are representative of three independent experiments. IB, immunoblot.

Fig. 2. Topological model of the \( \alpha_{1b} \)-AR. The YXX\( \Phi \) and dileucine motifs are highlighted by solid lines. The bars indicate the position of the stop codons introduced to construct the truncated C-tail fragments of the \( \alpha_{1b} \)-AR (T368, T380, T395, T425, T449, and T477), named after the number of the last encoded amino acid. The box includes the eight arginines deleted in the \( \Delta \)371–378 receptor mutant. The amino acids mutated to disrupt the YXX\( \Phi \) and dileucine motifs are also indicated.
found that the 1–157 and 282–435 fragments retained the ability to bind the C-tail of the \( \alpha_{1b}\)-AR, whereas the 158–257 fragment did not (Fig. 4B). These findings led us to conclude that the molecular determinants of \( \mu_2 \) involved in the recognition of the polyarginine motif on the C-tail of the \( \alpha_{1b}\)-AR are located on two domains at the N and C terminus, respectively, of the \( \mu_2 \) molecule. Interestingly, these domains are distinct from the \( \mu_2 \) region that binds tyrosine-based internalization signals, which is located between residues 157 and 282 (12).

**The \( \alpha_{1b}\)-AR/\( \mu_2 \) Interaction Occurs in the Cells and Is Regulated by Agonist-induced Receptor Activation**—To demonstrate that the \( \alpha_{1b}\)-AR and \( \mu_2 \) can form a complex inside the cells, we performed coimmunoprecipitation experiments from HEK-293 cells transiently expressing the HA-tagged \( \alpha_{1b}\)-AR. After immunoprecipitating the receptor using polyclonal anti-HA antibodies, monoclonal anti-HA as well as anti-\( \mu_2 \) antibodies were used to immunoblot the immunoprecipitated samples. The Western blots revealed that the \( \mu_2 \) endogenously expressed in HEK-293 cells could specifically co-immunoprecipitate with the \( \alpha_{1b}\)-AR, whereas no bands were immunoprecipitated by IgG (Fig. 5A, top panel, lanes 2 and 3). These findings demonstrate that beyond their ability to interact *in vitro*, the \( \alpha_{1b}\)-AR and \( \mu_2 \) can associate inside the cells.

To confirm that the \( \alpha_{1b}\)-AR/\( \mu_2 \) interaction in intact cells was mediated by the polyarginine motif identified above as the binding site for \( \mu_2 \), coimmunoprecipitation experiments were performed from HEK-293 cells overexpressing the HA-tagged receptor mutants truncated at residue 368 (T368) or carrying a deletion of the polyarginine motif (\( \Delta371–378 \)). The T368 and \( \Delta371–378 \) receptor mutants displayed pharmacological properties similar those of the wild type \( \alpha_{1b}\)-AR (results not shown). In addition, as previously shown (26), tagging the wild type or mutated receptors with HA or GFP at their N and C terminus, respectively, did not affect the pharmacological properties of the receptor (results not shown). As shown in Fig. 5A, the endogenous \( \mu_2 \) did not coimmunoprecipitate either with the T368 or with the \( \Delta371–378 \) receptor mutants, thus suggesting that the polyarginyl stretch between residues 371 and 378 represents the only binding site of the \( \alpha_{1b}\)-AR for \( \mu_2 \).

To investigate whether the whole heterotetrameric AP2 complex could interact with the \( \alpha_{1b}\)-AR, we determined whether additional subunits of the AP2 complex could be coimmunoprecipitated with the receptor. As shown in Fig. 5B, the \( \alpha \) and \( \beta_2 \) subunits endogenously expressed in HEK-293 cells were coimmunoprecipitated with the wild type receptor but not with the \( \Delta371–378 \) receptor mutant, thus suggesting that the whole AP2 complex can associate with the \( \alpha_{1b}\)-AR through the interaction mediated by its \( \mu_2 \) subunit.

To assess whether the \( \alpha_{1b}\)-AR/\( \mu_2 \) interaction could be modulated by the agonist-induced activation of the receptor, HEK-293 cells expressing the HA-tagged \( \alpha_{1b}\)-AR were incubated for 15 min in the absence or presence of \( 10^{-4} \text{ M} \) epinephrine prior to immunoprecipitation of the receptor. As shown in Fig. 6, treatment with epinephrine induced a 2-fold increase in the amount of endogenous \( \mu_2 \) coimmunoprecipitated with receptor when compared with untreated cells (Fig. 6, A (lanes 6 and 8) and B). This strongly suggests that the \( \alpha_{1b}\)-AR/\( \mu_2 \) interaction is dynamically regulated by the agonist-induced activation of the \( \alpha_{1b}\)-AR, which might increase the amount of \( \mu_2 \) associated with the receptor.

**Specificity of the \( \alpha_{1b}\)-AR/\( \mu_2 \) Interaction**—So far, four different adaptor protein complexes (APs) involved in sorting of membrane proteins have been identified and characterized (9). AP1 is involved in the formation of clathrin-coated vesicles from the trans-Golgi network and the trafficking of proteins from the trans-Golgi network to the plasma membrane (8). AP2 plays a role in the clathrin-mediated endocytosis of plasma membrane receptors (8). AP3 has been shown to mediate the sorting of proteins form early endosomes to lysosomes (27). Finally, AP4 has been shown to participate in the polarized transport of proteins to the basolateral membrane in Madin-Darby canine kidney cells (28). To assess whether the \( \alpha_{1b}\)-AR preferentially associates with the AP2 as compared with other AP complexes, we performed coimmunoprecipitation experiments from HEK-293 expressing the HA-tagged \( \alpha_{1b}\)-AR and GFP-tagged \( \mu_1 \), \( \mu_2 \), \( \mu_3 \), and \( \mu_4 \) subunits. Interestingly, Western blotting using anti-GFP antibodies indicated that, whereas \( \mu_2 \) could be immu-
noprecipitated with the HA-tagged α1b-AR, μ1, μ3, and μ4 could not (results not shown). This suggests that, inside the cells, the α1b-AR specifically associates with the μ2 subunit of the AP2 complex rather than with other AP complexes.

The α1b-AR/μ2 Interaction Is Involved in Clathrin-mediated Endocytosis of the Receptor—To confirm that the α1b-AR can
internalize in clathrin-coated vesicles, we assessed whether hypertonic sucrose and the overexpression of a dominant negative mutant of dynamin (K44A) could inhibit the agonist-induced internalization of the GFP-tagged α_{1b}-AR transiently expressed in HEK-293 cells. Incubation of cells with 0.45 M sucrose for 15 min prior to stimulation with epinephrine completely inhibited agonist-induced internalization (Fig. 8, C and D). Similarly, overexpression of the K44A dynamin mutant impaired receptor endocytosis when compared with control cells (Fig. 7, E and F). These results support the notion that in HEK-293 cells the α_{1b}-AR undergoes internalization through clathrin-mediated endocytosis.

It is well established that the AP2 complex controls the early steps of membrane receptor endocytosis, allowing clathrin to be recruited to the receptor. Since the α_{1b}-AR represents the first GPCR to be shown to directly interact with AP2, and since this interaction seems to occur through a noncanonical μ_{2} binding motif on the receptor, we sought to establish whether the α_{1b}-AR/μ_{2} interaction might play a role in the regulation of receptor endocytosis.

To test this hypothesis, we determined whether deleting the μ_{2} binding site on the C-tail of the α_{1b}-AR could affect agonist-induced receptor internalization. The GFP-tagged forms of the wild type α_{1b}-AR (WT-GFP) and of its mutants T368 (T368-GFP) and Δ371–378 (Δ371–378-GFP) were transiently expressed in HEK-293 cells and tested for their ability to undergo agonist-induced internalization. Cells expressing the different GFP-tagged receptors were treated with 10^{-4} M epinephrine for various times and subsequently incubated with a membrane-impermeant biotinylation reagent (sulfo-NHS-biotin) (see "Experimental Procedures"). Biotinylated cell surface receptors were precipitated using streptavidin-Sepharose beads and detected by Western blotting using anti-HA antibodies. The results of this biochemical assay indicated that the HA-tagged receptors could not be detected in streptavidin-Sepharose precipitates when cells were not incubated with the biotinylation reagent, thus confirming that, in our experimen-
One unexpected finding of our study was the observation that \( \mu_2 \) recognizes a noncanonical binding motif on the \( \alpha_{1b}\)-AR, which consists of eight consecutive arginines included between residues 371 and 378 of the C-tail of the receptor (Fig. 3). Deletion of this motif completely abolished the \( \alpha_{1b}\)-AR/\( \mu_2 \) interaction inside the cells measured in the communoprecipitation experiments (Fig. 5). Interestingly, the deletion of the \( \mu_2 \) binding motif also abolished the interaction of the \( \alpha_{1b}\)-AR with the \( \alpha \) and \( \beta \) subunits of the AP2 complex, suggesting that this motif represents the only point of contact between the AP2 complex and the receptor. The polyarginine motif of the \( \alpha_{1b}\)-AR is reminiscent of the binding site for \( \mu_2 \) previously identified in synaptotagmin, a neuronal AP2 binding proteins involved in recycling of synaptic vesicles, which contains six positively charged residues (KRLKKKK) (29).

The recent publication of the crystal structure of the entire AP2 complex has provided a better understanding of how the different subunits of the complex contact each other and of how the \( \mu_2 \) subunit interacts with tyrosine-based endocytic motifs located on membrane receptor (24). Whereas the YXXF motifs have been shown to bind to a hydrophobic pocket located in the subdomain A (residues 157–282) of \( \mu_2 \), recent studies indicate that the positively charged motif of synaptotagmin contacts the subdomain B (residues 283–435) of \( \mu_2 \) (25). Similarly, we could show that the subdomain B (residues 283–435) together with the N-terminal region of \( \mu_2 \) (residues 1–157) participate in the interaction with the polyarginine motif of the \( \alpha_{1b}\)-AR (Fig. 4).

Therefore, it appears that the N-terminal region and the subdomain B of \( \mu_2 \) might provide a docking surface for positively charged motifs exposed on the cytoplasmic face of membrane receptors. A systematic scanning mutagenesis of these domains will be required to determine whether the \( \alpha_{1b}\)-AR and synaptotagmin bind to similar structural determinants of the \( \mu_2 \).

The \( \alpha_{1b}\)-AR/\( \mu_2 \) interaction is dynamically regulated as demonstrated by the fact that activation of the receptor by the agonist increases the association of \( \mu_2 \) to the receptor by 2-fold (Fig. 6). This suggests that \( \mu_2 \) preferentially recognizes the agonist-occupied form of the \( \alpha_{1b}\)-AR. One can speculate that binding of epinephrine to the \( \alpha_{1b}\)-AR might trigger a conformational change that exposes the polyarginine motif, thus promoting the association of the receptor with the AP2 complex. A similar model has been proposed for the epidermal growth factor receptor-mediated recruitment of the AP2 complex in which the receptor exposes a high affinity binding site for \( \mu_2 \) only upon activation by epidermal growth factor (11, 12).

An important finding of our study is that the interaction of the \( \alpha_{1b}\)-AR with \( \mu_2 \) of the AP2 complex plays a role in receptor endocytosis. This was mainly demonstrated by the fact that deleting the \( \mu_2 \) binding motif in the C-tail of the \( \alpha_{1b}\)-AR markedly decreased agonist-induced receptor internalization as shown by confocal microscopy (Fig. 8) as well as by surface receptor biotinylation (Fig. 9). Hypertonic sucrose as well as the dominant negative dynamin mutant K44A almost completely inhibited epinephrine-induced endocytosis of the GFP-tagged \( \alpha_{1b}\)-AR, thus supporting the notion that the \( \alpha_{1b}\)-AR can internalize in clathrin-coated vesicles (Fig. 7). Since the AP2 complex links the clathrin coat to transmembrane proteins sorted into coated pits, the \( \alpha_{1b}\)-AR/\( \mu_2 \) interaction might represent a mechanism directly involved in targeting the \( \alpha_{1b}\)-AR to clathrin-coated vesicles.

In a previous study, we reported that agonist-induced internalization of the \( \alpha_{1b}\)-AR is, at least in part, mediated by \( \beta \)-arrestins. This was mainly demonstrated by two observations: (a) the stimulation of the \( \alpha_{1b}\)-AR with epinephrine induced a marked translocation to the cell surface of \( \beta \)-arrestin; (b) a dominant negative mutant of \( \beta \)-arrestin 1 (V53D) decreased
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FIG. 8. Internalization of the α₁b-AR and of its mutants monitored by confocal microscopy. Confocal microscopy of HEK-293 cells transfected with the cDNAs encoding the GFP-tagged forms of the α₁b-AR (WT-GFP) and of its mutants T368 (T368-GFP) and Δ371–378 (Δ371–378-GFP). After treatment with 10⁻⁴ m epinephrine for the indicated times, cells were incubated with 500 μM NHS-S-biotin for 30 min at 4 °C. Surface biotinylated receptors were precipitated using streptavidin-Sepharose beads, separated on SDS-PAGE, electroblotted onto nitrocellulose membranes, and detected by Western blotting using anti-HA monoclonal antibodies. The images are representative of five independent experiments.

FIG. 9. Internalization of the α₁b-AR and of its mutants monitored by biotinylation of the receptors at the cell surface. A, HEK-293 cells were transfected with the cDNAs encoding the HA-tagged forms of the α₁b-AR (lanes 1–4) and of its mutants T368 (lanes 5–8) and Δ371–378 (lanes 9–12). After treatment with 10⁻⁴ m epinephrine for the indicated times, cells were incubated with 500 μM NHS-S-biotin for 30 min at 4 °C. Surface biotinylated receptors were precipitated using streptavidin-Sepharose beads, separated on SDS-PAGE, electroblotted onto nitrocellulose membranes, and detected by Western blotting using anti-HA monoclonal antibodies. Western blot analysis performed on cell lysates using anti-HA monoclonal antibodies indicated that the receptor expression levels were similar in the different samples (results not shown). Results are representative of three independent experiments. B, densitometry of the bands corresponding to the biotinylated receptors precipitated by the streptavidin-Sepharose beads was performed as indicated under “Experimental Procedures.” Results are the mean ± S.E. of three independent experiments.

the internalization of the α₁b-AR (20). Therefore, in addition to the direct association of the receptor with the AP2 complex, β-arrestins also play an important role in the clathrin-mediated endocytosis of the α₁b-AR. This is supported by the observation that the deletion of the μ₂-binding motif did not completely abolish receptor internalization, suggesting that additional mechanisms regulate the endocytosis of the α₁b-AR (Figs. 8 and 9). Interestingly, overexpression of a dominant negative mutant of β-arrestin 1 (V53D) (30) abolished the residual internalization observed for the Δ371–378 mutant of the α₁b-AR lacking the μ₂-binding site (results not shown). Additional determinants involved in receptor endocytosis are likely to be localized on the C-tail of the α₁b-AR, since the T368 receptor mutant, lacking most of the C-tail, was almost totally impaired in its ability to undergo agonist-induced internalization (Figs. 8 and 9). Altogether, these findings suggest that agonist-induced endocytosis of the α₁b-AR results from multiple mechanisms involving the interaction of the receptor with both the AP2 complex and β-arrestins.

The findings of our study suggest that the molecular mechanisms underlying the internalization of the α₁b-AR seem to differ from those controlling the endocytosis of other GPCRs, like the β₂-AR, for which it is believed that their redistribution to clathrin-coated vesicles is mediated by β-arrestins. However, the possibility that GPCRs can directly interact with the AP2 complex has not been investigated so far, and it will be important to establish whether this interaction represents a common mechanism occurring at other GPCRs in addition to the α₁b-AR. The discovery that the AP2 complex can directly interact with the α₁b-AR raises several questions about the molecular mechanisms underlying receptor endocytosis. In particular, future studies will aim at elucidating the relationship between the structural determinants of the α₁b-AR involved in binding the AP2 complex versus β-arrestins, the respective role of the AP2 complex and β-arrestins in targeting the receptor to clathrin-coated vesicles as well as their interplay with other yet unidentified mechanisms regulating receptor trafficking and function.

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