

The ubiquitin-specific protease USP4 regulates the cell surface level of the A_{2A}-receptor

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Abbreviations used are: A_{2a}R, A_{2a} adenosine receptor; CFP, cyan fluorescent protein; ER, endoplasmic reticulum; UBPs, ubiquitin-specific processing proteases; USP, ubiquitin specific protease; YFP, yellow fluorescent protein

Abstract

Many membrane proteins incur a folding problem during biosynthesis; only a fraction thereof is exported from the endoplasmic reticulum (ER) because quality control is stringent. This is also true for G protein-coupled receptors. Here we identify the deubiquitinating enzyme Usp4 as an interaction partner of the A_{2a}-adenosine receptor, a G_s-coupled receptor. Usp4 binds to the carboxyl terminus of the A_{2A}-receptor and allows for its accumulation as de-ubiquinated protein. This relaxes ER quality control and enhances cell surface expression of functionally active receptor. The effect of Usp4 on the A_{2A}-receptor was specific because (i) it was not seen in C-terminally truncated versions of the receptor, (ii) it was not mimicked by Usp14, another member of the USP-family, and because (iii) it was not seen with the metabotropic glutamate receptor-5 (mGluR5), another G protein-coupled receptor with a high propensity for intracellular retention. These observations show that deubiquitinating enzymes can regulate quality control in the ER.

Introduction

Membrane proteins have to be inserted cotranslationally into the endoplasmic reticulum. This occurs via the translocon, a channel formed by the Sec61-subunits. During and after synthesis of membrane proteins in the endoplasmic reticulum, they undergo a strict quality control to ensure correct folding, before they are transported to their definitive site of action. Several aspects of this quality control are incompletely understood; nevertheless it is clear that incorrect folding of a membrane protein is sensed by the machinery of the endoplasmic reticulum (that is by chaperons, presumably). This leads to activation of ubiquitinating enzymes on the cytoplasmic side. These transfer ubiquitin to the cytoplasmic peptide chain of the incorrectly folded protein, which is retrotranslocated and degraded by the 26S proteasome (Kostova and Wolf, 2003). This scheme relies predominantly on observations that were made in *Saccharomyces cerevisiae*. Based on several pieces of experimental evidence, it is, however, reasonable to assume that higher eukaryotes employ a related machinery to eliminate misfolded proteins (Lilley and Ploegh 2004; Ye et al., 2004).

It has been increasingly appreciated that many human diseases can be linked to mutations, which result in the retention of the aberrant protein in the endoplasmic reticulum (ER). Cystic fibrosis is most commonly cited as the model disease: More than 1000 mutations have been identified in the gene encoding the CFTR (cystic fibrosis transmembrane conductance regulator) (Rowntree and Harris, 2003), but the majority of the patients (~70%) have the $\Delta F508$ -mutation of the CFTR. The resulting protein fails to reach the plasma membrane due to a stringent ER quality control mechanism, but it can function properly, if it reaches the plasma membrane (Pasyk and Foskett, 1995). There are many more examples that lead to defective ER-export of membrane proteins; these include mutations of the V_2 -vasopressin receptor (associated with diabetes insipidus;

Oksche and Rosenthal, 1998), of the LDL-receptor (resulting in hypercholesterolemia; Hobbs et al., 1990), of the HERG-K⁺-channel (resulting in long QT-syndrome-2; Kupersmidt et al., 2002) and many others. It is unclear why these mutated proteins are retained and eventually degraded although they are – at least in part - functionally active (see Pasyk and Foskett, 1995). However, the available evidence suggests that the quality control machinery in the endoplasmic reticulum is stringent, for it prefers to err on the side of rapidly degrading a protein that - when given time - may fold into a functionally active transporter or channel.

G protein-coupled receptors have been documented to incur a folding problem: a large portion of newly synthesized protein ($\geq 50\%$) is subject to degradation in the endoplasmic reticulum and does not reach the plasma membrane (Petaja-Repo et al., 2000 & 2001; Pankevych et al., 2003). This is similar to the situation with many other membrane proteins with multiple transmembrane spans, specifically with CFTR (Rowntree and Harris, 2003). During folding the intracellular carboxyl terminus of membrane proteins plays a prominent role, because it allows for docking of the ER-associated degradation machinery that eliminates incorrectly folded proteins. In addition, in several instances, segments within the carboxyl terminus of G protein-coupled receptors have been shown to be essential for proper folding of the protein: mutations within the carboxyl terminus of structurally unrelated G protein-coupled receptors result in receptors that fail to adopt a conformation capable of ligand binding (Krause et al., 2000; Pankevych et al., 2003; Duvernay et al., 2004). Here we identify the de-ubiquinating enzyme Usp4 as an interaction partner of the A_{2A}-adenosine receptor, a prototypical G_s-coupled receptor: Usp4 binds to the carboxyl terminus of the A_{2A}-adenosine receptor; de-ubiquitination of the receptor relaxes quality control in the ER and enhances cell surface expression. Hence, ER-associated ubiquitination is reversible, an observation that implies that quality control in the ER may be subject to dynamic regulation.

Materials and Methods

Materials and reagents. Guanine nucleotides including guanosine-5'-(3-*O*-thio)triphosphate (GTP γ S), adenosine deaminase, insulin-transferrin-sodium selenite supplement and Complete[®] protease inhibitor tablets were from Roche Diagnostics and Roche Molecular Biochemicals (Mannheim, Germany). CGS21680 and labeled 4-(2-[7-amino-2-[2-furyl][1,2,4]triazolo[2,3-a][1,3,5] triazin-5-yl-amino]ethyl)phenol (³H]ZM241385) (specific activity 27.4 Ci/mmol) were from Tocris Cookson Ltd. (Bristol, UK). Papain was from Worthington Biochemical Corp (USA), HEPES from Biomol (Munich, Germany), Xanthine amino congener (XAC) was from Research Biochemicals (Natick, MA). The materials required for SDS-PAGE were from BioRad (Richmond, CA). Fetal calf serum was from PAA Laboratories (Linz, Austria), Dulbecco's modified Eagle medium (DMEM), non-essential amino acids, β -mercaptoethanol, gentamicin, G418 (geneticin), Lipofectamine reagent and Lipofectamine Plus reagent from GIBCO-BRL (Grand Island, NY). Forskolin, chloroquine, kynurenate, progesterone, putrescin, lysozyme, 4-(3-butoxy-4-methoxybenzyl)imidazoline-2-one (RO201724), cAMP, L-glutamine, streptomycin, triton X-100, PMSF, ANTI-FLAG[®] M2 affinity gel and ANTI-FLAG monoclonal antibody-Peroxidase-conjugated, monoclonal ANTI-HA peroxidase-conjugated antibody were from Sigma Chemical Co. (St. Louis, MO). MG132 was from Calbiochem (Darmstadt, Germany), [³H]adenine from DuPont NEN (Boston, MA, USA), cytosine- β -D-arabinofuranoside (ARA-C) was ICN Biomedicals. ER-Tracker Blue-White DPX, a fluorescent dye that specifically stains the endoplasmic reticulum, was from Molecular Probes (Leiden, The Netherlands); pMAL vector and amylose resin for expression and purification of maltose binding protein (MBP) were from New England Biolabs Inc. (Beverly, MA, USA). The Micro BCA[®] protein assay reagent kit was from Pierce (Rockford, IL). Buffers and salts were from Merck (Darmstadt, Germany). Centrifuge tubes and tissue culture plates were from Greiner (Vienna, Austria) and from Corning

Costar (Acton, MA, USA). Plasmid preparation kits were from Qiagen (Hilden, Germany). Rabbit anti-GFP Living colors A.v. peptide antibody and the MATCHMAKER yeast two-hybrid system were from Clontech Laboratories, Inc (CA, USA). Horseradish peroxidase-conjugated anti-mouse- and anti-rabbit immunoglobulin antibodies were from Amersham Life Science (Buckinghamshire, England, U.K.). The immunoreactive bands on nitrocellulose blots were detected by chemoluminescence using SuperSignal chemoluminescence substrate from Pierce (Rockford, IL).

DNA constructs. To perform epitope tagging of the A_{2a} adenosine receptor, the sequence of the receptor, encompassing the coding region, was cleaved with *HindIII/SalI* from pEYFP-N1/human A_{2a}R cDNA (5) and ligated into *HindIII/SalI*-digested pCMV-Tag 2B vector (Stratagene), linking the A_{2a}R in frame to the N-terminus of FLAG-epitope. The DNA fragment encoding the C-terminal part of A_{2a}R was amplified by PCR from pcDNA3-A_{2a}R vector (5), which bears the full length of A_{2a}R, using the primers 5'-CCAGAATTCCGTATCCGCGAGTTC-3' and 5'-TTTAACTCGAGT TCAGGACACTCCTGC-3'. After restriction digestion of the PCR fragment with *EcoRI* and *XhoI*, A_{2a}R was inserted into pEG202. An identical strategy was employed for the generation of mutants in which the carboxyl terminus was truncated by using the following 3'-primers: 5'-CGC GCCTCGAGTTCATTGGTGCCTCAGGACGTGG-3' [for A_{2a}R_{ct}(1-311)] and 5'-TTTTTCTCG AGTTCAGCCATTGGGCCTCCGGTC-3' [for A_{2a}R_{ct}(1-360)]. The integrity of the engineered sequences was confirmed by fluorescent sequencing. The following plasmids were generous gifts: the plasmid for mammalian expression of HA-tagged ubiquitin (HA-Ubi) from J. Bies (Cancer Research Institute, Bratislava, Slovak Academy of Sciences), of GFP- and FLAG-tagged mouse homolog Usp4 from D. Gray (Ottawa Regional Cancer Center, Canada), of Usp14 from J. Blahos (UEM AVCR, Praha, Czech Republic).

Yeast two-hybrid interaction screen. Yeast two-hybrid analysis was performed using the MATCHMAKER yeast two-hybrid system. In this system, yeast strain EGY48 (MAT α *trp1 his3 ura3 leu2 6 LexAop-LEU2*) was used for transformation of bait, prey, and reporter plasmids pEG202, pJG4-5 and pSH18-34, respectively. Yeast strains were grown at 30 °C in either glucose minimal medium or induction medium; transformation was performed by using lithium acetate method in accordance with manufacturer's instruction. EGY48 containing the *lacZ* reporter plasmid (pSH18-34) and the A_{2a}R_{ct} bait plasmid (pEG202/A_{2a}R_{ct}) was transformed with prey plasmids bearing a human brain cDNA library. To verify the two-hybrid interactions, positive transformants grown on selection medium were assayed directly *in situ* for β -galactosidase activity assay by briefly exposure of the plates to chloroform followed by incubation with 100 μ M X-gal in the medium as a substrate.

Cell culture, cellular transfection and membrane preparations. HEK293 cells were maintained in Dulbecco's modified Eagle medium (DMEM) at 5% CO₂, 95% air and 37°C. Culture media were supplemented with 10% fetal calf serum, 2 mM L-glutamine, β -mercaptoethanol, non-essential amino acids, 50 mg/L gentamicin and - in order to maintain the selection pressure - with 0.2 mg/mL geneticin (G418) for the culture of stably transfected cells. PC12 cells were propagated as described (Kudlacek et al., 2001). One day before transfection, cells were replated to obtain subconfluent cultures either on glass coverslips (22 mm in diameter and placed into 6-well plates) or 10-cm diameter cell culture dishes. Transient transfections were done using the CaPO₄ precipitation method or LipofectAMINE PlusTM (Invitrogen). When required in cotransfections with several plasmids, the appropriate empty vectors were added to keep the amount of DNA/dish constant. The medium was changed to remove excess DNA precipitates in 5 h after

transfection. Serum starvation and incubation with adenosine deaminase was initiated on day 1 after transfection. The subsequent starvation under serum-free conditions lasted for 12-24 h; thereafter cAMP accumulation was stimulated as outlined below. Where indicated, cells were kept in the medium containing proteosomal inhibitor (50 μ M MG 132) or lysosomal inhibitor (100 μ M chloroquine) at 37 °C for 3 h prior to harvesting. Cells were harvested and membranes were prepared as described previously (Klinger et al., 2002a). Primary neuronal cultures were obtained from hippocampi of neonatal rats as described earlier (Boehm and Betz, 1997); four days after plating, the cultures were treated with 1 μ M cytosine- β -D-arabinofuranoside (ARA-C) to deplete glial cells. Neurons were seeded on polylysine-coated coverslips and transfected with LipofectAMINE 2000TM as outlined previously (Farhan et al., 2004).

Radioligand binding assays. PC12 cells and HEK293 cells transiently transfected with the appropriate plasmids were subjected lysed by three freeze-thaw cycles followed by brief sonication; membranes were collected by centrifugation (30 min at 50,000g) as outlined previously (Klinger et al., 2002a). Hence the particulate fraction contained intracellular membranes and the plasma membrane. Membranes were prepared from (100 μ g/assay) were incubated in a final volume of 0.3 ml containing 50 mM Tris.HCl (pH 8.0), 1 mM EDTA, 5 mM MgCl₂, 8 μ g/ml adenosine deaminase and concentrations of [³H]ZM241385 covering the range of 0.2 to 20 nM in the presence of 100 μ M GTP γ S. After 60 min at room temperature, the reaction was terminated by rapid filtration over glass fiber filters. Nonspecific binding was determined in the presence of 10 μ M XAC and amounted to 40% at the highest concentration of [³H]ZM241385. The data points were fitted by non-linear regression to the equation describing a rectangular hyperbola. Assays were performed in duplicate.

Agonist mediated cellular cAMP accumulation. Cells were grown in 6-well plates. The adenine nucleotide pool was metabolically labelled by incubating confluent monolayers for 16 h with [³H]adenine (1 µCi/well) as described (Kudlacek et al., 2001). After the preincubation, fresh medium was added that contained 100 µM RO201724 (a non-xanthine phosphodiesterase inhibitor) and adenosine deaminase (2 U/ml) to remove any endogenously produced adenosine. After 1 h, cAMP formation was stimulated by the A_{2A}-selective agonist CGS21680 (1 nM to 1 µM) for 15 min and the reaction was stopped by adding 2.5% perchloric acid with 100 µM cAMP (1 ml/dish). The supernatant (0.9 ml) was aspirated, neutralized with 100 µl of 0.4 M KOH, and diluted with 1.5 ml 50 mM Tris-HCl, pH 8.0; [³H]cAMP was isolated by sequential chromatography on Dowex AG 50W-X4 and neutral alumina columns. Assays were performed in triplicate.

Immunoprecipitation of the epitope-tagged A_{2A}-adenosine receptor. HEK293 cells stably expressing FLAG-tagged A_{2A}-adenosine receptor were washed three times with phosphate buffered saline; subsequently, the membranes were solubilized in ice-cold lysis buffer [50 mM Tris.HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl containing 1% Nonidet P-40 (vol/vol), protease inhibitors (Complete, Roche Molecular Biochemicals) and, where indicated, 10 mM N-ethylmaleimide (NEM)] for 1 h on ice. The insoluble material was collected by centrifugation at 16,000 × g for 10 min at 4 °C. The supernatant was processed for immunoprecipitation, each step of which was conducted with constant rotation at 4 °C. Then 40 µl of a 50 % (vol/vol) suspension of anti-Flag M2 Affinity Gel was added and the sample was incubated overnight. The beads were collected by centrifugation and washed three times in 1 mL Tris-buffered saline. Immune complexes were dissociated in SDS-polyacrylamide sample buffer containing 20 mM dithiothreitol by incubation for 1 h at 37 °C or, alternatively, for 5 min at 95 °C. Proteins were transferred to nitrocellulose membranes by using a semidry transfer system; immunodetection was achieved by using mono-

clonal peroxidase-conjugated anti-FLAG and anti-HA antibodies to detect the FLAG epitope of the A_{2a}R and the HA-epitope of ubiquitin respectively. The GFP moiety in Usp4 was detected with an anti-GFP antiserum and a horseradish peroxidase conjugated anti-rabbit IgG secondary antibody. The immunoreactive bands were visualized with by enhanced chemiluminescence.

Purification of fusion proteins and in vitro binding assays. The C-terminal part of the A_{2A}-receptor was amplified by PCR and subcloned into the *EcoRI* and *XhoI* sites in the multicloning site of the pMAL-cri vector. Expression of the resulting construct in *Escherichia coli* (BL21) produced an MBP-A_{2a}R-C-terminus fusion protein, which was purified by affinity chromatography on an amylose resin. MBP-A_{2a}R-C-terminus bound to the amylose resin was eluted with buffer A [20 mM Tris (pH 7,4), 200 mM NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol] containing 10 mM maltose. Maltose was removed by repeated cycles of concentration and dilution in a Centricon microconcentrator with a nominal cutoff of 30 kDa; the protein was concentrated to 1 to 2 mg/ml in buffer A, frozen in liquid nitrogen and stored at –80°C. Unfused MBP and a fusion protein comprising MBP and the C-terminus of the A₁-adenosine receptor were expressed and purified in an analogous way.

HEK293 cells were transiently transfected with a plasmid driving the mammalian expression of Usp4-Flag, harvested 48 h after transfection and lysed in Tris-buffered saline (20 mM Tris.HCl, pH 7.4, 150 mM NaCl), containing 1 mM EDTA, 1% Triton X-100 and protease inhibitors (Complete®, Roche Molecular Biochemicals). The lysate was subsequently centrifuged at 16,000 g for 10 min at 4 °C. The supernatant was applied to an anti-FLAG epitope immuno-affinity column with 1 ml of bed volume (Anti-FLAG M2 affinity Gel, Sigma). After washing the column with Tris-buffered saline (10 bed volumes), Usp4 was eluted with 6*1 mL of 0.1 M glycine.HCl, pH 3.5.

For pull-down assays, purified fusion proteins (3 μ g of MBP-A_{2a}R-C-terminus, MBP-A₁R-C-terminus or MBP) were incubated with 30 μ l of amylose resin (50% slurry); after removal of the unbound proteins, purified Usp4-Flag (0.3 μ g) was then added to the immobilized fusion proteins. Subsequently, the resin was washed three times with 1 mL MBP buffer (20 mM Tris (pH 7.4), 200 mM NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol); 10 mM maltose or the sample buffer for SDS-polyacrylamide gel electrophoresis was used to release the proteins bound to the resin.

Endoglycosidase H sensitivity. HEK293 cells were collected in buffer containing 20 mM HEPES, pH 7.2 and 0.37 M sorbitol. The cells were lysed by sonication on ice; after centrifugation (10 min at 40,000 g), the membrane pellet was resuspended in buffer (50 mM Tris.HCl, pH 7.4, 1 mM EDTA, 5 mM MgCl₂) and frozen in liquid nitrogen. Membranes (20 μ g/assay) assay were pelleted and resuspended in 20 μ l of buffer: 50 mM sodium citrate, pH 5.6; 0.6 % SDS; 0.15 M 2-mercaptoethanol]. Samples were incubated for 5 min at 95 °C. After cooling at 37 °C, PMSF (0.5 mM final concentration) and endo- β -N-acetylglucosaminidase-H [= endoglycosidase H = endoH, 10 mU) was added to the reaction; the mixture was incubated at 37 °C for 18 h prior to gel electrophoresis and immunoblotting. Western blot films were scanned by a BioRad scanning densitometer and processed with QuantiScan software (Biosoft[®]; Cambridge, UK).

Fluorescence microscopy. Transiently transfected HEK-293 cells and hippocampal neurons were investigated 1 day after transfection on an inverted epifluorescence microscope (Zeiss Axiovert 200M) using a 63-fold oil immersion objective and filter sets, which discriminate between CFP and YFP fluorescence (Chroma Technology Corp.; Brattleboro, VT). Images were captured with a cooled CCD-Kamera (CoolSNAP fx ; Photometrics, Roper Scientific, Tucson, AZ) and stored

in and processed with MetaSeries software (release 4.6 Metafluor and Metamorph; Universal Imaging).

Preparation of RNA, PCR and RNA-interference. Newborn rat pups were sacrificed, striata and hippocampi were dissected and frozen in liquid nitrogen. Cells (HEK293 & PC12 cells) or tissues were lysed in TriReagent (Sigma) and total RNA prepared according to the manufacturer's instruction. For HEK293 cells, reverse transcription (RT) for HEK cells was performed with 0.5µg total RNA; the primer pair for polymerase chain reaction (PCR) was ATGGCGGAAGGTGGAGGCTG (forward primer) and ACGCTCCGCAGGGATGTTGA (reverse primer). The reverse transcription for hippocampi, striata and PC12 cells was performed with 1µg total RNA; the subsequent polymerase chain reaction was done with the forward primer was AGCAGAAATTTCACTACCTCTTCAAAC and the reverse primer: CAGGAAGACCTCCATAATCCG. For RNA interference, 3×10^5 HEK 293 cells stably expressing YFP-tagged A_{2A}-receptors at low levels were seeded in a 6 well plate and transfected with 500 pmol stealth siRNA (Invitrogen) with Lipofectamine 2000 according to the manufacturer's instructions. Several siRNA sequences were employed: CCGAGGCGUAUAAACUACUAAA (referred to as siRNA-1) and CCAUUUCAGCAAGGCAGACACCAUU (siRNA2) were investigated in several experiments as a pair of inactive (siRNA1) and active (siRNA2) molecules. Forty-eight hours after transfection, RNA was isolated to quantify the knockdown of mRNA coding for Usp4 by RT-PCR. At the same time, the distribution of YFP-tagged A_{2A}-receptor was visualized by fluorescence microscopy.

Results

The A_{2A}-receptor is subject to proteasomal rather than lysosomal degradation

The rat pheochromocytoma cell line PC12 expresses the A_{2A}-receptor endogenously. At steady state, substantial amounts of receptor can be visualized within the cell (Aslan & Fredholm 2000; see also below). These may represent recycling receptors, receptors en route to lysosomal degradation or receptors subjected to ER-associated degradation. In order to discriminate between these possibilities, we treated PC12 cells with the proteasome inhibitor MG132 and with the lysosomal inhibitor chloroquine and determined the number of binding sites with the antagonist radioligand antagonist [³H]ZM241385 (Fig. 1A). Addition of the proteasome inhibitor MG132 resulted in an increase in the membrane levels of the A_{2A}-receptor (▲ in Fig. 1A); on average, binding increased by about 50% ($B_{\max} = 2.04 \pm 0.29$ and 2.9 ± 0.37 pmol/mg in the absence and presence of MG132, respectively). In contrast, the presence of chloroquine did not affect the A_{2A}-receptor level (▼ in Fig. 1B). The model of quality control in the endoplasmic reticulum predicts a prominent role of the carboxyl terminus of membrane proteins, because it serves as the docking site for the machinery that targets the protein for proteasomal degradation (Kostova and Wolf, 2003). Thus, a truncation of the carboxyl terminus of the A_{2A}-receptor ought to render the receptor insensitive to the action of MG132. We tested this prediction by transiently expressing the wild type (human) A_{2A}-receptor and a truncated version thereof, A_{2A}-receptor (1-311), in HEK293 cells. In the A_{2A}-receptor (1-311), the last 101 amino acids have been eliminated, but the 20 amino acid adjacent to the seventh transmembrane segment have been retained; the signaling properties of this receptor only differ in a subtle manner from the wild type receptor (Klinger et al., 2002a). As can be seen from Fig. 1B, the expression level of the A_{2A}-receptor(1-311) was not increased by the addition of MG132. Thus, the carboxyl terminus (i.e. the last 100 amino acids) of the A_{2A}-receptor render the expression levels sensitive to a proteasome inhibitor.

Yeast-two hybrid screen for a binding partner

Taken together, the observations summarized in Fig. 1 suggest that proteasomal degradation of the receptor is preceded by one (or several) reversible step(s) in which the receptor committed to degradation can nevertheless be rerouted to increase the number of available receptor. In order to understand the underlying mechanism, we searched for a candidate protein that bound to the carboxyl terminus of the A_{2A}-adenosine receptor by using the last 120 amino acids as a bait in yeast-two hybrid interaction hunt. Because the A_{2A}-receptor is abundantly expressed in several brain regions, most notably the basal ganglia (Fredholm et al., 2005), we screened a human brain library and identified more than 5 different interaction partners that reproducibly bound to the fusion protein comprising lexA and the carboxyl terminus of the A_{2A}-receptor. We focused on Usp4, because Usp4 is a de-ubiquinating enzyme, albeit of ill-defined function. It was the only interactor that was related to proteasomal degradation; we surmised that its deubiquinating activity may be relevant for the effects observed with the proteasome inhibitor MG132 summarized in Fig. 1.

Usp4 is the human homolog of murine UNP (>90% amino acid identity), in spite of the eponymous description (ubiquitous nuclear protein; Gupta et al., 1994), in all cell lines that we tested, Unp/Usp4 was found in the cytosol rather than in the nucleus (see also below). The ratio of nuclear to cytosolic distribution is dependent on the cell type (Soboleva et al., 2005). The data shown in Fig. 1B predict that truncation of the bait construct ought to eliminate the interaction with Usp4, if there were any link between binding of Usp4 to the A_{2A}-receptor and the regulation of receptor levels by inhibition of proteasomal degradation. This was the case: the interaction between the Usp4-containing prey construct and the C-terminus of the A_{2A}-receptor induced β -galactosidase activity to levels that approached that of the constitutive and inducible controls (cf. 1st and 2nd streak with 3rd and 4th streaks in Fig. 2A). In contrast, truncation of the bait by 101

amino acids eliminated induction of β -galactosidase (7th and 8th streak in Fig. 2A); in fact, truncation of bait by the last 52 amino acids sufficed to abolish the interaction between prey and bait (5th and 6th streak in Fig. 2A). It is worth noting that other interactors were still capable of binding to the truncated bait constructs and thus to induce β -galactosidase activity to levels comparable to those seen with the wild type carboxyl terminus (Gsandtner et al., 2005).

Interaction of the A_{2A}-receptor with Usp4

In order to obtain independent evidence for an interaction between the A_{2A}-receptor and Usp4, we coexpressed the FLAG-epitope tagged A_{2A}-receptor and GFP-tagged Usp4 in HEK293 cells and subjected cellular lysates to immunoprecipitation with a anti-FLAG antibody immobilized on sepharose beads. The eluate contained about 5-10% of the total Usp4 - as detected by an antiserum directed against GFP – provided that Usp4 and the A_{2A}-receptor were coexpressed (Fig. 2B, *lane 3*). In contrast, no immunoreactive material was recovered from cells that did not express the A_{2A}-receptor (Fig. 2B, *lane 2*) or USP4-GFP (Fig. 2B, *lane 1*). These findings indicate that the A_{2A}-receptor and Usp4 do not only interact when expressed as fragments in yeast but that they are also capable of forming a complex when coexpressed as full length proteins in mammalian cells.

Both, the yeast two hybrid interaction assay and the coimmunoprecipitation from mammalian cell extracts, do not prove that the binding of Usp4 to the carboxyl terminus of the A_{2A}-receptor occur via a direct interaction. We have therefore also detected the physical association between the carboxyl terminus and USP4 by using a pull-down assay. Because a fusion protein comprising glutathione-S-transferase (GST) the carboxyl terminus of the A_{2A}-receptor only yielded insoluble inclusion bodies upon expression in E.coli, we fused the carboxyl terminus to the maltose binding protein (MBP) to obtain MBP-A_{2A}R-CT. As a control we employed the MBP-fusion protein

containing the carboxyl terminus of the A₁-adenosine receptor (MBP-A₁-R-CT). The MBP-fusion proteins were immobilized on amylose resin and subsequently incubated with immuno-purified FLAG-tagged Usp4. The beads were washed three times and the last wash was analyzed for the presence of Usp4 (Fig. 2C, upper blot, *lanes 1 and 2*, for suspensions containing MBP-A_{2A}R-CT and MBP-A₁R-CT). Usp4 was specifically recovered in the eluate (to about 30-50% of the input) of immobilized MBP-A_{2A}R-CT (Fig. 2C, upper blot, *lane 3*) but not of MBP-A₁R-CT (Fig. 2C, upper blot, *lane 4*). We verified that comparable amounts of MBP-fusion protein had been employed by immunoblotting with an antiserum directed against MBP (Fig. 2C, lower blot).

USP4 enhances the cell surface expression of the A_{2A}-receptor

As mentioned above, in living cells, a large portion of A_{2A}-adenosine receptors was visualized within the cell rather than at the plasma membrane (Fig. 3A). This was true irrespective of whether stably or transiently transfected cells were employed. If Usp4 was co-expressed, the A_{2A}-receptor level in the membrane increased at the expense of the intracellular fraction of the receptor (Fig. 3B). The following observations show that the effect of Usp4 was specific: (i) It was not mimicked by coexpression of another deubiquitinating enzyme, Usp14 (Fig. 3C). Usp14 is the functional homolog of yeast Ubp6p, associates with the 26S proteasome complex (Borodovsky et al., 2001; Chernova et al., 2003) and is apparently specific for monoubiquitinated substrates (Wilson et al., 2002). (ii) We employed another G protein-coupled receptor that has a high propensity to accumulate within the cell, namely the type-5 metabotropic glutamate receptor (mGluR5); CFP-tagged mGluR5 was predominantly found within the transfected cells, regardless of whether the receptor was expressed in the absence (Fig. 3D) or presence of Usp4 (Fig. 3E) or Usp14 (Fig. 3F). (iii) Based on the yeast two-hybrid screen (see Fig. 2A), Usp4 is not expected to affect the subcellular distribution of carboxyl terminally truncated versions of the A_{2A}-recep-

tor; this prediction has been verified: Usp4 did neither decrease the intracellular levels of the A_{2A}-receptor(1-360) (Fig. 3G,H) nor of the A_{2A}-receptor(1-311) (Fig. 3I,J). Finally, we tested the effect of combining expression of Usp4 and inhibition of proteosomal degradation. MG132 ought to relax quality control and thus allow the receptor to escape from the endoplasmic reticulum. Addition of the proteasome inhibitor MG132 augmented the amount of receptor at the cell surface (*cf.* Fig. 3K and Fig. 3A); in the presence of both, Usp4 and MG132, essentially all of the receptor was found at the cell surface (Fig. 3L).

In order to test whether endogenous levels of Usp4 sufficed to regulate cell surface expression of A_{2A}-receptors, we employed RNA-interference. We were not successful in reducing mRNA levels for Usp4 by RNA-interference in PC12 cells (three primers tested). When expressed at levels comparable to those seen in PC12 cells or in striatal membranes, the A_{2A}-receptor accumulates in large amounts within HEK293 cells (see Fig. 3A). Under these conditions, RNA-interference is unlikely to yield any detectable increment in intracellular accumulation. Therefore, we selected a HEK 293 cell line that expressed A_{2A}-receptors at low levels. In these cells the A_{2A}-receptor is predominantly found at the plasma membrane (Fig. 4B). Of several siRNAs, siRNA2 caused a significant reduction in the mRNA encoding Usp4 (Fig. 4A). In cells, in which Usp4 mRNA had been lowered by RNA-interference (*cf.* Fig. 4A), the A_{2A}-receptor appeared within the cell (Fig. 4C).

USP4 raises the cell surface level of A_{2A}-receptors in hippocampal neurons

The intracellular accumulation of the A_{2A}-receptor in HEK293 cells may arise from the fact that these cells do not endogenously express the receptor and are more likely to retain the receptor. USP4 is abundantly expressed in hippocampus, striatum and PC12 cells (Fig. 4D). We therefore

expressed the CFP-tagged A_{2A}-receptor in hippocampal neurons, where the receptor is endogenously expressed (Rosin et al. 1998), and visualized the distribution of receptors in living cells in the absence and presence of co-expressed GFP-tagged Usp4. In the absence of Usp4, the A_{2A}-receptor was mainly found within the soma (Fig. 5A). Coexpression of Usp4 (visualized in Fig. 5C) increased the level of the fluorescence at the cell surface (Fig. 5B). In contrast, the presence of Usp4 (visualized in Fig. 5F) did not enhance the cell surface level of the truncated version A_{2A}-receptor(1-311), which was predominantly detected within the somata of the neurons (cf. Fig. 5D and 5E). Finally, we also visualized CFP-tagged mGluR5 which was found within the neuronal soma (Fig. 5G); the coexpression of Usp4 (visualized in Fig. 5I) did not lead to the appearance of mGluR5 at the cell surface (Fig. 5H).

Coexpression of Usp4 results in the accumulation of deubiquitinated A_{2A}-receptor

In order to show that Usp4 utilized the A_{2A}-receptor as substrate, we transiently cotransfected HEK293 cells with plasmids encoding the Flag-tagged A_{2A}-receptor, HA-tagged ubiquitin and GFP-tagged Usp4. The A_{2A}-receptor was immunoprecipitated with anti-Flag antibodies from detergent lysates of cells that either coexpressed only HA-tagged ubiquitin (Fig. 6A, lanes 1,2) or the combination of HA-tagged ubiquitin and Usp4 (Fig. 6B, lanes 4,5): Receptor bands were detected with anti-Flag antibody (blots shown on top); in the absence of Usp4, the FLAG-reactive immunostaining was seen in the range of ~48-50 kDa (Fig. 6A top, lanes 1,2); in the presence of Usp4, the FLAG-tagged receptor migrated at ~40-42 kDa (Fig. 6B top, lanes 1,2). Lanes 3 and 6 represent the negative controls, that is immunoprecipitation was carried out with cellular lysates that lacked the A_{2A}-adenosine receptor but contained HA-tagged ubiquitin and – in lane 6 - Usp4. Regardless of the conditions, immunoreactivity was neither recovered in the ~40-42 kDa nor in the ~48-50 kDa range. Thus, the immunostaining was specific. The nitrocellulose membranes

were stripped and stained with anti-HA antibodies (Fig. 6A&B, bottom blots). In cells cotransfected with the plasmids encoding the Flag tagged A_{2A}-receptor and HA-tagged ubiquitin, the HA-antibody stained a ~48-50 kDa band. This corresponded to the ubiquitinated form of the A_{2A}-receptor, because this band was also stained with the anti-HA antibody (cf. Fig. 6A top and bottom blots). In contrast, when coexpressed with Usp4, the A_{2A}-receptor, which migrated as a band of 40-42 kDa (Fig. 5B, top, lanes 4&5), was not detected with the anti-HA antibody (Fig. 6B, bottom, lanes 4&5). This band therefore represents the deubiquitinated species of the receptor.

Coexpression of USP4 increases the surface expression of A_{2A}-receptors

As documented in Fig. 3, Usp4 caused a redistribution of the CFP-tagged A_{2A}-receptor to the cell surface. Two scenarios can be envisaged: Usp4 enhances ER-export of the *de novo* synthesized A_{2A}-receptor. Alternatively, the A_{2A}-receptor may undergo rapid constitutive endocytosis such that – even in the absence of agonist – a high proportion of the receptor is found within the cell at steady state. By contrast with newly synthesized receptors which are either en route to the Golgi or to ER-associated degradation, recycling receptors have already passed through the Golgi; thus, they ought to be fully glycosylated. As can be seen from Fig. 6C and D, the bulk of the A_{2A}-receptor that was recovered from cellular lysates was cleaved by endoglycosidase H (Fig. 6C & D, *cf.* first and second left hand lanes and set of bars respectively). However, in cells that coexpressed Usp4, the A_{2A}-receptor became resistant to deglycosylation by endoglycosidase H (Fig. 6C & D, *cf.* third and fourth lanes and set of bars respectively). Thus, the presence of Usp4 allows the A_{2A}-receptor to escape the endoplasmic reticulum and rapidly traffic through the Golgi to the plasma membrane.

It is conceivable that relaxing quality control by coexpressing Usp4 allowed unfolded receptors to

escape from the endoplasmic reticulum. In order to rule out this possibility, binding assays were performed with [³H]ZM241385, a specific and selective A_{2A}-receptor antagonist (Palmer et al., 1995). Fig. 7A shows a set of representative saturation curves for specific binding of [³H]ZM241385 to membranes from HEK293 cells that were either solely transfected with a plasmid driving the expression of (either the CFP or the FLAG-tagged) A_{2A}-receptor or of the receptor and Usp4. The coexpression of Usp4 (Fig. 7A, triangles) increased B_{max} but did not affect the affinity of the radioligand. K_D-values were for the wild type A_{2A}-receptor 1.97±1.2 and 2.12±0.96 nM (n=7), for A_{2A}-receptor(1-360) 2.17±1.16 and 1.84±0.57 nM (n=5), for A_{2A}-receptor(1-311) 2.70±1.157 and 3.26±1.51 nM (n=4), in the absence and presence of USP4, respectively. The effect of Usp4 depended on the carboxyl terminus of the A_{2A}-receptor, for it was not seen with the truncated forms A_{2A}-receptor-(1-311) or A_{2A}-receptor(1-360): representative saturation curves are shown in Fig. 7B; B_{max} averaged from several saturation experiments are shown in the bar diagram in Fig. 7C.

The model of quality control in the endoplasmic reticulum predicts that all steps are reversible provided that the carboxyl terminus of the membrane protein has not yet been engulfed by the proteasome (Kostova and Wolf, 2003). Accordingly the action of Usp4 and of proteasome inhibition is expected to be additive. This was the case. As can be seen from the average B_{max}-values summarized in Fig. 7C., sole addition of MG132 caused a pronounced increase in the amount of A_{2A}-receptor binding sites. This was associated with a substantial increase in ubiquitinated receptor (inset in Fig. 7C). The combined presence of both, Usp4 and MG132 resulted in an even more dramatic increase in the number of receptors.

Coexpression of USP4 enhances the A_{2A}-receptor-mediated cAMP accumulation

The A_{2A}-receptor is a prototypical G_s-coupled receptor (Klinger et al., 2002b); thus activation of the receptor leads to stimulation of adenylyl cyclase. The binding data showed that coexpression of Usp4 increased the number of cell surface binding sites. We verified that this translated into an increase in functional receptors by measuring agonist-induced cellular cAMP accumulation. In cells that expressed Usp4 (triangles in Fig. 8A), the agonist CGS21680 elicited a larger maximum effect than in cells that only expressed the A_{2A}-adenosine receptor (circles in Fig. 8A). We stress that this is not a non-specific effect that can, for instance, be accounted for by an increased responsiveness of the catalytic moiety of adenylyl cyclase in the presence of Usp4. Control experiments revealed that cells expressing solely the A_{2A}-receptor or the A_{2A}-receptor and Usp4 did not differ in their responsiveness to forskolin (Fig. 8B). When expressed in HEK293 cells, the A_{2A}-receptor displays some appreciable constitutive activity, which is magnified in the presence of forskolin (Klinger et al., 2002a). Accordingly, the antagonist ZM241385 (1 μM) was included to eliminate any possible confounding effect, which may result from the fact that A_{2A}-receptor levels differ substantially in the absence and presence of coexpressed Usp4.

DISCUSSION

The family of deubiquitinating enzymes (acronym = DUB) is categorized into three subfamilies, the smaller ubiquitin C-terminal hydrolase (UCH) subfamily, the larger ubiquitin-specific processing protease (USP/UBP) subfamily and recently described subfamily of OTU-domain containing proteins. UCH enzymes are generally small, containing only a proteolytic core domain that functions to cleave small molecular weight adducts from the ubiquitin C-terminus. In contrast, USPs/UBPs – such as Usp4 - are physically much larger and contain substantial and divergent sequences beyond their catalytic core. The analysis of genomic sequences predicts the presence of more than 90 deubiquitinating enzymes in a given mammalian genome (Chung and Baek, 1999), the vast majority of which belong to the USP/UBP-family. In fact, cDNAs have recently been cloned that encode 22 additional members of the human USP-family (Quesada et al., 2004). While the catalytic activity has been tested using artificial substrates, very little is known about their physiological substrates and thus their physiological functions. In fact, the number of identified substrates is remarkably small: the largest list exists for *fat facets*/FAM/USP9X, which deubiquitinates the ras effector AF-6 (Taya et al., 1998), β -catenin (Taya et al., 1999), Liquid facets (a drosophila homolog of the mammalian endocytotic protein epsin, Chen et al., 2002) and Vasa (an RNA-helicase, Liu et al., 2003). The substrates of the mammalian enzymes USP7/HAUSP (herpes virus associated USP) and USP2a are p53 (Li et al., 2002) and fatty acid synthase (Gadner et al., 2004), respectively. Membrane-embedded receptors, in general, and G protein-coupled receptors, in particular, are subject to ubiquitination (Hicke, 1999, Wojcikiewicz, 2004). However, to the best of our knowledge, specific deubiquitination reactions have not yet been reported for transmembrane receptors: the A_{2A}-receptor is the first example of a G protein-coupled receptor which is shown to be a specific substrate for a deubiquitinating enzyme of the USP/UBP-family.

In many instances, deubiquinating enzymes accelerate proteasomal degradation by removing ubiquitin from proteasome-bound substrate or proteolytic intermediates thereof. Deubiquinating enzymes may thereby allow the proteasome to engulf the substrate more efficiently and to operate at the maximal rate. However, there is also precedent for an antagonism between proteasomal degradation and deubiquinating enzymes: the developmental defect, which is caused by *fat facet* mutants in the drosophila compound eye, can be suppressed by a reduction in proteasomal activity (Huang et al., 1995). Similarly, USP7/HAUSP prevents the degradation of p53 (Li et al., 2002). The effect of Usp4 on the A_{2A}-receptor is also consistent with a preproteasomal action of Usp4: Usp4 rescues the receptor from degradation and thereby enhances its cell surface levels. This conclusion is based on the following sets of observations: (i) Usp4 binds to the carboxyl terminus of the A_{2A}-receptor; the interaction requires the most distal portion of the receptor, *i.e.* the last 50 amino acids. (ii) When heterologously expressed, a substantial portion of the A_{2A}-receptor is detected as ubiquitinated protein. The coexpression of Usp4 and the A_{2A}-receptor allows for the accumulation of deubiquinated receptor; this is associated with enhanced levels of the A_{2A}-receptor at the cell surface, increased amounts of functional receptor that can bind ligand and an augmented biological response. (iii) As an additional control, we employed the metabotropic glutamate receptor-5 (mGluR5), a group I mGluR, because – like the A_{2A}-receptor - mGluR5 also has a propensity to accumulate within the cell. In addition, group I-metabotropic glutamate receptors are subject to modification by the seven-in-absentia homolog 1A (SIAH1), a ring domain containing E3 ligase. This modification accelerates their rate of degradation, while blockage of the proteasome prevents their degradation (Moriyoshi et al., 2004). Usp4 does not act on mGluR5. (iv) Finally, we employed another USP/UBP-family member, namely Usp14. The rationale for choosing Usp14 was as follows: murine Usp14 is encoded by the *ataxia* (ax)-gene, the mutated form of which is associated with ataxia in mice

(Wilson et al., 2002); the absence of the metabotropic glutamate receptor-1 (mGluR1), a G_q-coupled receptor, also causes ataxia in mice (Aiba et al., 1994). We thus surmised that Usp14 may act on group I mGluRs, which it did not. More importantly, Usp14 did not affect the subcellular distribution of the A_{2A}-receptor. Thus, raising deubiquinating activity in the cell did not per se suffice to enhance the accumulation of the A_{2A}-receptor at the cell surface.

Ubiquitination plays a prominent role in supporting endocytosis of membrane receptors (Hicke, 1999). A more recent survey of several GPCRs indicates that ubiquitination can either target the receptors during biosynthesis to proteasomal degradation or redirect them on the endocytotic pathway to the lysosome (Wojcikiewicz, 2004). Our experiments were not designed to address a possible role of Usp4 in regulating the rate of endocytosis of the A_{2A}-receptor and it is conceivable that Usp4 also affects the rate of receptor recycling. It is, however, unlikely that the effects observed in the present study are accounted for by a Usp4-induced inhibition of endocytosis. Firstly, endocytosed receptors are still capable of binding antagonist radioligands. In contrast, a large fraction of newly synthesized receptor in the endoplasmic reticulum fails to bind antagonist with appreciable affinity (Pankevych et al., 2003). Thus, the finding that Usp4 (and of the proteasome inhibitor) raised the level of antagonist binding is consistent with an action during synthesis and subsequent quality control in the ER. Secondly, coexpression of Usp4 affected the proportion of receptor that was resistant to the action of endoglycosidase H: in the absence of Usp4, the bulk of the receptor was sensitive to deglycosylation by endoglycosidase H and, thus, had not yet been delivered to the Golgi apparatus. In contrast, in the presence of Usp4 a substantial portion of the receptor was resistant to endoglycosidase H and had thus left the ER-compartment and trafficked through the Golgi. Finally, our experiments were done in the absence of agonist, *i.e.* under conditions where endocytosis of G protein-coupled receptors is not prominent.

Similar to other misfolded membrane proteins, incorrectly folded G protein-coupled receptors are retrotranslocated through the sec61 translocon and degraded by the proteasome (Petaja-Repo, 2001). The latter step is contingent on ubiquitination. From a teleological perspective, it appears most plausible to target the carboxyl terminus of a G protein-coupled receptor for ubiquitination. This allows for cotranslational quality control because the accumulation of misfolded intermediates can be sensed and trigger the ubiquitination of the carboxyl terminus that is still tethered to the Sec61 channel. Point mutations are known to render G protein-coupled receptors more prone to misfold. Mutations in the V₂-vasopressin receptor, which lead to *diabetes insipidus*, are among the most prominent examples (Oksche and Rosenthal, 1998). Attempts to rescue these mutant receptors have been originally inspired by non-specific manipulations that were successful in CFTR, including DMSO, glycerol and other polyols, trimethylamine-*N*-oxide and phenylbutyrate (Gelman and Kopita, 2002). G protein-coupled receptors, however, have the advantage that folding may be aided by receptor ligands provided that they are membrane permeable (Bernier et al., 2004). The current observations suggest another mechanism that may represent a viable alternative, namely to target the deubiquinating enzyme that can recognize the receptor as a substrate. As shown here for the A_{2A}-receptor, this approach is - in principle - also capable of rescuing a receptor from ER-associated degradation and of thereby increasing its cell surface level. We assume that the current observations are of relevance for G protein-coupled receptors, in general, and for other membrane proteins that are subject to stringent quality control in the endoplasmic reticulum.

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FIGURE LEGENDS:

Fig. 1. **Proteasome inhibition increases expression of the A_{2A}-receptor.** *A*, Saturation hyperbolae for specific binding of [³H]ZM241385 to membranes from PC12 cells (50 μg/assay) endogenously expressing the A_{2A}-receptor. Membranes prepared from PC12 cells, which were kept in the presence or in the absence of 50 μM MG132 or 100 μM chloroquine for 3 h, were incubated in buffer containing the indicated concentrations of [³H]ZM241385 in the presence of 100 μM GTPγS. *B*, Saturation hyperbolae for specific binding of [³H]ZM241385 to membranes from transiently transfected HEK293 cells. Membranes (50 μg/assay) were prepared from HEK293 cells that expressed the full-length A_{2A} receptor and the truncated version A_{2A}-receptor(1-311) [A_{2A}R(1-311)] and that were maintained in the presence or in the absence of MG132 for 3 h. These membranes (50 μg/assay) were incubated in buffer containing the indicated concentrations of [³H]ZM241385 in the presence of 100 μM GTPγS. Data are means from duplicate determinations in a representative experiment which was repeated three times.

Fig. 2. **The A_{2A}-receptor interacts with Usp4 *in vitro*.** *A*, The yeast indicator strain pSH18-34 was co-transformed with various combinations of bait and prey vectors. The left semicircle denotes as a mirror image the individual conditions: Streaks 1 and 2, a constitutive and a galactose-inducible control, respectively, *i.e.* yeast cells transformed with a plasmid encoding the lexA binding domain fused to the transactivation domain and with plasmids encoding the regulatory Bα protein phosphatase 2A fused to lexA and Aα subunit of protein phosphatase fused to the transactivation domain (Yeong et al., 2003), respectively. Streaks 3 and 4, pEG202-A_{2A}R_{ct} and pJG45-Usp4; streaks 5 and 6, pEG202-A_{2A}R_{ct}(291-311); streaks 7 and 8, pJG45-Usp4; pEG202-A_{2A}R_{ct}(291-360) and pJG45-Usp4. All transformants were grown on selection medium and β-galactosidase assays were performed with these yeast colonies. Note that the yeast

expressing wild type and truncated A_{2A}-receptor C-termini were streaked in duplicate. *B*, Coimmunoprecipitation of the A_{2A}-receptor and Usp4: Flag-tagged A_{2A}-receptor (A_{2A}R-Flag) and GFP-tagged Usp4 (Usp4-GFP) were transiently expressed either individually (lanes 1 and 2, respectively) or co-expressed (lane 3) in HEK293 cells. Cellular lysates were subjected to immunoprecipitation with the anti-Flag M2 antibody (*lane 3*). Lane 4 shows the cellular lysate prepared from cotransfected HEK293 cells (=input to reaction in lane 3). Immunoblotting was done with an anti-GFP antibody. *C*, MBP-pull down assay. Purified fusion protein (3 μg/reaction) comprising the C-terminus of the A_{2A}-receptor (MBP-A_{2A}R_{ct}, *lanes 1 and 3*) or of the A₁-receptor (MBP-A₁R_{ct}, *lanes 2 and 4*) were incubated 30 μl of amylose beads (50% slurry); purified Flag-tagged Usp4 (0.3 μg) was added and the mixtures were incubated for 3 h at 4 °C. The slurry was washed and the last wash was applied onto the gel (*lanes 1&2*; devoid of MBP in the bottom panel), SDS sample buffer was used to release the proteins bound to the beads (*lanes 3 and 4*; containing MBP in the bottom panel). Immunoreactive bands were detected with the anti-Flag antibody (top) or with an antiserum directed against MBP (bottom). The experiments were carried out three times with similar results.

Fig. 3. Cell surface of the A_{2A}-receptor after coexpression of Usp4 or of Usp14 in HEK293 cells. HEK293 cells were transiently transfected with plasmids encoding the following sets of proteins: CFP-tagged A_{2A}R (A, K); CFP-tagged A_{2A}R and GFP-tagged Usp4 (B, L); CFP-tagged A_{2A}R and GFP-tagged Usp14 (C); CFP-tagged mGluR5 (D); CFP-tagged mGluR5 and Usp4 (E); CFP-tagged mGluR5 and Usp14 (F); CFP-tagged (A_{2A}R_{ct}(1-360) (G); CFP-tagged (A_{2A}R_{ct} (1-360) and GFP-tagged Usp4 (H); CFP-tagged (A_{2A}R_{ct} (1-311) (I); CFP-tagged (A_{2A}R_{ct} (1-311) and GFP-tagged Usp4 (J). Cells were incubated in the presence of MG132 (50 μM) for 3 h (K, L). Images were captured 24 h later with the appropriate filter settings. The bar diagram summarizes

the data from three independent experiments: cells were photographed as in Panels A-L. Prints were assessed by a blinded observer who scored the number of cells indicated on the y-axis for predominant expression of the receptor at the plasma membrane (black bar; M) or within the cell (white bar; C); data are from three separate transfections each.

Fig. 4. siRNA-mediated knockdown of Usp4 mRNA in HEK293 cells (A-C) and detection of Usp4 mRNA in hippocampus, striatum and PC12 cells (D). A, HEK293 cells (3×10^5 cells) stably expressing low levels of YFP-tagged A_{2A} -receptors were transiently transfected with lipofectamine 2000 (lane 0), the combination of lipofectamine 2000 and 500 pmol each siRNA directed against lamin (= irrelevant siRNA, lane Lam), siRNA1 (directed against Usp4, but inactive) and siRNA2. After 48 hours, cells were lysed and the level of mRNA coding for Usp4 examined by reverse transcription followed by polymerase chain reaction (RT-PCR; Panel A). The right most lane is the marker; the approximate size of the amplicon was 500 bp. In parallel, sham transfected cells (Panel B) and cells treated with siRNA2 were examined by fluorescence microscopy (C). D, reverse transcription followed by polymerase chain reaction (RT-PCR) to document the expression of Usp4 in hippocampus (hip) striatum (str) and PC12 cells. Data are representative for at least three separate transfections.

Fig. 5. Expression of the A_{2A} -receptor or of mGluR5 in the absence and presence of Usp4 in cultured hippocampal neurons. Neurons were prepared from rat neonatal hippocampus and cultured as outlined under Experimental Procedures, transfected using LipofectAMINE PlusTM with CFP-tagged A_{2A} -receptor (A_{2A} R-CFP; A), A_{2A} R-CFP and GFP-tagged Usp4 (Usp4-GFP) (B&C); A_{2A} R(1-311)-CFP (D), A_{2A} R(1-311)-CFP and Usp4-GFP (E&F); mGluR5-CFP (G), mGluR5-CFP and Usp4 (H&I). All images were captured 24 h later with the appropriate filter

settings, that is CFP filter settings in panels A, B, D, E, G and H (excitation at 430 nm, emission at 476 nm) and YFP-filter settings in panels C, F & I were taken using (excitation at 510 nm; emission at 530 nm) to document the expression of Usp4-GFP in the neurons shown in panels B, E and I.

Fig. 6. The A_{2A}-receptor is deubiquitinated by Usp4 *in vitro* and insensitive to cleavage by Endo-β-N-glucosaminidase H when coexpressed with Usp4. (A&B), Immunoprecipitation of A_{2a}R from HEK293 cells, transiently transfected with next sets of plasmids: Flag-tagged A_{2a}R, HA-tagged ubiquitin (*lines 1, 2*); Flag-tagged A_{2a}R, HA-tagged ubiquitin and GFP-tagged Usp4 (*lines 4,5*); GFP-tagged Usp4 and/or HA-tagged ubiquitin (*lines 6, 3*). Cells were collected 48 h after transfection and membrane preparation, immunoprecipitation were made as described in *Methods*. After the transfer membranes with proteins were stained with anti-Flag antibody (1:500 dilution) to reveal A_{2a} receptor (upper panel), than stripped for 30 min at 50° C and incubated with anti-HA antibody to stain ubiquitin (lower panel). Data are from a representative experiment that was reproduced 3 times. *C*, Immunoblot of membranes from HEK 293 cells, transiently expressing A_{2a}R-Flag, Usp4-GFP and treated with Endo-β-N-glucosaminidase H (EndoH). Cells were collected 48 h after transfection, membranes prepared and extracts thereof subjected to EndoH treatment. After the transfer, immunoreactive bands were revealed with the anti-Flag antibody. Data are from a representative experiment that was reproduced 3 times. Images were scanned and processed with QuantiScan software. The average densities in arbitrary units (a.u.) are shown in *D* for these four independent experiments; error bars indicate s.e.m.

Fig. 7. Usp4 enhances the expression of A_{2A}-receptor binding sites. Saturation hyperbolae for

specific binding of [³H]ZM241385 to membranes from transiently transfected HEK 293 cells. Membranes prepared from HEK293 cells expressing the full-length A_{2A} receptor (A), and truncated versions A_{2a}R(1-360) and A_{2a}R(1-311) with or without Usp4 (B), in the presence or in the absence of MG132 were incubated in buffer containing the indicated concentrations of [³H]ZM241385 in the presence of 100 μM GTPγS. The B_{max} values from these binding experiments are summarized in Panel C. Results are means ± SD from 4 independent experiments that were carried out in parallel and done with duplicate determinations. Asterisk indicates a significant difference from the full length A_{2a}R at p= 0.001 (unpaired t-test). *Inset*: HEK 293 cells expressing YFP-tagged A_{2A}-receptor (same cell line as used in Fig. 4) were transfected with a plasmid driving the expression of HA-ubiquitin. On the next day, the cells were incubated for 3 h in the presence of 50 μM MG132, the receptor was immunoprecipitated by employing an anti-GFP antiserum. The amount of ubiquitinated receptor recovered in the immunoprecipitate was visualized by blotting with an antibody directed against the HA-epitope.

Fig. 8. Usp4 enhances A_{2A}-receptor-mediated cAMP accumulation. Stimulation of cAMP accumulation in transiently transfected HEK293 cells by the A_{2A}-agonist CGS21680 (A) or by 10 μM forskolin in the presence of the antagonist ZM241385 (1 μM). Cells expressing solely the full-length A_{2A} receptor (circles) and together with Usp4 (triangles) were seeded in 6-well dishes, the cellular adenine nucleotide pool was metabolically prelabelled for 16 h with [³H]adenine. After a preincubation of 30 min in fresh medium containing adenosine deaminase (2 U/ml), cAMP production was stimulated by the indicated concentrations of the A_{2A}-selective agonist CGS 21680 (A) or 10 μM forskolin in the presence of 1 μM ZM241385. Data are means ±SD from 4 (A) and 3 (B) independent experiments that were done in triplicate and carried out in parallel.

Figure 1

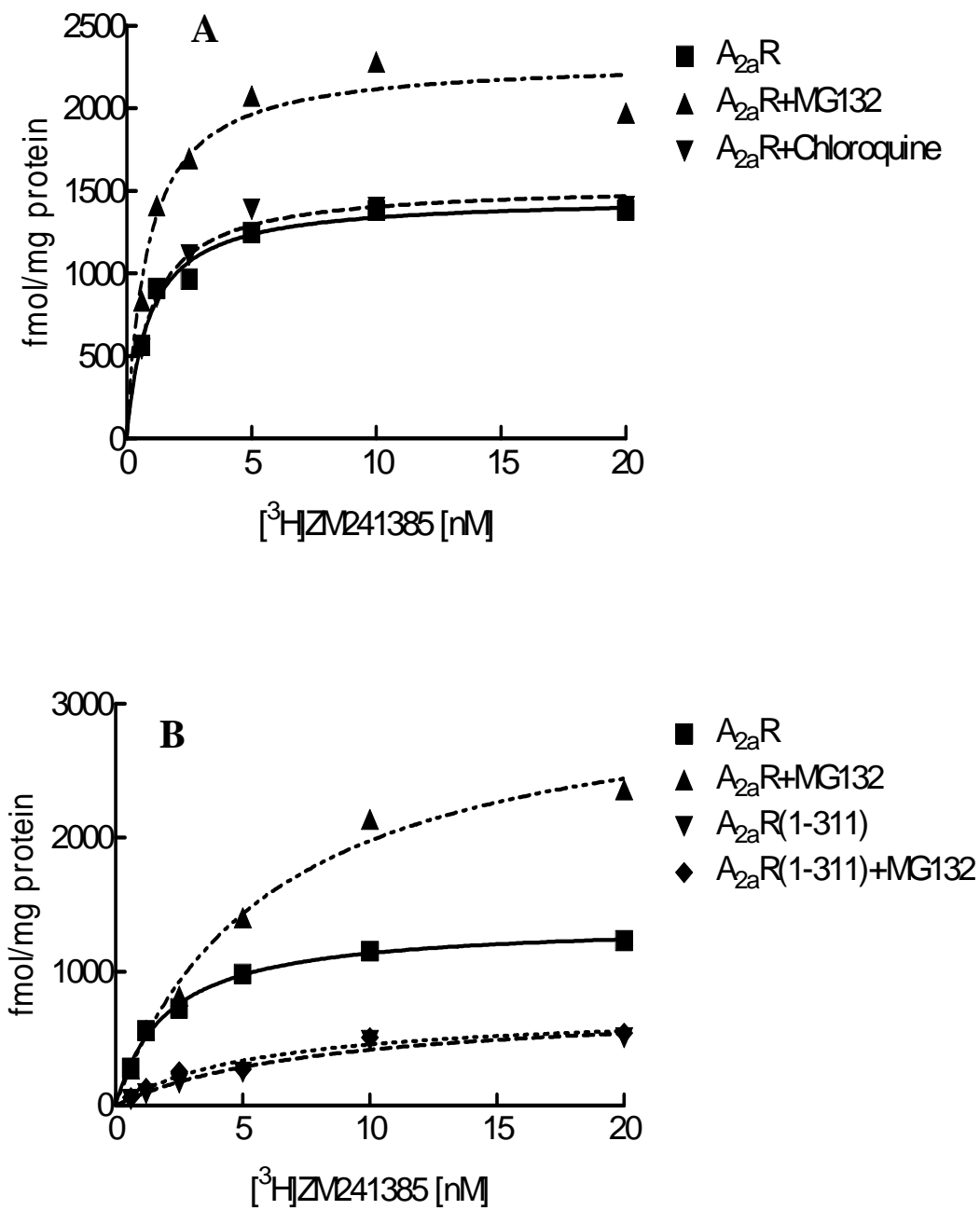


Figure 2

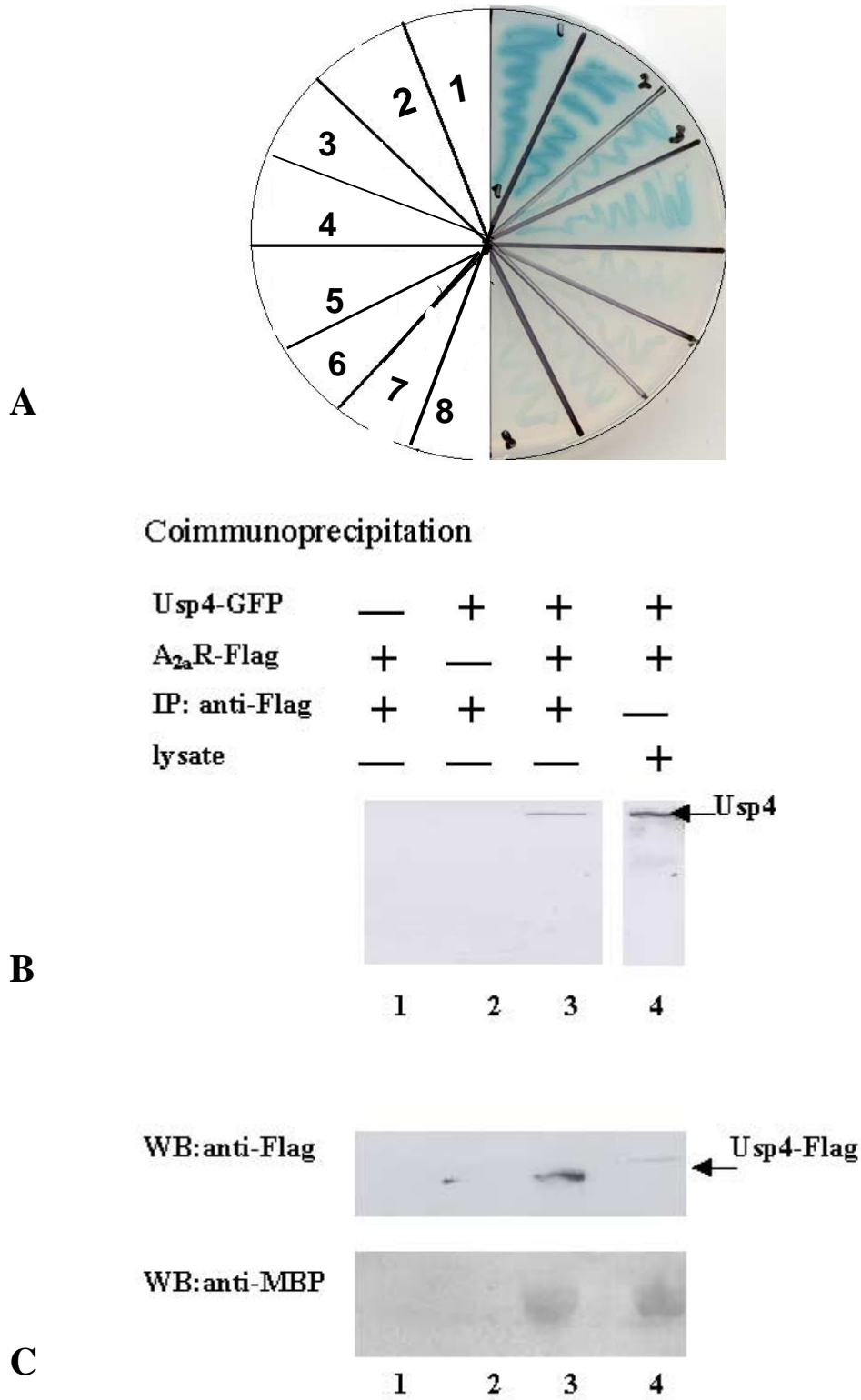


Figure 3

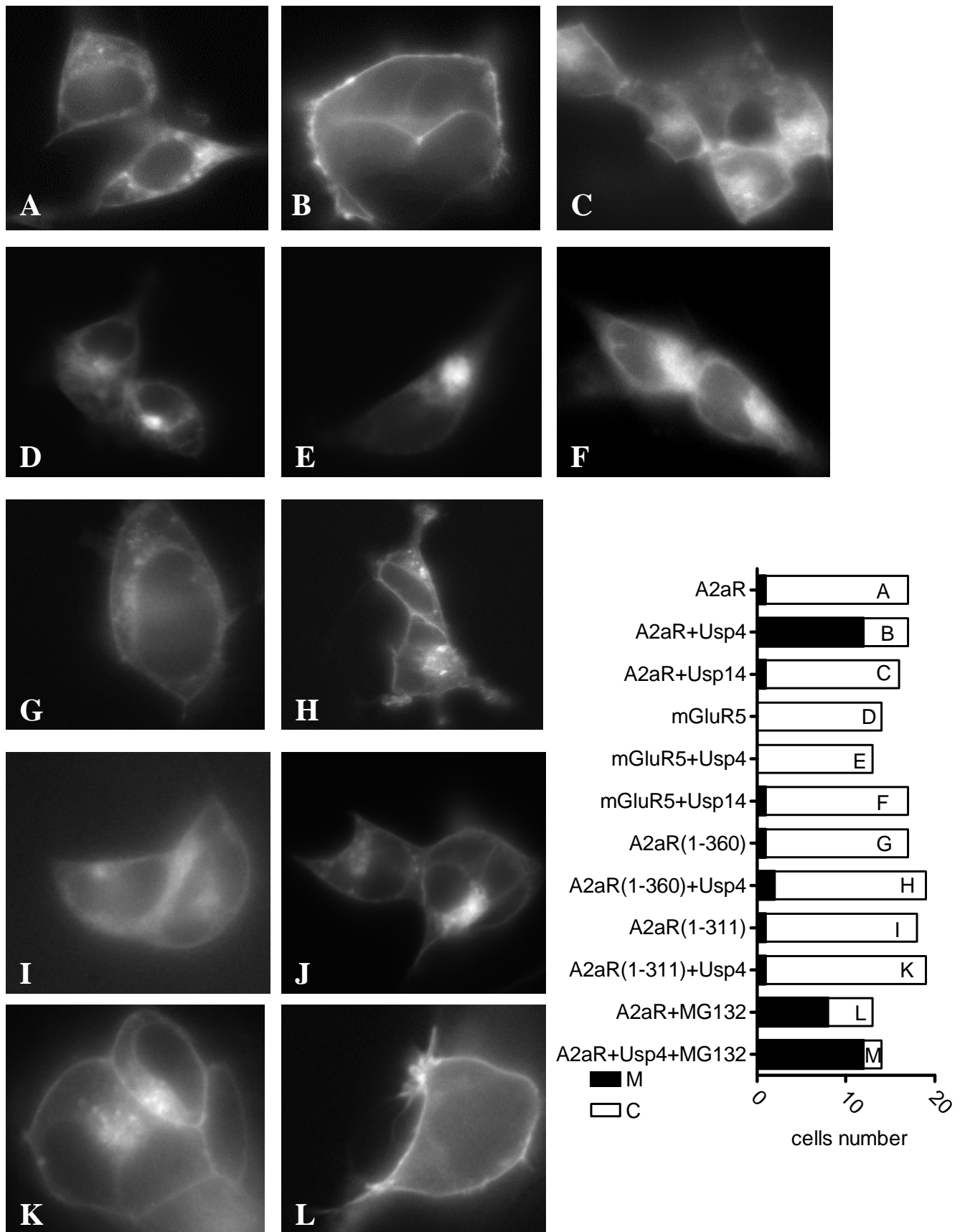


Figure 4

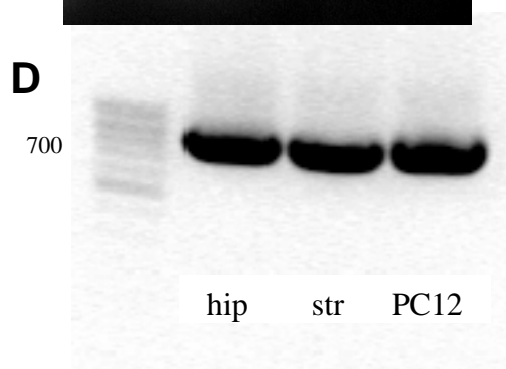
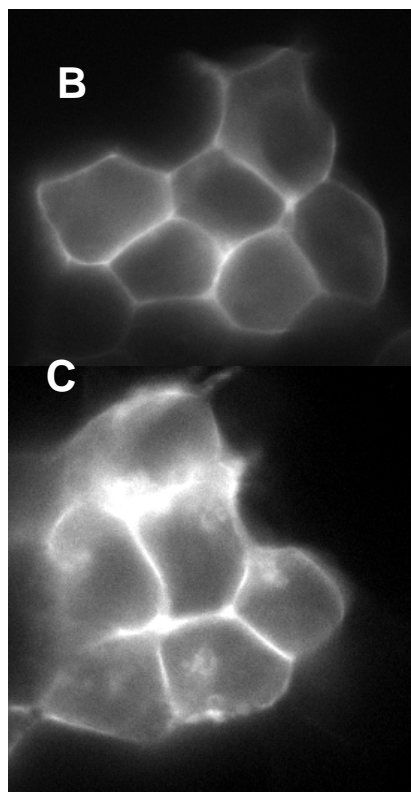
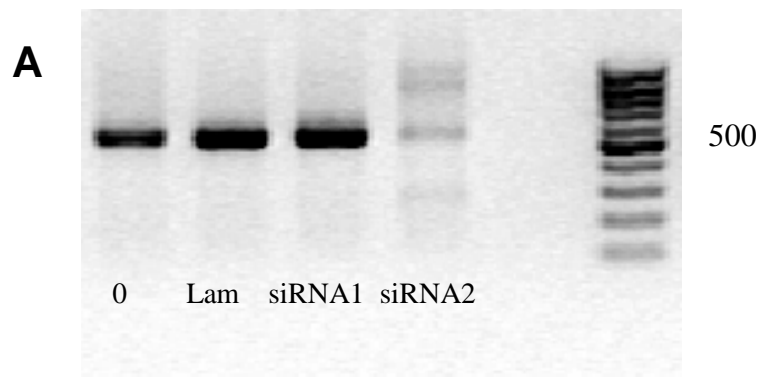


Figure 5

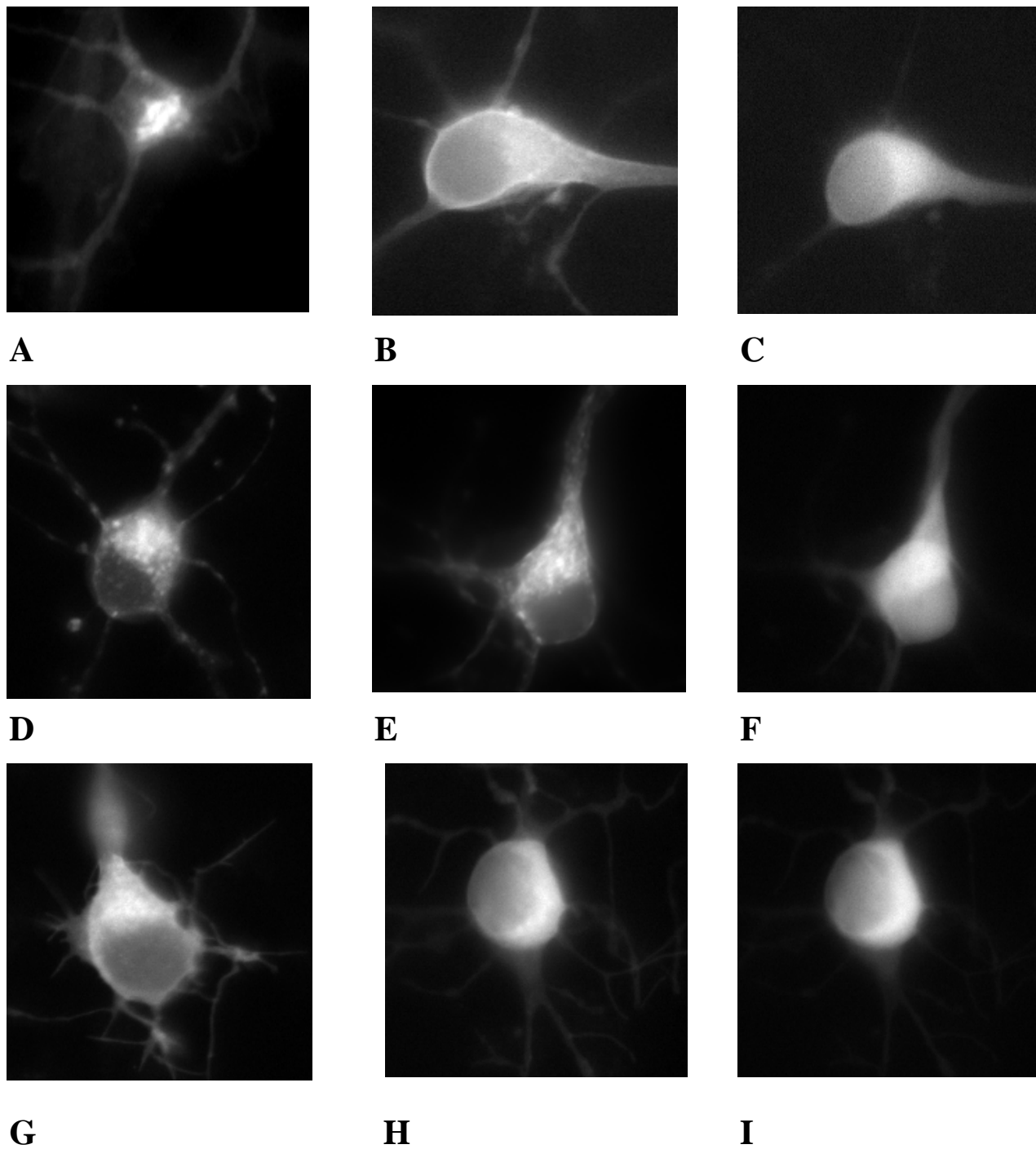


Figure 6

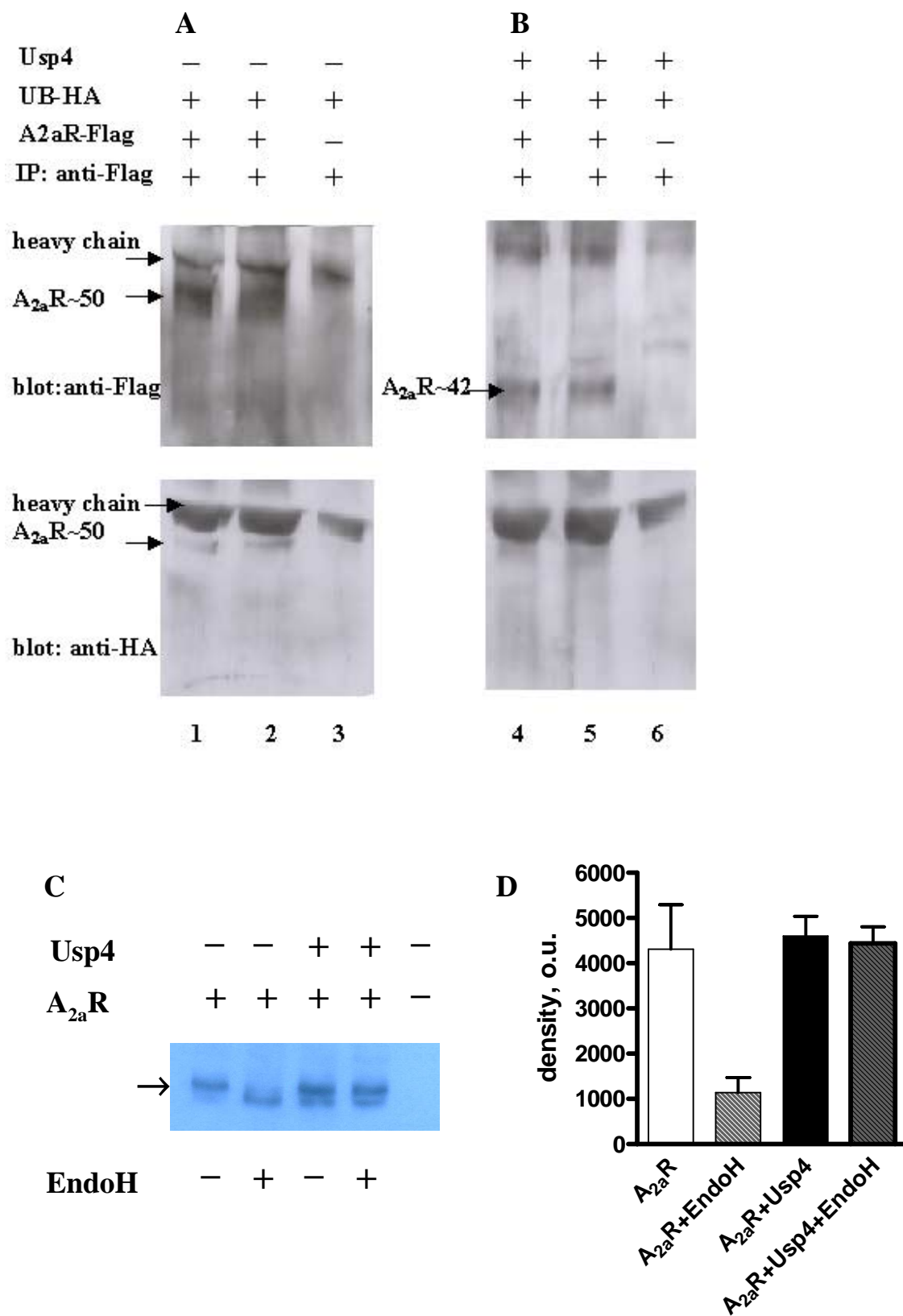


Figure 7

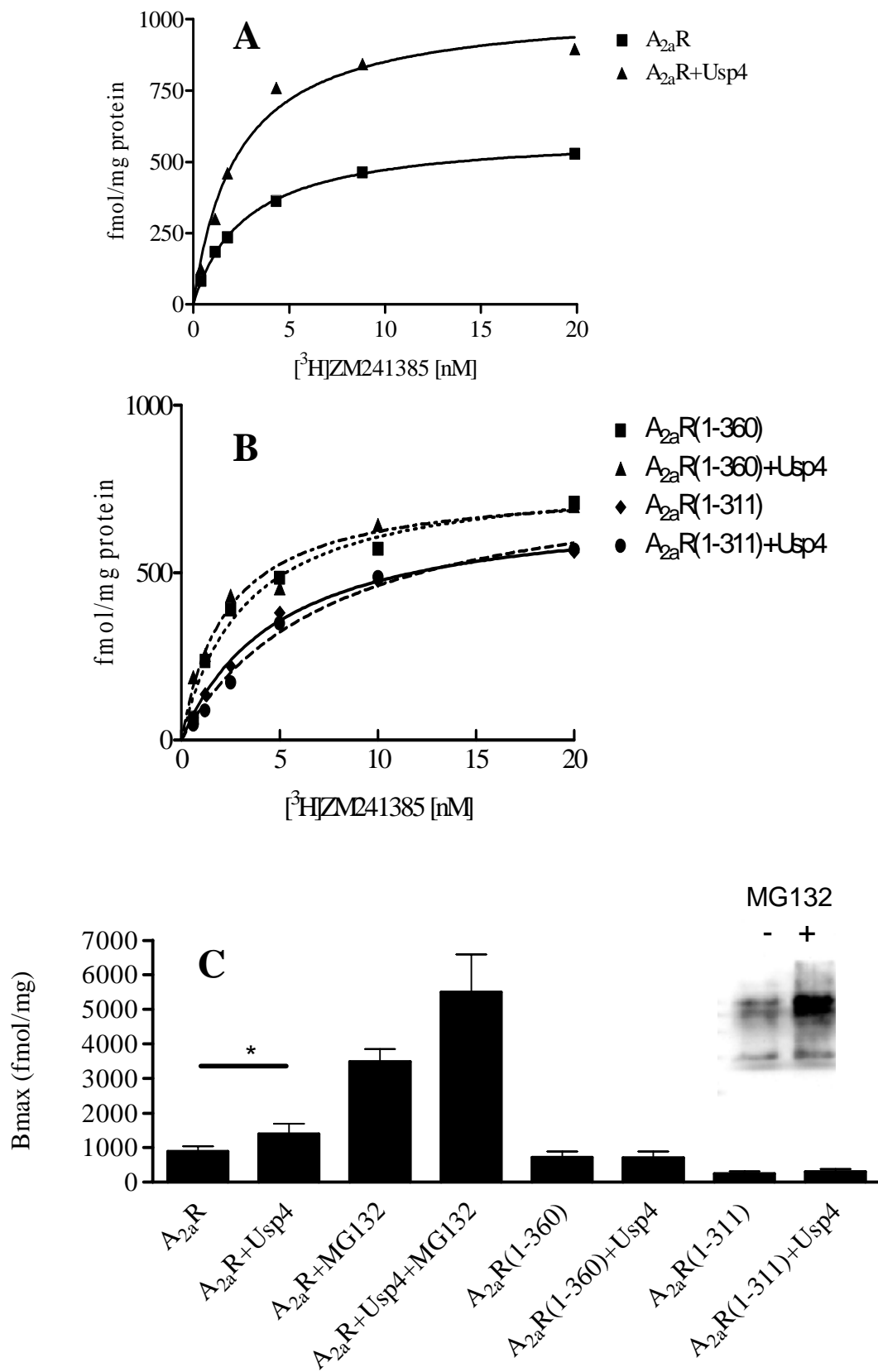


Figure 8

