Identification of the invariant chain (CD74) as an angiotensin AGTR1-interacting protein

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Abstract

Little is known about the protein–protein interactions that regulate the trafficking of the angiotensin II type I receptor (AGTR1) through the biosynthetic pathway. The membrane–proximal region of the cytoplasmic tail of the AGTR1 has been identified by site–directed mutagenesis studies as an essential site for normal AGTR1 folding and surface expression. Based on yeast two–hybrid screening of a human kidney cDNA library with the AGTR1 carboxyl-terminal tail as a bait, we identified the invariant chain (CD74) as a novel interacting protein. This association was confirmed by co-immunoprecipitation and co-localization studies. The binding site for CD74 on the AGTR1 carboxyl-terminal tail was localized to a site previously identified as important for the exit of the AGTR1 from the endoplasmic reticulum (ER), and conserved in many G protein–coupled receptors. Transient co-expression of CD74 with the AGTR1 in CHO-K1 cells consistently reduced the AGTR1 density at the cell surface. Furthermore, the interaction of CD74 with the carboxyl-terminal tail of the AGTR1 caused its retention in the ER and promoted its proteasomal degradation. These observations indicate that CD74 and the AGTR1 become associated in the early biosynthetic pathway, and that CD74 is a negative regulator of AGTR1 expression.


Introduction

The angiotensin II type I receptor (AGTR1) is the primary effector in the physiological actions of the octapeptide hormone, angiotensin II (AngII), on blood pressure regulation, and salt/water balance. The AGTR1 is also involved in the pathogenesis of renal diseases and several cardiovascular disorders, including hypertension, cardiac hypertrophy, and atherosclerosis, and has been implicated in the Marfan syndrome and cancer. In addition to its classical target tissues, local AngII formation has important physiological roles in a number of other cell types, including immune cells (Hunyady & Catt 2006). Binding of AngII to AGTR1s initiates conformational changes that lead to an activation of G proteins, predominantly Gq11, and several signaling pathways including stimulation of phospholipase C (PLC), Ca2+ signal generation, activation of protein kinase C isoforms and small G proteins, stimulation of receptor and non-receptor tyrosine kinases, and activation of mitogen–activated protein kinases (MAPKs; de Gasparo et al. 2000, Hunyady & Catt 2006). In parallel with these signaling events, AngII also causes internalization of the AGTR1 (Thomas 1999). These diverse signaling pathways, and the intracellular trafficking of the receptors, are modulated by a variety of protein–protein interactions.

The carboxyl-terminal tail of the AGTR1 is recognized as the main site of such interactions, and site–directed mutagenesis studies have identified motifs that are important in receptor signaling (Shibata et al. 1996, Oliveira et al. 2007), internalization (Hunyady et al. 1994), trafficking (Anborgh et al. 2000, Duvernay et al. 2004), and desensitization (Smith et al. 1998). However, there is a dearth of information about the direct protein–protein interactions of the carboxyl-terminal tail of the AGTR1 and the several proteins that bind to this region of the receptor. These include PLCγ (Venema et al. 1998), JAK2 (Ali et al. 1997), AGTR1–interacting proteins termed ATRAP (also known as AGTRAP; Daviet et al. 1999, Guo et al. 2003), as well as thimet oligopeptidase 1 (EP24.15; Shivakumar et al. 2005), GDP/GTP exchange factor–like protein (Guo et al. 2004), RAB5A (Seachrist et al. 2002), caveolin (Wyse et al. 2003), and β-arrestin (Qian et al. 2001). In searching for other interacting proteins with specific regulatory functions, we utilized yeast two–hybrid screening with the AGTR1 carboxyl–terminal tail as bait, and observed that the invariant chain (CD74) is a novel interacting protein for the AGTR1 carboxyl terminus.

CD74 is a type II transmembrane protein that is primarily expressed in antigen–presenting cells, and acts as a chaperone for the major histocompatibility complex (MHC) class II
molecules. Shortly after synthesis, CD74 forms trimers and associates with the MHC class IIz and β2-microglobulin in the ER, forming a nanomer complex. This association prevents peptide binding in the ER. The fully assembled MHC class II–CD74 complex exits the ER, transits the Golgi, and is directed to the endosomal–lysosomal compartments. There, CD74 is degraded and released from MHC class II molecules, which form dimers that bind the antigenic peptides and traffic to the cell surface for presentation to the T-lymphocytes (Lamb et al. 1991, Lamb & Cresswell 1992). In addition to its location in the ER and in lysosomes, 2–5% of cellular CD74 is expressed at the cell surface, where it acts as a high-affinity binding protein for the macrophage migration inhibitory factor (MIF; Leng et al. 2003). MIF binds to the extracellular domain of CD74 and is required for the activation of MAPK cascades and cell proliferation, as well as PTGER2 production (Lue et al. 2006). The CD74 protein is also expressed independently of MHC class II expression in a number of specific cell types (Momburg et al. 1986, Badve et al. 2002).

Our studies indicate that CD74 associates with the AGTR1 early in the biosynthetic pathway, and impedes its intracellular trafficking. Consequently, co-expression of CD74 causes AGTR1 accumulation in the ER, and its proteasomal degradation via the ER-associated pathway.

### Materials and Methods

#### Materials

The cDNA of the human AGTR1, used for yeast two-hybrid screen, was provided by Dr Bergsma (SmithKline Beecham Pharmaceuticals). The cDNA of the rat vascular smooth muscle AGTR1α-receptor, used for co-immunoprecipitation, binding, internalization assays, and confocal microscopy studies, was provided by Dr Bernstein (Emory University, Atlanta, GA, USA). The cDNA of the human CD74 was provided by Dr Eric O Long (NIAID, Bethesda, MD, USA). Anti-HA.11 monoclonal antibody was from Covance (Berkeley, CA, USA). Anti-Xpress monoclonal antibody was from Invitrogen, anti-calreticulin polyclonal antibody was from Abcam (Cambridge, MA, USA), horseradish peroxidase-conjugated goat anti-mouse antibody was from KPL (Gaithersburg, MD, USA), Alexa 594-conjugated goat anti-mouse IgG and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG were from Molecular Probes (Eugene, OR, USA), protease inhibitor cocktail set III and MG-132 were from Calbiochem (La Jolla, CA, USA), and leupeptin was from Bachem (T orrance, CA, USA). Unless otherwise stated, all other chemicals and reagents were from Sigma. Chinese hamster ovary (CHO)-K1 and human embryonic kidney (HEK) 293 cells were from ATCC (Manassas, VA, USA) and CHO-K1 Tet-On cells were from Clontech. Human adrenocortical carcinoma cell line, NCI-H295R, was from ATCC. pcDNA4/HisMax C plasmid was from Invitrogen, and pTRE-Tight and pEGFP-C3 plasmids were from Clontech.

#### Two-hybrid screen

The cDNA library screening was performed using the MATCHMAKER LexA Two-Hybrid System (Clontech). The mutant human AGTR1 carboxyl terminus (T332D, S335D, and T336D) bait used for the yeast two-hybrid screen contained amino acids 301–359 fused in-frame with the pLexA DNA-binding domain (BD) in vector pGilda. This region of AGTR1 encompassing the entire cytoplasmic carboxy-terminal segment was synthesized by PCR with restriction sites (EcoRI and XhoI) at 5' and 3' ends. The resulting construct pGilda-mhAGTR1 (C) was co-transformed into yeast EGY48 [p8op-lacZ] with a human kidney MATCHMAKER Lex A cDNA library (Clontech) that contained the pB42AD activation domain (AD). From over 9×10⁷ primary colonies screened, 46 putative positive colonies were identified. To eliminate duplicate colonies bearing the same AD/library plasmid, the insert-screening procedure was applied. Plasmids from 13 out of 46 Leu⁺, LacZ⁺ yeast transformant colonies were isolated and the AD/library inserts amplified by PCR. The PCR products were characterized by digesting with a frequent-cutter restriction enzyme, Hae III, and fragment sizes analyzed by agarose gel electrophoresis. One representative activating plasmid was rescued and the cDNA insert sequenced.

#### Plasmid constructs, mutagenesis, and transfection

The green fluorescent protein (GFP)-tagged AGTR1 (AGTR1-GFP) was constructed by linking the cDNA of enhanced green fluorescent protein (EGFP) carboxy terminally to the coding region of the AGTR1 as described earlier (Hunyady et al. 2002). The hemagglutinin epitope (HA)-tagged AGTR1 (HA-AGTR1) was amplified by PCR and subcloned into the pTRE-Tight expression vector. CD74 was amplified by PCR and subcloned into the pcDNA4/HisMax C expression vector. The CD74 N-terminal deletion mutants were constructed by PCR and subcloned into the pcDNA4/HisMax C vector. The sequences of the mutants were verified by sequencing. CHO-K1 cells were transiently transfected in 24-well plates with the indicated constructs using 3 µg/ml Lipofectamine (Carlsbad, CA, USA) as described previously (Hunyady et al. 1994). For confocal microscopy, cells were grown on glass coverslips and transfected with the indicated constructs using 3 µl/ml FuGENE 6 (Roche Diagnostics). CHO-K1 cells were maintained in NaHCO₃-buffered Ham’s F-12 medium containing 10% (v/v) fetal bovine serum, 100 µg/ml streptomycin, and 100 IU/ml penicillin. HEK 293 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (v/v) fetal bovine serum, 100 µg/ml streptomycin, and 100 IU/ml penicillin. Stably transfected HEK 293 cells were maintained in 200 µg/ml G418, which was removed before the commencement of experiments. H295R cells were cultured in a 1:1 mixture of DMEM:F-12, containing 10 ml/l percentin/streptomycin, 10 µg/ml aminoglycoside.
were washed twice with ice-cold PBS, and lysed in 0.5M NaOH, and 0.05% (w/v) SDS solution. The cell-associated radioactivity was measured by γ-spectrometry as previously described (Hunyady et al. 1994). The percentage of internalized ligand at each time point was calculated from the ratio of the acid-resistant specific binding to the total (acid-resistant + acid-released) specific binding.

Proteasomal and lysosomal inhibitor studies

For inhibitor studies, CHO-K1 cells were transiently transfected with CD74 and the HA-AGTR1 or the empty pcDNA4/HisMax C vector and the HA-AGTR1 as described above. Forty-eight hours after transfection, cells were treated with the indicated inhibitors (20 μM MG-132, 1 mM leupeptin, or 20 mM NH₄Cl) for 6 h before the binding assay was performed.

Western blot analysis

For immunodetection of expressed proteins, forty-eight hours after transfection, cells were scraped into 50 μl Laemmli buffer containing protease inhibitor cocktail. After sonication and centrifugation, the supernatant proteins were analyzed on 8–16% or 12% Tris–glycine gels and transferred to polyvinylidene difluoride membranes. Blots were then probed with primary antibodies and detected with horseradish peroxidase-conjugated secondary antibodies using the SuperSignal West Pico or Dura detection kits (Pierce). For western blot analysis of phospho-p44/42 MAPK, the Odyssey infrared imaging system was used. H295R cells cultured in 6-well plates (10⁶ cells/well) at 60% confluence were washed with serum-free medium and incubated overnight. After incubation, cells were washed with serum-free medium before treatment with AngII (100 nM) for the indicated times. Cells were then washed in ice-cold PBS and scraped into 100 μl Laemmli buffer. After sonication, centrifugation, and heating to 95 °C for 5 min, 35 μl of the extract sample was loaded onto 8–16% gel and western blot analysis was performed. Blots were blocked for 1 h in Odyssey blocking buffer (LI-COR Biosciences; Lincoln, NE, USA) and probed with phospho-p44/42 MAPK monoclonal antibody (Cell Signaling Technology; Danvers, MA, USA) and RPS6KA1 (Ribosomal S6 kinase 1) polyclonal antibody (Santa Cruz Biotechnology; Santa Cruz, CA, USA) overnight at 4 °C. After washing four times for 5 min at room temperature in PBS containing 0.1% Tween 20 with gentle shaking, blots were incubated with IRDye 680 Goat Anti-Rabbit IgG (LI-COR Biosciences) and IRDye 800CW Goat Anti-Mouse IgG (LI-COR Biosciences) in 1: 20 000 dilution for 1 h at room temperature. Immunofluorescence of phospho-MAPK and RPS6KA1 was analyzed in an Odyssey infrared imaging system (LI-COR Biosciences).

Confocal laser-scanning microscopy

CHO-K1 cells transiently transfected with the AGTR1-GFP or HEK 293 cells stably expressing the AGTR1-GFP were...
transiently transfected with either CD74 or deletion mutants of CD74 as described above, and were grown on glass coverslips. After 48 h, the cells were washed twice with PBS prior to fixation with 4% (w/v) paraformaldehyde. The cells were then incubated with sodium borohydride (1 mg/ml) for 15 min and permeabilized with 0.1% (v/v) Triton X-100 in PBS. Incubation with anti-HA antibody (1:100) for 1 h at room temperature was followed by two 15-min washes with 25 mM Tris–HCl (pH 7–4) containing 0.14 M NaCl, 2.7 mM KCl, 0.1% (v/v) Tween 20, and incubation for 1 h with Alexa 594–conjugated goat anti-mouse antibody (1:1000). The coverslips were mounted using Aquapolymount (Polysciences, Inc., Warrington, PA, USA) fluorescence mounting medium, and images were detected with a Zeiss LSM 510 confocal laser-scanning microscope. GFP or FITC and Alexa 594 were excited with argon (488 nm) and helium/neon (543 nm) lasers, and emitted fluorescence was detected in multi-track mode with 500–550 and 565–615 nm bandpass filters respectively. Image analysis was performed by using LSM Image Examiner. The value for fluorescence intensity ratio of AGTR1-GFP localized at the plasma membrane and in the cytosol was calculated by averaging the fluorescence intensity of pixels of defined plasma membrane and cytosolic areas (excluding the nucleus) and subtracting the non-specific background from outside the cell.

Results

Identification of CD74 as an interacting protein for the AGTR1 carboxyl-terminal tail in yeast two-hybrid assay

To identify novel proteins interacting with the human AGTR1, a human kidney cDNA library was screened using the AGTR1 carboxyl-terminal tail (amino acids 301–359) as a bait that was modified by replacing residues T332, S335, T336 to aspartic acid to mimic the phosphorylation state of the receptor. Screening of $9 \times 10^7$ transformants resulted in 13 independent clones that were putatively positive. One of the cDNAs isolated was 1200 bp long and encoded an open reading frame for 206 amino acids. Blast homology searches revealed 99% DNA sequence identity with the human invariant chain of the MHC, class II antigen associated (CD74).

Association of the AGTR1 and CD74 in transfected cells

In order to determine whether the AGTR1 interacts with CD74 in mammalian cells, HEK 293 cells stably expressing the HA-AGTR1 were transiently transfected with CD74. The cells were harvested, and the HA-AGTR1 was precipitated with μMACS anti-HA MicroBeads. The precipitates were analyzed by SDS-PAGE and CD74 was detected using antibody against its Xpress tag. As shown in Fig. 1A, co-immunoprecipitation of CD74 with the HA-AGTR1 was observed when the HA-AGTR1 was permanently expressed in the HEK 293 cells. Figure 1B shows the expression of HA-AGTR1 and CD74 in the cell lysates and arrows indicate the non-glycosylated and glycosylated forms of the HA-AGTR1 and the monomer and trimer forms of CD74 and its lysosomal degradation product. The data are representative of three similar experiments.

The intracellular localization of transiently expressed CD74 in HEK 293 cells stably expressing the AGTR1-GFP was analyzed by immunostaining. The AGTR1-GFP protein used for confocal microscopy studies has been previously shown to have similar signaling, trafficking, and phosphorylation characteristics in HEK 293 cells as the non-tagged or HA-tagged AGTR1, despite the relatively large GFP tag on its carboxyl terminus (Hunyady et al. 2002). Immunostaining of the Xpress epitope tag of CD74 revealed co-localization with the AGTR1-GFP in a region adjacent to the nucleus, consistent with that of the ER (Fig. 2A). Co-localization in the ER region was also observed in CHO-K1 cells transiently co-transfected with the AGTR1-GFP and CD74 (Fig. 2B, upper panels). In these cells, the localization of CD74 was confirmed by its co-localization with the ER marker protein, calreticulin (Fig. 2C).
CD74 interacts with the AGTR1  ·  M Szaszk, H-D Chen and others

Down-regulation of AGTR1 cell surface expression by CD74

To investigate the possible functional role of a direct interaction between CD74 and the AGTR1, cell surface expression of the HA-AGTR1 was determined. Analysis of the 125I-[Sar₁,Ile⁸]AngⅡ binding to CHO-K1 cells transiently co-expressing the HA-AGTR1 and CD74 revealed a significant reduction in cell surface expression of the HA-AGTR1 (Fig. 3A). To confirm that the changes in the 125I-[Sar₁,Ile⁸]AngⅡ binding are due to decreased expression of the AGTR1 at the cell surface, western blot analysis was performed on lysates of CHO-K1 cells transiently co-expressing the HA-AGTR1 and CD74. The cells were fixed and stained with anti-Xpress antibody and Alexa 594-conjugated secondary antibody to visualize CD74. The fluorescence of AGTR1-GFP is shown in green (left panel) and localization of Xpress-tagged CD74 is shown in red (middle panel). (C) CHO-K1 cells were transiently co-transfected with Xpress-tagged CD74 (left panel) and calreticulin antibody as an ER marker (left panel). Typical cells are shown from a representative example of three experiments with identical results.

**Figure 2** Co-localization of AGTR1-GFP and CD74 in the ER. (A) HEK 293 cells stably transfected with the AGTR1-GFP were transiently co-transfected with Xpress-tagged CD74. The cells were fixed and stained with anti-Xpress antibody and Alexa 594-conjugated secondary antibody to visualize CD74. The fluorescence of AGTR1-GFP is shown in green (left panel) and localization of Xpress-tagged CD74 is shown in red (middle panel). (B) CHO-K1 cells were transiently co-transfected with the AGTR1-GFP (left panel) and Xpress-tagged CD74. The cells were fixed and stained with anti-Xpress antibody to visualize CD74 (middle panel). (C) CHO-K1 cells were transiently co-transfected with Xpress-tagged CD74. The cells were fixed and stained with anti-Xpress antibody (middle panel) and calreticulin antibody as an ER marker (left panel).

**Figure 3** Cell surface expression of AGTR1 in CHO-K1 cells transiently co-transfected with CD74. (A) CHO-K1 cells were grown in 24-well plates and co-transfected with 0.5 μg HA-AGTR1 cDNA and 0.5 μg empty pcDNA4/HisMax C vector (control) or 0.5 μg HA-AGTR1 cDNA and 0.5 μg CD74. Cell surface expression levels of the AGTR1 were measured by 125I-[Sar₁,Ile⁸]AngⅡ binding. Data are shown as means ± S.E.M. of seven independent experiments, each performed in duplicate. ***P<0.001 versus control. (B) Representative immunoblot showing expression levels of HA-AGTR1 in cells co-transfected with the empty pcDNA4/HisMax C vector or CD74. Anti-HA antibody was used to detect the HA-AGTR1 level by western blot analysis. Lower arrow indicates the non-glycosylated and upper arrow indicates the glycosylated forms of the HA-AGTR1. The data are representative of three such experiments performed.

**Inducible AGTR1 expression in CHO-K1 Tet-On cells**

To determine whether the reduction of the surface expression of AGTR1 caused by CD74 expression occurs on the biosynthetic pathway or after the export of AGTR1 to the plasma membrane and its internalization to endosomal compartments, the Tet-On system was used for inducible, regulated AGTR1 expression. CHO-K1 Tet-On cells transiently transfected with the tetracycline-responsive plasmid containing the HA-AGTR1 were co-transfected with either CD74 or the empty vector cDNA. The cells were exposed to 1 μg/ml concentration of doxycycline for increasing time intervals, to control transcription of the HA-AGTR1. The cell surface receptor expression was determined by 125I-[Sar₁,Ile⁸]AngⅡ binding at each time point. The cell-bound radioactivity is shown in Fig. 4 as percentage of binding after 24 h of doxycycline induction. In the Tet-On system, the doxycycline-controlled expression of the HA-AGTR1 was markedly inhibited beginning from the earliest stage of receptor expression, when CD74 was co-expressed with the HA-AGTR1. No HA-AGTR1 was detected at the cell surface during the first 6 h of CD74 co-expression, indicating that down-regulation of the AGTR1 occurs in the biosynthetic pathway and not after its expression at the plasma membrane.

**CD74 does not influence internalization of the AGTR1 from the plasma membrane**

Although our microscopy studies did not show the presence of CD74 at the plasma membrane, 2–5% of CD74 was expressed at the cell surface in other reports (Sant et al. 1985, Wraith et al. 1990). This raised the possibility that an interaction between the AGTR1 and CD74 may occur at the plasma membrane, and that CD74 may increase the internalization of the AGTR1 and its trafficking to lysosomal compartments for degradation.
To further investigate the possibility that CD74 interacts with the AGTR1 at the plasma membrane, receptor internalization studies were performed. Transient co-expression of CD74 with the HA-AGTR1 had no effect on receptor internalization (Fig. 5), consistent with our results with the tetracycline-regulated AGTR1 expression system and our observation by immunostaining that the AGTR1 and CD74 are associated in the biosynthetic pathway.

**CD74 binding site in the membrane-proximal region of the AGTR1**

The CD74 binding region within the AGTR1 carboxyl-terminal tail was determined by studies on AGTR1 mutants.

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**The N-terminal region of CD74 is necessary for AGTR1 binding**

We sought to identify the AGTR1 binding site on CD74 by using a series of deletion constructs of CD74, and testing for changes in $^{125}$I-[Sar$^1$,Ile$^8$]AngII binding in cells co-expressing AGTR1 with the different truncated CD74 mutants. CD74 has a short cytoplasmic N-terminal tail containing two dileucine-based endosomal targeting motifs that mediate its interaction with the clathrin adaptor AP1 and AP2 proteins (Kongsvik et al. 2002), and its internalization from the plasma membrane to the trans-Golgi network (Hofmann et al. 1999). It also contains an arginine-based ER retention signal that maintains type II membrane proteins in the ER, and is followed by a single transmembrane domain and a long carboxyl-terminal extracytoplasmic region (Michelsen et al. 2005). This region of CD74 is required for class II assembly, whereas the 80–104 region (CLIP) mediates association with class II molecules, giving rise to nanomers, and the 153–183 region is involved in the formation of CD74 trimers (Bertolino & Rabourdin-Combe 1996). The schematic of the deletion mutants of CD74 used in the binding experiments is shown in Fig. 7A. The binding of $^{125}$I-[Sar$^1$,Ile$^8$]AngII to the cells transfected with the constructs with the deletion of residues 123 (D123-CD74) and 72 (D72-CD74), which remove the CLIP domain and transmembrane domain respectively, was

![Figure 5](image_url) Effect of overexpressed CD74 on doxycycline-induced expression of AGTR1 in CHO-K1 Tet-On cells. CHO-K1 Tet-On cells were grown in 24-well plates and co-transfected with 0.5 μg AGTR1 cDNA containing pTRE-Tight plasmid and 0.5 μg empty pcDNA4/HisMax C (● control) or 0.5 μg AGTR1 cDNA containing pTRE-Tight plasmid and 0.5 μg CD74 (■ CD74). Forty-eight hours after transfection, cells were incubated with 1 μg/ml doxycycline for the indicated times. Cell surface expression levels of the AGTR1 were measured by $^{125}$I-[Sar$^1$,Ile$^8$]AngII binding. Data are shown as means ± S.E.M. of three independent experiments, each performed in duplicates.

![Figure 6](image_url) Effect of overexpressed CD74 on the surface expression of carboxyl-terminal AGTR1 deletion mutants. Cell surface expression levels of wild-type (AGTR1) and deletion mutant AGTR1 (AGTR1Δ319) co-expressed with empty pcDNA4/HisMax C vector or CD74 were measured by $^{125}$I-[Sar$^1$,Ile$^8$]AngII binding in CHO-K1 cells. $^{125}$I-[Sar$^1$,Ile$^8$]AngII binding of AGTR1 was considered as control. Data are shown as means ± S.E.M. of three independent experiments, each performed in duplicate. **P<0.01 and ***P<0.001 versus AGTR1. + P<0.01 versus AGTR1Δ319.
unchanged (Fig. 7B). This indicates that these mutations abolish the interaction with the HA-AGTR1, suggesting that the region downstream of residue 72 is necessary for HA-AGTR1 association. Deletion of the N-terminal region, and removal of the two dileucine-based endosomal targeting signal, increases the level of CD74 at the plasma membrane (Nakatsu et al. 2000). Co-expressing the CD74 deletion mutant lacking the first 33 residues of the amino-terminal part decreased HA-AGTR1 binding (Fig. 7B). This indicates that this mutant still interacts with the HA-AGTR1 and that the HA-AGTR1 binding site on CD74 is located on its amino-terminal region between residues 34 and 71. The expression levels of CD74 mutants were determined by western blot analysis (Fig. 7C).

Co-localization studies by immunostaining of the Xpress epitope tag of the mutant CD74 proteins co-expressed with the AGTR1-GFP confirmed our conclusions from the binding studies (Fig. 7A). The Δ72-CD74 and Δ123-CD74 deletion mutants showed diffuse cytoplasmic localization and no co-localization with the AGTR1-GFP. However, the proximal amino-terminal deletion mutant CD74 (Δ33-CD74), which showed increased plasma membrane localization, still co-localized with the AGTR1-GFP in the ER region and aggregates were seen in the ER. For the semi-quantitative analysis of the AGTR1-GFP number at the plasma membrane in these cells, the fluorescence intensity ratio of AGTR1-GFP localized at the plasma membrane and in the cytosol was calculated (Fig. 8B). The fluorescence intensity of the AGTR1-GFP at the plasma membrane is significantly decreased in the cells co-expressing CD74 or Δ33-CD74 with the AGTR1-GFP which is in agreement with the binding data.

**AGTR1s in the presence of CD74 are targeted to the proteasomal degradation pathway**

To analyze the possible degradation pathways of the AGTR1 in cells expressing CD74, inhibitors of proteasomal and lysosomal degradation were used. Proteasomal activity was suppressed by addition of MG-132, a specific proteasome inhibitor. To analyze lysosomal contribution, we used both leupeptin, an inhibitor of cysteine proteases, and NH4Cl, a weak base that increases
lysosomal pH and thereby inhibits the proteolytic activity of lysosomal enzymes. Leupeptin inhibits the trafficking of MHC class II molecules and degradation of CD74 in the lysosome, causing accumulation of the MHC class II–CD74 complex therein and reduction of its cell surface expression (Loss & Sant 1993). Transiently transfected CHO-K1 cells were incubated in the absence or presence of inhibitors for 6 h, and HA-AGTR1 expression was determined by 125I-[Sar1,Ile8]AngII binding assay. Inhibition of proteasomal activity by MG-132 enhanced HA-AGTR1 accumulation at the cell surface when CD74 was co-expressed, compared with the expression of the receptor only. By contrast, the lysosomal inhibitor had no significant effect on the cell surface level of the HA-AGTR1 (Fig. 9). Collectively, these data suggest that degradation by the proteasome system limits AGTR1 expression when CD74 is co-expressed.

**Down-regulation of endogenous AGTR1 signaling by CD74**

The human adrenocortical cell line H295R expresses endogenous AGTR1 and secretes aldosterone in response to AngII. In these cells AngII activates p44/42 MAPK with a peak of 5 min lasting up to hours (Natarajan et al. 2002). To prove that CD74 is capable of interfering with the plasma membrane expression of

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**Figure 8** Subcellular localization of co-transfected CD74 mutants and AGTR1-GFP. (A) CHO-K1 cells were transiently co-transfected with the AGTR1-GFP and Xpress-tagged CD74 mutants. The cells were fixed and stained with anti-Xpress antibody. The fluorescence of AGTR1-GFP is shown in green (middle panels) and localization of Xpress-tagged CD74 mutants are shown in red (upper panels). Typical cells are shown from a representative example of three experiments with identical results. (B) Fluorescence intensity ratio of AGTR1-GFP localized at the plasma membrane and in the cytosol was calculated by averaging the fluorescence intensity of pixels of defined plasma membrane and cytosolic areas (excluding nucleus) and subtracting the non-specific background from outside the cell. Each value is means ± S.E.M. of at least four determinations in each experiment. ***P<0.001 versus control.
CD74 overexpression on the downstream signaling events of the HA-AGTR1 and 0.5 HA-AGTR1 binding of HA-AGTR1 in non-treated samples. Data are means ± S.E.M. of three independent experiments, each performed in duplicate. *** 0.001 versus control.

Figure 9 Effects of inhibitors of the proteasomal and lysosomal pathways on the surface expression of the AGTR1. CHO-K1 cells were grown in 24-well plates and co-transfected with 0.5 µg HA-AGTR1 and 0.5 µg empty pcDNA4/HisMax C vector or 0.5 µg HA-AGTR1 and 0.5 µg CD74. Forty-eight hours after transfection, the cells were incubated with 20 µM MG-132, 1 mM leupeptin, or 20 mM NH₄Cl, or were untreated for 6 h. Cell surface expression levels of the HA-AGTR1 were measured by 125I-[Sar¹,Ile⁸]AngII binding and are shown as percentage of 125I-[Sar¹,Ile⁸]AngII binding of HA-AGTR1 in non-treated samples. Data are means ± S.E.M. of seven independent experiments, each performed in duplicate. *P<0.05 versus control.

the endogenous AGTR1, H295 cells stably expressing CD74 were used for 125I-[Sar¹,Ile⁸]AngII binding and signaling pathway analysis. Stable overexpression of CD74 in H295R cells significantly decreased 125I-[Sar¹,Ile⁸]AngII binding compared with the cells overexpressing the empty pcDNA4/HisMax C vector (Fig. 10A). We next investigated the effect of CD74 overexpression on the downstream signaling events of the endogenous AGTR1 stimulation. Phosphorylation of the p44/42 MAPK was detected by western blotting with specific antibodies at several time points after stimulation of H295R cells with 100 nM AngII (Fig. 10B). The phospho-p44/42 MAPK signal was decreased when CD74 was stably expressed in H295R cells due to the reduced amount of AGTR1 on the cell surface. The signal of RPS6KA1 used as a control for equal protein loading was unaffected.

Discussion

The AngII-activated AGTR1 is phosphorylated by specific G protein-coupled receptor kinases (GRKs) at the plasma membrane (Oppermann et al. 1996, Thomas 1999, Hunyady et al. 2000, Olivares-Reyes et al. 2001). To mimic the phosphorylation state of the AGTR1, we replaced residues T332, S335, and T336 with aspartic acid and used this modified AGTR1 carboxyl-terminal tail in yeast two-hybrid studies to identify AGTR1-associated proteins. This revealed that the MHC class II chaperone, CD74, undergoes association with the carboxyl-terminal region of the AGTR1. However, our studies of co-localization, binding assay, and co-immunoprecipitation with the non-phosphorylated native AGTR1 indicate that the non-phosphorylated AGTR1 interacts with CD74. This is consistent with the location of the binding site for CD74 above these phosphorylation sites. These findings have demonstrated that interaction of CD74 with the AGTR1 occurs in the biosynthetic pathway before the receptor reaches the plasma membrane and prior to its phosphorylation. However, our studies do not exclude the possibility that the phosphorylation state of the AGTR1 may influence its interaction with CD74.

Although relatively little is known about the transport of G protein coupled receptors (GPCRs) to the cell surface, a crucial role for the membrane-proximal region of the carboxyl-terminal tail has been shown for several GPCRs. This corresponds to the putative helix 8, which is an α-helix with amphipathic properties. Several GPCRs lacking the carboxyl terminus are not expressed at the cell surface, suggesting the presence of an export motif in this domain and roles of chaperone proteins that bind to this region and regulate receptor trafficking in the biosynthetic pathway (Gaborik et al. 1998). The sequence F(X)₆L is highly conserved in the membrane-proximal carboxyl terminus of many GPCRs and functions as a common motif mediating receptor transport from the ER to the cell surface (Duvernay et al. 2004, Thielen et al. 2005). It is also necessary for receptor folding in the ER and to pass the quality control system. It is interesting that the site of CD74 binding to the AGTR1 is located in this region, which contains the F(X)₆L motif that has been previously found to be important in AGTR1 exit from the ER and folding (Gaborik et al. 1998), and is recognized as a caveolin-like binding motif (Leclerc et al. 2002). In the case of the dopamine D1-receptor, the membrane-associated EK protein, DRIP78 (also known as DNAJC14) was found to interact with this membrane-proximal region of the
receptor. Overexpression of DRIP78 led to retention of the D1 receptor in the ER (Bermak et al. 2001). Overexpression of DRIP78 with the AGTR1 increased the plasma membrane expression of the receptor (Leclerc et al. 2002). In several GPCRs, homo- or hetero-dimerization is a prerequisite for their exit from the ER, and their membrane expression. For example, the formation of heterodimers between GABBR1 (also known as GABRB1) and GABBR2 (also known as GABRB2) is required for functional expression. Also, 14-3-3 proteins were found to be associated with the GABBR1 carboxyl terminus, and to impede the interaction between the two receptor carboxyl termini (Couve et al. 2001). Olfactory GPCRs are inefficiently expressed at the plasma membrane of heterologous cells due, in part, to degradation of the ER-retained receptors by the ubiquitin–proteasome system (Lu et al. 2003). These findings suggest the necessity of endogenous chaperones for the expression of GPCRs at the plasma membrane.

Our data indicate that the AGTR1 co-expressed with CD74, like the carboxyl-terminal-deficient mutants and misfolded receptors, aggregates in the ER and undergoes degradation by the proteasomal system. The role of the proteasomal system in AGTR1 degradation during CD74 co-expression was demonstrated by the effect of its inhibition with MG-132. That co-immunoprecipitation could be performed only in stably transfected cells confirms the finding that CD74 directs the proteasomal degradation of the receptor. In transiently co-transfected cells, the receptor cannot reach a sufficiently high level of expression for successful co-immunoprecipitation when co-expressed with CD74.

The targeting of MHC class II molecules to the endocytic compartments, where they encounter processed antigen, and their exit from the ER, are determined by CD74. This trafficking pathway is mediated by the cytoplasmic tail of CD74, which contains two dileucine-based targeting motifs that are required for its lysosomal targeting and endocytosis from the plasma membrane (Kang et al. 1998). It also contains an arginine-based ER retention motif at the proximal N-terminus, which is conserved in type II membrane proteins (Michelsen et al. 2005). Phosphorylation of two serine residues in the neighborhood of the arginine-based signals regulates the exit of the MHC class II–CD74 complex from the ER (Anderson & Roche 1998, Kuwana et al. 1998). CD74 associates with 14-3-3 proteins in a phosphorylation-dependent manner, and this interaction results in shielding of the arginine-based signal. When the N-terminus is not phosphorylated, βCOP binds even in the presence of the 14-3-3β protein. Upon phosphorylation, 14-3-3β binds and βCOP association is suppressed, stimulating forward trafficking of the MHC class II–CD74 complex (O’Kelly et al. 2002). Inhibition of CD74 phosphorylation impairs the trafficking of newly synthesized MHC class II molecules to antigen-processing compartments. Protein kinase C is responsible for the constitutive phosphorylation of 50% of the total cellular pool of CD74. Stimulation of protein kinase C activity in antigen-presenting cells significantly enhances the kinetics of degradation of class II–associated CD74 in the antigen-processing compartments, and the binding of antigenic peptides to the class II molecules. In cells expressing a CD74-phosphorylation mutant, trafficking of class II molecules to endosomes is impaired and CD74 proteolysis is inhibited, consistent with a direct effect of CD74 phosphorylation on MHC class II trafficking (Anderson et al. 1999).

In our study, N-terminal truncation and removal of the two dileucine- and arginine-based targeting signals of CD74 (A33–CD74) reduced the surface expression of the AGTR1, indicating its binding to the receptor. Interestingly, although N-terminal-deleted CD74 still binds the AGTR1 in the ER, it has increased plasma membrane localization according to our confocal microscopy studies and as reported previously (Nakatsu et al. 2000).

The fraction of CD74 that is not associated with MHC class II molecules is transported to the plasma membrane and rapidly internalized with a half-life of 3–4 min (Roche et al. 1993). About 2–5% of the total CD74 is expressed at the plasma membrane, where it acts as a receptor for MIF and is required for its activation of MAPK (Leng et al. 2003). MHC class II molecules appear to reach the lysosomal compartment together with CD74 via the plasma membrane (McCormick et al. 2005). Nonetheless, our confocal microscopy experiments did not reveal CD74 expression and its co-localization with the AGTR1-GFP at the plasma membrane. In addition, CD74 is a member of the regulated intramembrane proteolysis-processed protein family. After intramembranous cleavage in the endocytic compartments, the cytosolic CD74 fragment is released and induces the activation of transcription mediated by the NFXBp65/RELA homodimer and the B-cell-enriched co-activator, TAF(2)105, by translocation to the nucleus (Becker-Herman et al. 2005).

In summary, we have identified CD74 as an interacting protein with the carboxyl-terminal domain of the AGTR1 in the biosynthetic pathway. Based on our results, CD74 appears to function as a negative regulator of AGTR1 expression at the cell surface. Because of the wide expression of CD74, dominant negative approaches could be helpful to elucidate the function of the endogenous CD74 protein in the expression of the AGTR1. Since CD74 can be expressed separately from MHC class II molecules in various tissues, and its expression is increased in inflammatory processes (Momburg et al. 1986), changes in CD74 expression may have a significant role in the regulation of AGTR1 expression at the cell surface under specific conditions. This could result from its ability to direct certain of the newly synthesized AGTR1 molecules from the ER to their degradation in the proteasome.

Declaration of interest

The authors have nothing to declare.

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