THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 30, Issue of July 29, pp. 27866–27871, 2005

Printed in U.S.A.

Cell Surface Expression of CD147/EMMPRIN Is Regulated by Cyclophilin 60*

Received for publication, April 7, 2005, and in revised form, April 29, 2005
Published, JBC Papers in Press, June 9, 2005, DOI 10.1074/jbc.M503770200

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CD147, also known as extracellular matrix metalloproteinase inducer, is a regulator of matrix metalloproteinase production and also serves as a signaling receptor for extracellular cyclophilins. Previously, we demonstrated that cell surface expression of CD147 is sensitive to cyclophilin-binding drug cyclosporin A, suggesting involvement of a cyclophilin in the regulation of intracellular transport of CD147. In this report, we identify this cyclophilin as cyclophilin 60 (Cyp60), a distinct member of the cyclophilin family of proteins. CD147 co-immunoprecipitated with Cyp60, and confocal immunofluorescent microscopy revealed intracellular colocalization of Cyp60 and CD147. This interaction with Cyp60 involved proline 211 of CD147, which was shown previously to be critical for interaction between CD147 and another cyclophilin, cyclophilin A, in solution. Mutation of this proline residue abrogated co-immunoprecipitation of CD147 and Cyp60 and reduced surface expression of CD147 on the plasma membrane. Suppression of Cyp60 expression using RNA interference had an effect similar to that of cyclosporin A: reduction of cell surface expression of CD147. These results suggest that Cyp60 plays an important role in the translocation of CD147 to the cell surface. Therefore, Cyp60 may present a novel target for therapeutic interventions in diseases where CD147 functions as a pathogenic factor, such as cancer, human immunodeficiency virus infection, or rheumatoid arthritis.

Cyclophilins are a family of proteins which share peptidyl-prolyl *cis-trans* isomerase (PPIase)[1] activity and serve as receptors for the immunosuppressive drug cyclosporin A (CsA). The PPIase activity of cyclophilins is the basis for their proposed role in protein folding (1). Another established activity of cyclophilins is regulation of protein trafficking in cells. Cyclophilin A has been shown to be a component of the transport complexes that regulate surface expression of asialoglycoprotein receptor expression in human cells (2). The *Drosophila* cyclophilin homolog, NinaA, participates in trafficking of rhodopsin, the most abundant subclass of photoreceptors (3–5). NinaA and its mammalian counterparts are retina-expressed integral membrane proteins with the cyclophilin-homologous domain located in the lumen of the endoplasmic reticulum and intracellular transport vehicles (4, 6, 7). In all these examples cyclophilins were found to form a specific stable complex with the substrate, suggesting that they function as a chaperone escorting its protein substrate through the traffic pathway.

Recent reports suggest that cyclophilins may be involved in cell surface externalization of three other proteins, namely, insulin receptor (8), Flt3 ligand (13), and CD147 (25). In all these cases, treatment of the cells with CsA reduced the cell surface expression of these proteins (insulin receptor, Flt3L, CD147) without altering their total cellular levels, suggesting a block at the level of transition from the endoplasmic reticulum to the plasma membrane. The cyclophilin(s) involved in regulation of trafficking of these proteins have not yet been identified.

The mechanisms by which cyclophilins regulate protein trafficking remain unknown. Molecular studies of *Drosophila ninaA* mutants have shown that CsA has only a minor effect on rhodopsin transport, and *ninaA* mutations need not be within the PPIase/CsA-binding domain to cause severe reductions in rhodopsin levels (10–12). On the other hand, cell surface expression of the Flt3 ligand, insulin receptor, and CD147 was greatly reduced by CsA, suggesting involvement of the PPIase/CsA-binding domain of cyclophilin in the regulation of trafficking of these proteins (8, 13, 25). The cyclophilin-interacting site has been identified in CD147 as the proline-containing region at the end of the transmembrane helix adjacent to the extracellular domain (25).

CD147 is a type 1 integral membrane protein shown to regulate intercellular adhesion pathways through an intracellular signaling-mediated mechanism (14). It is also known as extracellular matrix metalloproteinase inducer (EMMPRIN) and EMMPRIN expressed by cultured tumor cells stimulates fibroblasts to produce very high levels of collagenase activity, which likely facilitates tumor metastasis (15, 16). Supporting its key role in the processes of tumorigenesis and metastasis, EMMPRIN was reported as one of the most constantly upregulated mRNA in metastatic cells (17). Another physiologic role of CD147 relates to its activity as a receptor for extracellular cyclophilins (18, 19). Since cyclophilins emerge as medi-

* This work was supported in part by National Institutes of Health Grants R03 A1057018 and R21 A1060720 (to M. I. B.) and R01 AI029110 (to B. S.) and by funds from George Washington University. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: PPIase, peptidyl-prolyl *cis-trans*-isomerase; CypA, cyclophilin A; Cyp60, cyclophilin 60; EMMPRIN, extracellular matrix metalloproteinase inducer; GFP, green fluorescent protein; CHO, Chinese hamster ovary; HEK, human embryonic kidney; CsA, cyclosporin A; PE, phycoerythrin; PBS, phosphate-buffered saline; siRNA, small interfering RNA; FAM, carboxyfluorescein; MFI, mean fluorescent intensity.

ators of intercellular communication that may regulate chemotactic responses in many physiologic and pathologic processes, such as cell-mediated immunity and inflammation (20), regulation of CD147 expression may determine the outcome of these events. A related activity of CD147 is its role as a cofactor in human immunodeficiency virus type 1 infection, where CD147 likely functions as a receptor for virion-associated CypA, thus increasing virus entry into target cell (21).

In this report, we identify the cyclophilin regulating cell surface expression of CD147 as cyclophilin 60 (Cyp60), a previously described 60-kDa member of the cyclophilin family of proteins (22). These results suggest that Cyp60 may be an important regulator of intracellular transport.

MATERIALS AND METHODS

Antibodies and Reagents—Fluorescein isothiocyanate- or PE-conjugated anti-CD147 monoclonal antibodies were purchased from Ancell (Bayport, MN), peridinin-chlorophyll protein-cojugated anti-CD4 monoclonal antibody was from BD Biosciences, and PE-labeled anti-CD71 monoclonal antibody was from CALTAG Laboratories (Burlingame, CA). Anti-GM130 mouse monoclonal antibody was from BD Biosciences, mouse monoclonal anti-FLAG antibody was from Chemicon (Temecula, CA), rabbit polyclonal anti-CypA antibody was from United States Biological (Swampscott, MA), and rhodamine and Cy5-labeled goat anti-mouse and anti-rabbit IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA). Cyclosporin A was purchased from Sigma, TOPRO-3 iodide was from Molecular Probes (Eugene, OR).

Co-immunoprecipitation—CHO cells were transfected with GFP- or CD147.wt-GFP-expressing vectors, cultured for 15 h, and harvested with 2 mM EDTA. Approximately 6×10^5 cells were lysed in TNEP lysis buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1% Triton X-100), spun at 14,000 rpm at 4 °C for 20 min in an Eppendorf microcentrifuge, and the supernatant (300 μl) was collected and incubated on ice for 1 h with mouse monoclonal anti-GFP antibody (Roche Diagnostics Corp., Mannheim, Germany). Immune complexes were precipitated by adding protein G beads (50 μl of 50% slurry in PBS) for 1 h at room temperature. Proteins were eluted from beads by adding non-reducing sample buffer to each sample and heating at 60 °C for 3 min. Supernatants were loaded on 12% SDS-PAGE gel. Western blotting was performed using rabbit polyclonal anti-CypA (1:1000; Affinity Bioreagents) or anti-GFP (1:5000; Abcam, Cambridge, MA) antibodies and developed by the ECL procedure (Amersham Biosciences, Uppsala, Sweden).

HEK 293T cells were co-transfected with vector expressing FLAG-Cyp60 (23) together with CD147-GFP or CD147.P211A-GFP-expressing vector. Cells were cultivated for 15 h and harvested with 2 mM EDTA. Approximately 6×10^5 cells were lysed in TNEP lysis buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1% Triton X-100), and cell lysates were spun at 14,000 rpm at 4 °C for 20 min in an Eppendorf microcentrifuge and incubated with mouse IgG for 1 h at 4 °C, followed by a 1-h incubation at room temperature with protein G-conjugated Sepharose beads to remove nonspecifically binding material. NaCl concentration was then adjusted to 300 mM, and mouse monoclonal anti-FLAG antibody (Chemicon) or mouse monoclonal anti-GFP antibody (Roche Diagnostics Corp.) was added to the lysates. After an overnight incubation at 4 °C, protein G-conjugated beads were added and immune complexes collected, resolved on SDS-PAGE, and analyzed by Western blotting using rabbit polyclonal anti-FLAG (Affinity BioReagents, Golden, CO), goat polyclonal anti-CD147 (Santa Cruz Biotechnology, Santa Cruz, CA), or rabbit polyclonal anti-GFP antibody (Abcam).

Immunofluorescent Microscopy—Cells grown on glass coverslips were fixed for 20 min at room temperature with 4% formaldehyde in PBS. HeLa cells transfected or not with FLAG-Cyp60 were incubated for 1 h at room temperature with a rabbit polyclonal antibody (1 μg/ml) raised against recombinant Cyp60 or commercially available polyclonal anti-FLAG antibody (Affinity BioReagents) in PBS containing 0.1% bovine serum albumin and 0.1% saponin. They were then incubated for 1 h at room temperature with Alexa546-labeled goat polyclonal antibodies to rabbit immunoglobulin (Molecular Probes) at a dilution of 1:1000 and stained with Hoechst 33258 (Wako, Osaka, Japan). Cells were covered with a drop of Gel/Mount (Biomeda, Foster City, CA) and then viewed and photographed with a Nikon Eclipse E800M microscope equipped with a color chilled 3CCD camera (model C5810; Hamamatsu Photonics, Hamamatsu, Japan). CHO K1 cells transfected with CD147-GFP were fixed, permeabilized by incubation in 100 mM glycine in Tris-buffered saline for 15 min at room temperature, washed, and incubated for 2 h at room temperature using primary mouse monoclonal antibody against Golgi marker GM130 (BD Biosciences) in 4% goat serum in PBS, followed by staining with rhodamine-labeled goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) at 1:100 dilution. HEK 293T cells co-transfected with CD147-GFP and FLAG-Cyp60 were stained using mouse monoclonal anti-FLAG antibody (Chemicon) and rhodamine-labeled goat anti-mouse IgG (Jackson ImmunoResearch Laboratories). TOPRO dye (Molecular Probes) was added together with the secondary antibody to counterstain the nuclei. All images of CHO K1 and HEK 293T cells were acquired using a BX-60 Olympus Epifluorescence microscope (Melville, NY) with an Evolution MP digital camera using Image-Pro Plus Version 4.5 acquisition software (Media Cybernetics, Silver Spring, MD).

Analysis of CD147 Expression after Subtilisin Treatment—HEK 293T (human embryonic kidney epithelial) cells (2×10^6 cells/ml) were treated with 0.3 mg/ml of subtilisin B (Sigma) for 30 min at 37 °C. Cells were washed and incubated in RPMI/10% FBS with or without CsA (5 μg/ml). Samples were collected for analysis before the treatment, immediately after the treatment, and following various incubation times. Cells were stained with PE-conjugated anti-CD147 or anti-CD71 antibodies or isotype control and analyzed by flow cytometry.

Suppression of Cyp60 Expression by RNA Interference—siRNAs labeled with 6-FAM at the 5′ end were synthesized by IDT Inc. (Coralville, IA). The sequence of the positive strands of siRNAs was as follows: siRNA.wt, 5′-CAGCAACAGGUCUCAAUUCUU-3′ (wild type); siRNA.mut, 5′-CAGCAACAGcagUCAAUUCUU-3′. The small letters denote three changed nucleotides in the mutant siRNA. siRNAs were transfected into HEK 293T cells using Oligofectamine (Invitrogen).

RESULTS

Cyclophilin 60 Co-immunoprecipitates with CD147—Our recent results suggested that cell surface expression of CD147 is regulated by a cyclophilin (25). We also showed that mutation of proline 211 in the transmembrane domain of CD147 (CD147.P211A) abrogates binding of CypA to CD147 in solution and abolishes sensitivity of CD147 surface expression to CsA. Our preliminary experiments demonstrated that CD147 has extended half-life on the cell surface (data not shown), suggesting that its turnover is a relatively slow process. Since CD147 interaction with cyclophilin regulates delivery of CD147 to the plasma membrane (25), we reasoned that the best chances to detect it are in *CD147*-transfected cells characterized by unregulated high level expression of CD147.

To test whether CD147 associates intracellularly with cyclophilin, we performed a co-immunoprecipitation analysis. As our previous studies (25) were done in CHO cells, we used the same cells in the first experiments. CHO cells were transfected with the GFP-CD147- or GFP-CD147.P211A-expressing constructs and cultivated in the presence or absence of CsA for 48 h. Untransfected CHO cells or cells transfected with the GFP-expressing vector were used as negative controls. GFP-containing complexes were immunoprecipitated with anti-GFP antibody and analyzed by Western blotting using antibodies to CypA (the most abundant cellular cyclophilin) or GFP. It should be noted that the polyclonal anti-CypA antibody used here recognizes most cyclophilins in different species (data not shown). Therefore, it was expected to react with hamster CypA, should it co-precipitate with CD147. To our surprise, no CypA-specific band (~18 kDa) was revealed (Fig. 1*A*, *upper panel*). Instead, in the immunoprecipitate from cells transfected with *GFP-CD147*, the antibody to CypA revealed a band (marked with a *star* on the *upper panel* of Fig. 1*A*) corresponding to a protein with an approximate molecular mass of 60 kDa. The intensity of this band was substantially weaker in the immunoprecipitate from CsA-treated *GFP-CD147*-transfected cells (consistent with demonstrated sensitivity of CD147 cell surface expression to CsA (9)), and no such band was observed in the immunoprecipitate from cells transfected with the *GFP-*

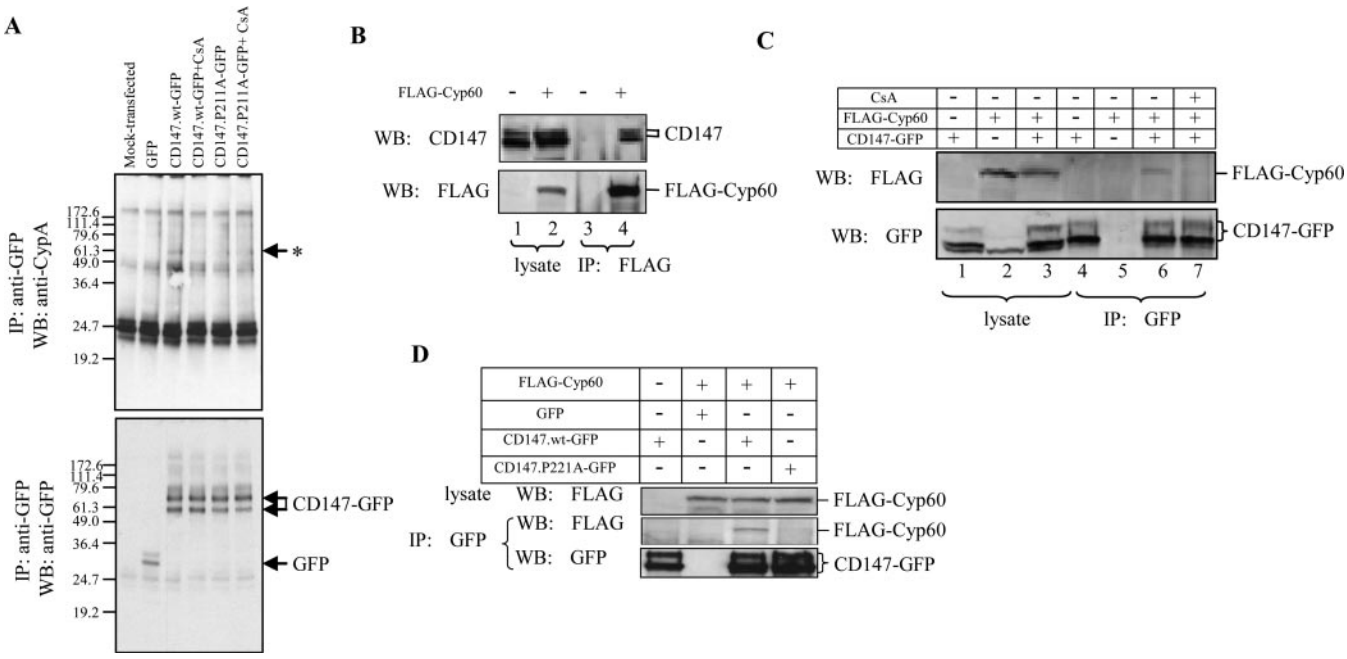

FIG. 1. **Cyclophilin 60 co-immunoprecipitates with CD147.** *A*, 60-kDa cyclophilin co-immunoprecipitates with CD147 in CHO cells. CHO K1 cells were mock-transfected or transfected with vectors expressing GFP alone (*GFP*) or GFP-fused wild-type human CD147 (*CD147.wt-GFP*) or CD147 construct carrying a P211A substitution (*CD147.P211A-GFP*). Cells transfected with CD147-GFP were treated or not with CsA (5 μM). Lysates containing both cytosol and membrane proteins were immunoprecipitated with a monoclonal antibody to GFP and analyzed by Western blotting using a polyclonal antibody to CypA (*upper panel*) or to GFP (*bottom panel*). The *asterisk* in the *upper panel* marks the 60-kDa protein recognized by the anti-CypA antibody. CD147-GFP migrates as non-glycosylated and variably glycosylated forms (*bottom panel*). The 25- and 50-kDa bands present in all six lanes correspond to the light and heavy (respectively) chains of the mouse antibody used for immunoprecipitation and are due to cross-reactivity of the secondary anti-rabbit IgG antibody with mouse immunoglobulins. *B*, HEK 293T cells were transfected with FLAG-tagged Cyp60 and immunoprecipitated using anti-FLAG antibody. Blots were developed using anti-FLAG (*bottom panel*) or anti-CD147 (*upper panel*) antibody. *C*, HEK 293T cells were co-transfected with FLAG-Cyp60 and CD147-GFP constructs, cultured for 48 h in the presence or absence of CsA (5 μg/ml), and immunoprecipitated using anti-GFP antibody. Blots were developed using anti-GFP (*bottom panel*) or anti-FLAG (*upper panel*) antibody. *D*, the experiment was performed as described for *C*, except that CD147-GFP construct carrying P211A mutation was added.

CD147.P211A mutant construct (defective in binding to CypA (9)) or in untransfected or *GFP*-transfected cells (Fig. 1*A*, *upper panel*).

To verify that the same amount of proteins was loaded in each lane, we stripped the blot and re-developed it using anti-GFP antibody. Two bands (57 and 76 kDa) and a smear above the 76-kDa band revealed in cells transfected with wild-type or mutant GFP-CD147 correspond to variously glycosylated forms of the GFP-CD147 fusion protein (Fig. 1*A*, *bottom panel*). We conclude that CD147 interacts intracellularly with a cyclophilin that has a molecular mass of 60-kDa.

Among various cyclophilins described in the literature, only one species, Cyp60, has been reported to have a molecular mass close to 60 kDa (22). To determine whether Cyp60 associates with CD147 in human cells, we transfected HEK 293T cells with FLAG-tagged *Cyp60*, immunoprecipitated it using anti-FLAG antibody, and tested for CD147 in the immunoprecipitate using anti-CD147 mAb. Indeed, CD147 was found in the precipitate (Fig. 1*B*), while β-actin, used to control the specificity of interaction, was not precipitated (data not shown). Several CD147-specific bands on the blot relate to differently glycosylated forms of the protein. In a reverse pull-down analysis, FLAG-Cyp60 was revealed in the immunoprecipitate with the anti-GFP antibody from HEK 293T cells co-transfected with *FLAG-Cyp60* and *CD147-GFP* (Fig. 1*C*). Cyclosporin A greatly reduced the amount of FLAG-Cyp60 co-immunoprecipitating with CD147-GFP, supporting the hypothesis that CD147-Cyp60 interaction involves the enzymatic site of Cyp60. Interaction between CD147 and Cyp60 was also abrogated by the mutation of the proline residue 211 (Fig. 1*D*), which was shown previously to be critical for interaction between CD147 and cyclophilin A in solution (25). Taken together, these results indicate that Cyp60 interacts with CD147 in a CsA-dependent fashion; this interaction also involves the Pro211 residue of CD147.

Cyp60 Regulates the Cell Surface Expression of CD147—Previously, we demonstrated that cell surface expression of CD147 is sensitive to CsA (25). To check whether Cyp60-CD147 interaction accounts for the observed sensitivity to CsA, we suppressed Cyp60 expression in HEK 293T cells using the RNA interference approach. Cyp60-specific siRNA (siRNA.wt) greatly reduced expression of Cyp60 without affecting expression of CypA (Fig. 2*A*). This effect of siRNA was eliminated by changing three nucleotides in the Cyp60 siRNA sequence (siRNA.mut), thus demonstrating the specificity of the observed inhibition.

We next studied the effect of Cyp60 suppression on reconstitution of cell surface CD147 after treatment with subtilisin, which is commonly used as a plasma membrane protein stripping agent (24, 25). As shown in Fig. 2*B*, CD147 surface expression on HEK 293T cells was greatly reduced (although not completely eliminated) by subtilisin treatment (peak with light shading marked "*sub 0h*") but was almost completely restored after 12 h of incubation in complete medium (this peak was identical to the peak showing fluorescence distribution in cultures treated with CsA solvent, ethanol, which is labeled "*sub 12h*"). In the presence of CsA, reconstitution of CD147 on the cell surface was delayed: at the 12 h time point, expression of CD147 (presented as mean fluorescent intensity (MFI)) was almost 2-fold lower in CsA-treated cells (peak labeled "*sub 12h + CsA*") than in cells treated with ethanol (relative mean fluorescence intensity (R_{MFI}) = 1.9, Fig. 2*B*). Importantly, no

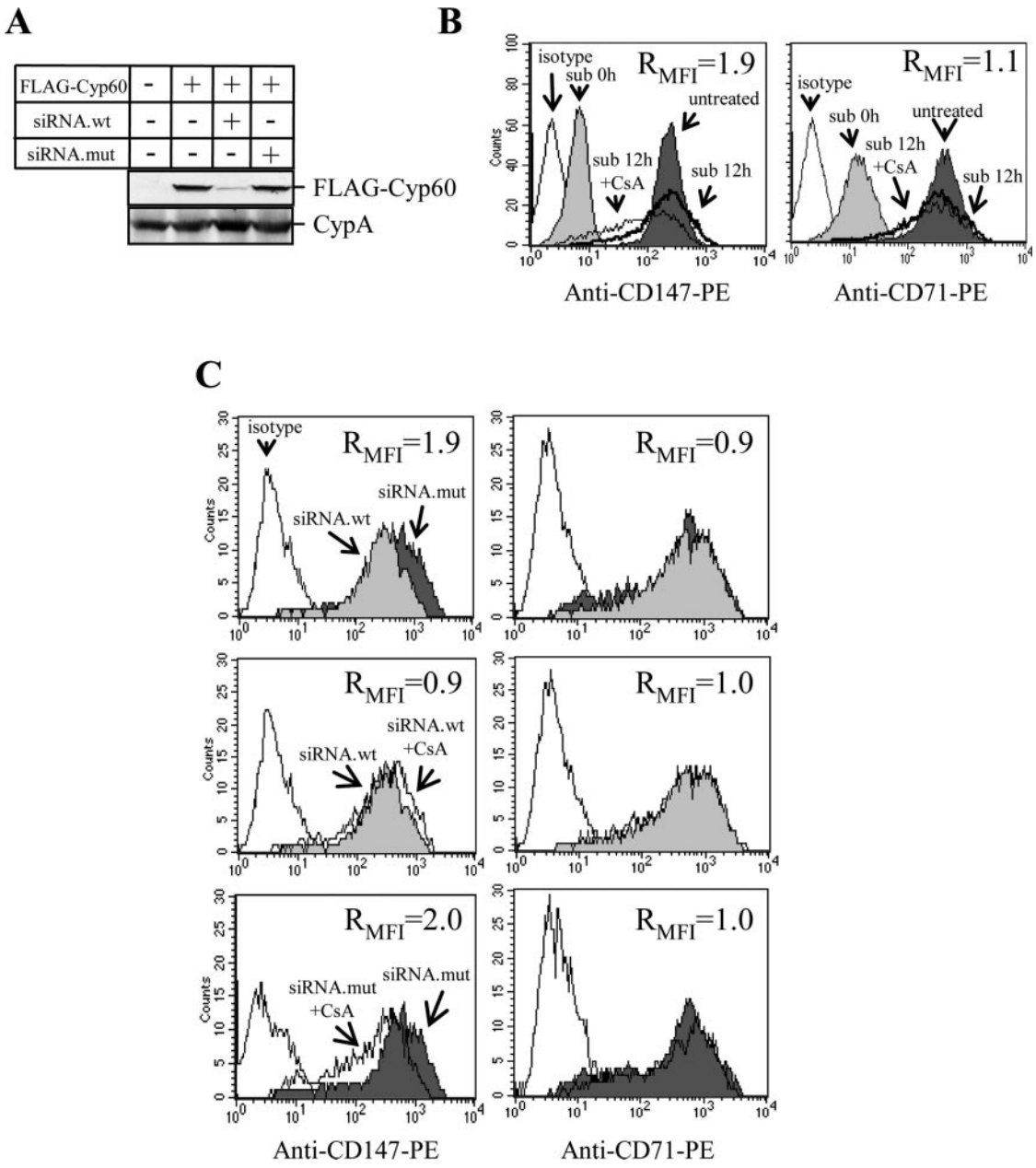

FIG. 2. **Cyp60 regulates CD147 surface expression.** *A*, HEK 293T cells were co-transfected with FLAG-tagged Cyp60 (*FLAG-Cyp60*) and Cyp60-specific siRNA (*siRNA.wt*). siRNA with a three-nucleotide substitution (*siRNA.mut*) was used as a control. Expression of FLAG-Cyp60 and endogenous CypA was tested by Western blotting using anti-FLAG and anti-CypA antibodies, respectively. *B*, HEK 293T cells were treated with subtilisin and cultured in the presence or absence of CsA (5 μM) for 12 h. Cell surface expression of CD147 (*left panel*) and CD71 (*right panel*) was measured prior to subtilisin treatment (*peaks* with *dark shading* marked "*untreated*"), immediately after the treatment (*peaks* with *light shading* marked "*sub 0h*"), and following a 12-h cultivation (*unshaded peaks* marked "*sub 12h*" and "*sub 12h + CsA*") by flow cytometry after staining with PE-conjugated anti-CD147 or anti-CD71 antibody, respectively. Isotype peaks (*unshaded peaks* marked "*isotype*") show staining with PE-conjugated isotype controls. Results are presented as an overlay of histograms obtained with subtilisin-treated and -untreated cells. R_{MFI} values show the ratio of mean fluorescence intensity of cells cultured without (*peaks* marked "*sub 12h*") and with CsA (*peaks* marked "*sub 12h + CsA*") (R_{MFI} = MFI (sub 12 h) ÷ MFI (sub 12 h + CsA)). Note that subtilisin-treated cells cultured in the presence of CsA (*sub 12h + CsA*) express less CD147 (but not CD71) than cells cultured in the presence of CsA solvent, ethanol (*sub 12h*). *C*, the experiment was performed as described for *B*, except that 24 h prior to subtilisin treatment, the cells were transfected with FAM-labeled Cyp60-targeting siRNA.wt (*light shading*) or siRNA.mut (*dark shading*). CD147 and CD71 expression was analyzed by flow cytometry gating on FAM-expressing cells. R_{MFI} shows the MFI ratio between cells transfected with siRNA.mut and siRNA.wt (*upper row panels*), untreated and CsA-treated cells transfected with siRNA.wt (*middle row panels*), and untreated and CsA-treated cells transfected with siRNA.mut (*bottom row panels*). Note the shift in CD147-specific (but not CD71-specific) fluorescence distribution of cells transfected with siRNA.wt relative to cells transfected with siRNA.mut (*top row*); a similar shift was observed when cells transfected with siRNA.mut were cultured in the presence of CsA (*bottom row*); no shift was seen when cells transfected with siRNA.wt were cultured in the presence of CsA (*middle row*).

such reduction was observed with another transmembrane protein, transferrin receptor (CD71), thus indicating the specificity of the CsA effect.

We then analyzed CD147 re-expression in HEK 293T cells transfected with siRNA.wt or siRNA.mut. The siRNAs used for this analysis were labeled with FAM, thus allowing to gate on transfected cells. Results presented in Fig. 2*C* (*upper left panel*) demonstrate that surface expression of CD147 was reduced in cultures transfected with siRNA.wt as compared with cultures transfected with siRNA.mut. The reduction (~2-fold) was similar to that observed with CsA (Fig. 2*B* and the *left panel* in the *bottom row*), suggesting that Cyp60 is the main (and likely the

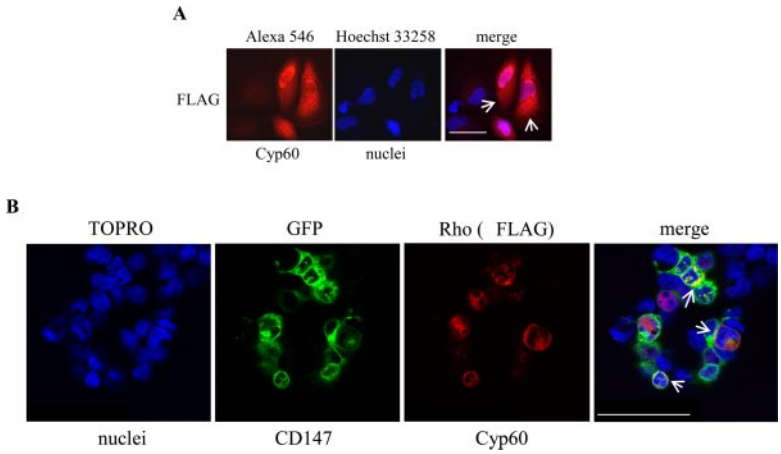

FIG. 3. **Intracellular localization of Cyp60 and CD147.** *A*, HeLa cells were transfected with *FLAG-Cyp60*, fixed, and incubated with anti-FLAG monoclonal antibody followed by Alexa546-labeled goat antibody to mouse IgG. Nuclei were counterstained using DNA-specific dye Hoechst 33258. Cells were viewed on a fluorescent microscope. *Arrows* point to cells where Cyp60 is visible in the cytoplasm. *Scale bar* is 50 μm. *B*, HEK 293T cells were co-transfected with *FLAG-Cyp60* and *CD147-GFP*. Cells were permeabilized and stained using primary mouse monoclonal anti-FLAG antibody followed by secondary rhodamine-conjugated goat anti-mouse IgG. Nuclei were counterstained using TOPRO. *Arrows* point to cells where Cyp60 co-localizes with CD147 (*yellow* staining in the merge panel). *Scale bar* is 50 μm.

only) cyclophilin that regulates CD147 intracellular trafficking. This conclusion was supported by our finding that treatment with CsA did not further reduce CD147 surface expression in cells transfected with siRNA.wt (*middle left panel* in Fig. 2*C*). The observed effects were specific for CD147, as expression of the transferrin receptor (CD71) was not affected by Cyp60-specific siRNAs (*right panel* in the *top row* in Fig. 2*C*), consistent with its insensitivity to CsA (*right panels* in Fig. 2*B* and in the *bottom row* in Fig. 2*C*). Similarly, no shift in CD71 reconstitution was induced by a combination of CsA and Cyp60-specific siRNA (*right panel* in the middle row in Fig. 2*C*).

Cyp60 Partially Co-localizes with CD147—Cyp60 was previously reported to localize to the cell nuclei when overexpressed in transfected cells (22). Using anti-FLAG antibody, we analyzed the intracellular localization of FLAG-Cyp60 in transfected HeLa cells. As shown in Fig. 3*A*, in many cells FLAG-Cyp60 was found to be almost evenly distributed between the nuclear and cytoplasmic compartments.

In HEK 293T cells co-transfected with FLAG-Cyp60 and CD147-GFP, partial co-localization of Cyp60 and CD147 was observed (Fig. 3*B*). Importantly, the most prominent co-localization of these proteins occurred at the plasma membrane (*yellow* staining marked by *arrows* in the merged image on Fig. 3*B*). Therefore, Cyp60 appears to accompany CD147 to the plasma membrane, thus functioning as a classical chaperone molecule.

Taken together, these results support the model where cyclophilin 60, via interaction with the proline-containing region adjacent to the transmembrane domain of CD147, regulates CD147 transport from the Golgi network to the plasma membrane.

DISCUSSION

The experiments presented in this report demonstrate that the surface expression of CD147 is regulated by a distinct member of the cyclophilin family, Cyp60. The mechanism of this activity involves interaction of Cyp60 with the proline-containing region within or adjacent to the predicted transmembrane domain CD147. When this interaction was blocked, either by CsA or by mutating the critical proline residue, expression of CD147 on the cell surface was significantly delayed. This effect is consistent with the proposed role of cyclophilins in assisting the transport of the proteins from the endoplasmic reticulum to the plasma membrane (4, 8).

Cyp60 has been previously cloned from a Raji B lymphocyte library in a two-hybrid screen using serine proteinase inhibitor eglin c as bait (22). Wang and co-authors reported nuclear localization of Cyp60 and suggested that it may chaperone nuclear proteins or be involved in their proper folding (22). Our analysis demonstrated that in cells overexpressing Cyp60 it localizes both to the nuclei and the cytoplasm (Fig. 3*A*). Localization of endogenous Cyp60 awaits preparation of a specific antibody that would distinguish this cyclophilin from the other members of this family of proteins.

Cyp60 co-localized with CD147 at the plasma membrane (Fig. 3*B*), suggesting that this cyclophilin may function as a chaperone escorting CD147 through the secretory pathway. Previously, a similar function was described for Drosophila cyclophilin homolog NinaA, which regulates secretion of its protein target rhodopsin (4). Cyp60, however, is the first mammalian cyclophilin implicated in protein transport to the plasma membrane. It appears to be the only cyclophilin involved in CD147 membrane transport, as cyclosporin A treatment did not add anything to the effect of Cyp60-specific siRNA (Fig. 2*C*).

Cyp60 interaction with CD147 involves proline 211 in the transmembrane domain. This proline residue is close to the extracellular domain of CD147 and is likely to be accessible in the lumen of the Golgi vesicles (25). Therefore, the CD147-interacting domain of Cyp60 must be on the luminal side of the Golgi membrane to access this site. Since, unlike NinaA, Cyp60 is not predicted to be an integral transmembrane protein, it is likely secreted into the lumen. In contrast, CypA is a cytosolic protein and thus may not have access to Pro^{211} of CD147, which may explain why no CypA was co-immunoprecipitated with CD147, despite CypA binding to CD147 in solution (25).

CD147 has been shown to interact with several other proteins that may influence its localization. For example, when association of CD147 with monocarboxylate transporter MCT1 was disrupted by mutating a critical glutamic acid residue 218 in the transmembrane domain of CD147, neither CD147 nor MCT1 reached the plasma membrane (26). CD147 also interacts with caveolin-1 on a cell surface and this interaction seems to negatively regulate clustering and activity of CD147 (27), although its role in CD147 cell surface expression has not been investigated. Finally, leucine 252 in the cytoplasmic domain of CD147 was identified as a basolateral signal which targets CD147 to the basolateral membrane in extraocular epithelia

(28). This signal seems to function only in some cell types (*e.g.* it was not recognized in human retinal pigment epithelium cells (28)), suggesting that it mediates interaction with some cell-specific regulator of protein trafficking. Future studies will hopefully integrate these findings to suggest a comprehensive model of CD147 trafficking.

Our finding that CD147 expression is regulated by Cyp60 opens new opportunities for treating diseases where CD147 functions as a pathogenic factor. One example is rheumatoid arthritis, where levels of CD147 on reactive neutrophils and cyclophilin in synovial fluids have been shown to correlate with disease severity (29). Another is cancer where CD147 (EMMPRIN) was shown to be enriched on the surface of tumor cells and may play a key role in matrix metalloproteinase production and tumor cell invasion (30, 31). Yet another example is human immunodeficiency virus infection, which depends on CD147 for high virus replication (21). Our results demonstrate that expression of CD147 can be reduced by agents targeting Cyp60, such as CsA or Cyp60-specific siRNA, suggesting new approaches to treatment of rheumatoid arthritis, AIDS, or prevention of metalloproteinase-dependent cancer metastasis.

In conclusion, our results identify a novel mechanism for regulation of plasma membrane expression of CD147/EMMPRIN and, by inference, a family of related integral membrane proteins. This mechanism involves interaction between the proline-containing transmembrane region of CD147 and cyclophilin 60, which leads to efficient trafficking of the protein from the Golgi to the plasma membrane. Future studies will identify other proteins regulated by Cyp60 and will determine the molecular events involved in this regulation.

Acknowledgments—We thank Dr. Robyn Rufner and the George Washington University Medical Center Imaging Facility for help with confocal image analysis. We acknowledge members of the Bukrinsky laboratory for helpful discussions. We are especially grateful to Dr. Anthony Cerami for valuable suggestions during the course of this work.

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