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Regulation of CD147 Cell Surface Expression

INVOLVEMENT OF THE PROLINE RESIDUE IN THE CD147 TRANSMEMBRANE DOMAIN*

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CD147, also known as extracellular matrix metalloproteinase inducer, is a regulator of matrix metalloproteinase production and serves as a signaling receptor for extracellular cyclophilins. Here we demonstrate that the cell surface expression of CD147 is regulated by cyclophilins via the transmembrane domain of CD147. Solution binding experiments demonstrated that the transmembrane domain was both necessary and sufficient for CD147 binding to cyclophilin A (CypA). Treatment with cyclosporin A significantly reduced surface expression of CD147 and of CD8-CD147 fusion protein carrying the extracellular domain of CD8 fused to the transmembrane and cytoplasmic domains of CD147, but did not affect expression of CD8. Peptide binding studies demonstrated specific interaction between CypA and the proline-containing peptide from the CD147 transmembrane domain. Mutation of this proline residue reduced binding of CD147-derived peptides to CypA and also diminished transport of CD147 to the plasma membrane without reducing the total level of CD147 expression. These results suggest involvement of a cyclophilin-related protein in CD147 cell surface expression and provide molecular details for regulation of CD147 trafficking by cyclophilins.

CD147 is a type 1 integral membrane protein shown to regulate intercellular adhesion pathways through an intracellular signaling mediated mechanism (1). It is also known as extracellular matrix metalloproteinase inducer (EMMPRIN),[1] and studies by Biswas and co-workers (2, 3) demonstrated that EMMPRIN expressed by cultured tumor cells stimulates fibroblasts to produce very high levels of collagenase activity, which likely facilitates tumor metastasis. To support its key role in the processes of tumorigenesis and metastasis, EMMPRIN was reported as one of the most constantly up-regulated mRNAs in metastatic cells (4). Another physiologic role of CD147 relates to its activity as a receptor for extracellular cyclophilins (5, 6). Because cyclophilins emerge as mediators of intercellular communication that may regulate chemotactic responses in many physiologic and pathologic processes, such as cell-mediated immunity and inflammation (7), regulation of CD147 expression may determine the outcome of these events.

The fact that CD147 interacts with extracellular cyclophilins in a cyclosporin-sensitive fashion (5) suggests that such an interaction may also occur intracellularly, where cyclophilins are abundant. Cyclophilins are a family of proteins that share peptidyl-prolyl *cis-trans*-isomerase (PPIase) 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 (8). However, cyclophilins also bind folded proteins, raising the possibility that certain functions of mature proteins may be regulated by cyclophilins. An example of such activity is the inhibitory effect of cyclophilin A (CypA), a prototypic member of the cyclophilin family, on the tyrosine kinase Itk (9), which is essential for the maintenance of proper CD4+ T cell responses (10).

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 (11). In yeast, CypA was shown to mediate the import of fructose-1,6-bisphosphatase into intermediate transport vesicles for vacuole delivery (12) and to promote nuclear export of Zpr1, an essential zinc finger protein (13). The *Drosophila* cyclophilin homolog NinaA participates in trafficking of rhodopsin, the most abundant subclass of photoreceptors (14–16). 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 (15, 17, 18). In all these examples, except interaction with Zpr1, 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 (19, 20) suggest that cyclophilins may be involved in cell surface externalization of two other proteins, namely insulin receptor and Flt3 ligand. In both cases, treatment of the cells with CsA reduced surface expression of the proteins without altering their total cellular levels, suggesting a block at the level of transition from the endoplasmic reticulum to trans-Golgi network. 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*

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[1] The abbreviations used are: EMMPRIN, extracellular matrix metalloproteinase inducer; CypA, cyclophilin A; PPIase, peptidyl-prolyl *cis-trans*-isomerase; CHO, Chinese hamster ovary; CsA, cyclosporin A; HPLC, high pressure liquid chromatography; PE, phycoerythrin; MIF, migration inhibitory factor; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; GFP, green fluorescent protein; HEK, human embryonic kidney; CHO, Chinese hamster ovary; PerCP, peridinin chlorophyll protein.

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 (21–23). Cell surface expression of the Flt3 ligand and insulin receptor, on the other hand, was greatly reduced by CsA, suggesting involvement of the PPIase/CsA-binding domain of cyclophilin in the regulation of trafficking of these proteins (19, 20). However, the cyclophilin-interacting domain within these target proteins has not been determined.

Analysis of CD147 cell surface expression revealed its unexpected sensitivity to CsA, suggesting involvement of a cyclophilin in the regulation of CD147 intracellular trafficking. In this report, we present evidence that sensitivity to CsA is eliminated by mutating a single proline residue in the transmembrane domain of CD147. Mutation of this residue abrogates binding of cyclophilin to CD147 and also reduces the cell surface expression of CD147. These results suggest that intracellular trafficking of CD147/EMMPRIN is regulated by a cyclophilin.

MATERIALS AND METHODS

Antibodies and Reagents—FITC- or PE-conjugated anti-CD147 monoclonal antibodies were purchased from Ancell (Bayport, MN); PerCP-conjugated anti-CD4 monoclonal antibody was from BD Biosciences, and PE-labeled anti-CD71 monoclonal antibody was from Caltag (Burlingame, CA). Rabbit polyclonal anti-CypA antibody was from US Biological (Swampscott, MA). Cyclosporin A was purchased from Sigma.

Plasmids and Transfection—Complementary DNAs for human CypA and CypB were cloned into the pET14b vector (Novagen, Madison, WI). Cyclophilins were expressed in *Escherichia coli* and purified by using the histidine-binding methodology as described (24). For expression of CD147, a DNA fragment encoding the full-length human CD147 was cloned into the pcDNA3.1 vector (Invitrogen). CD147-GFP construct (25) was kindly provided by Dr. Zucker (State University of New York, Stony Brook). All mutations were generated using the QuikChange kit (Stratagene, La Jolla) and verified by DNA sequence analysis. For protein expression in mammalian cells, cells were transfected using Metafectene (Biontex, Munich, Germany), according to the manufacturer's protocol. Efficiency of transfection of CHO K1 and HEK 293T was routinely 20–30%.

For solution binding studies, DNA fragments encoding the full-length (lacking only the signal peptide) or truncated versions of CD147, CD8 α-chain, and CD8/CD147 chimeric protein were cloned into the pT7Blue2 vector (Novagen) and expressed using the TnT Coupled Reticulocyte Lysate System (Promega, Madison, WI).

Solution Binding Assay—Affinity resins were prepared by coupling recombinant human CypA or CypB to CNBr-activated Sepharose (Pharmacia, Piscataway, NJ) according to the manufacturer's instructions. Control resin was prepared similarly, except that cyclophilins were either replaced by the macrophage migration inhibitory factor (MIF) or omitted entirely during the coupling step. Binding reactions were performed using [^{35}S]methionine/[^{14}C]leucine-labeled proteins for 90 min at room temperature in the binding buffer (20 mM HEPES, pH 6.8; 150 mM KOAc; 2 mM $Mg(OAc)_2$; 2 mM dithiothreitol; 0.1% Tween 20; 0.1% casamino acids; protease inhibitors mixture (Roche Applied Science)). Unbound proteins were removed by washing the resin three times with the binding buffer, and then bound proteins were eluted with the Laemmli buffer and revealed by electrophoresis and autoradiography.

HPLC Analysis—Peptides (2 μM each) were incubated with CypA (1 μM) overnight in the HPLC binding buffer (100 mM NH_4OAc, pH 8.5) at 4 °C. Unbound peptides were removed by centrifugation through the gel filtration Micro Bio-Spin P6 chromatography column (Bio-Rad). Material that passed through the column was loaded on the C18 column and analyzed by the reverse phase HPLC (running gradient program from 20 to 50% acetonitrile with 0.1% trifluoroacetic acid for 60 min).

Mass Spectral Analysis by MALDI-TOF—Peptides (50 μM) were incubated at room temperature in the presence or absence of CypA (10 μM) for 45 min, at which time the reaction mixture was run through a Micro Bio-Spin P-6 column (Bio-Rad) to remove unbound peptide. Samples of the starting material and the flow-through fraction from the column (containing CypA and bound peptide) were lyophilized, dissolved in 0.1% trifluoroacetic acid, desalted by passing through a ZipTip C18 (Millipore, Billeric, MA) according to manufacturer's instructions, and loaded on mass spectrometer for analysis. Mass spectrometry analysis was performed on the Kratos Kompact SEQ (Kratos Analytical, Chestnut Ridge, NY) machine using α-cyano-4-hydroxycinnamic acid (Aldrich) as a matrix. Measurements were taken in the linear delayed extraction mode, with external calibration.

Analysis of CD147 Expression after Subtilisin Treatment—U937 (human monocytic cell line) or HEK 293T (human embryonic kidney epithelial) cells (2×10^6 cells/ml) were treated with 0.3 mg/ml subtilisin B (Sigma) for 30 min at 37 °C. Cells were washed and incubated in RPMI, 10% fetal bovine serum with or without CsA (5 μg/ml). Samples were collected for analysis before the subtilisin treatment, immediately after the treatment, and following various incubation times. Cells were stained with anti-CD147-FITC or anti-CD4-PerCP (U937 cells) or PE-conjugated anti-CD147 or anti-CD71 (HEK 293T cells) antibodies (or fluorescently labeled isotype controls) and analyzed by flow cytometry.

Confocal Laser-scanning Microscopy—HEK 293T cells cultured on polylysine-coated coverslips (BD Biosciences) were transfected with the vector encoding the CD147-GFP fusion protein (25). 16–18 h after transfection, cells were washed and fixed with 3.7% formaldehyde solution in PBS for 15 min at room temperature, followed by a 15-min incubation at room temperature in 100 mM glycine in Tris-buffered saline (10 mM Tris, pH 7.5; 150 mM NaCl). Following three washes with PBS, slides were blocked with 4% goat serum in PBS for 1 h at room temperature and then stained with mouse monoclonal antibody against the Golgi matrix protein GM130 (BD Biosciences) followed by rhodamine-labeled goat anti-mouse IgG (Jackson ImmunoResearch). Slides were viewed in an Olympus IT-2 microscope attached to a Bio-Rad 1024MRC scanning confocal system. Images were collected with a $\times$60 objective. Z sections 0.5 μm thick were taken and processed using Adobe Photoshop 7.0 and Bio-Rad plug-ins.

RESULTS

Cyclosporin A Inhibits Cell Surface Expression of CD147—Our recent studies (5, 6) demonstrated that CD147 functions as a signaling receptor for extracellular cyclophilins A and B in a cyclosporin A-sensitive fashion. This result implies the presence of a cyclophilin-binding site on CD147, which could also bind cyclophilin intracellularly. Previous reports (14, 19, 20) implicated cyclophilins in regulation of cell surface expression of several transmembrane proteins, *e.g.* rhodopsin, insulin receptor, or Flt3 ligand. We therefore hypothesized that surface expression of CD147 could be regulated by a cyclophilin. To start addressing this question, we tested the effect of cyclosporin A on the cell surface localization of CD147. Because we failed to find a human cell line that does not express CD147, we used hamster CHO cells and transfected them with a vector expressing human CD147. As shown in Fig. 1 (*panels* in the *left column*), a marked decrease in CD147 expression was observed in the presence of CsA. In contrast, the expression of human CD8 in CHO cells transfected with a CD8-expressing vector was not affected by CsA (*panels* in the *right column*). This result suggests that CD147 expression is regulated by cyclophilin and involves its PPIase/CsA-binding domain.

This effect could be due to diminished expression of the constructs or inefficient transport to the plasma membrane. To discriminate between these two possible mechanisms, we investigated the effect of CsA on expression of the CD147 construct carrying GFP linked at the C-terminal end, which has been shown previously (25) to be physiologically similar to the wild-type CD147. Analysis of CD147 expression demonstrated that CsA significantly reduced the surface expression of CD147-GFP without affecting total GFP expression (Fig. 2).

Results presented so far show the effect of CsA on CD147 expression in transfected CHO cells. To test whether CsA blocked surface expression of endogenous CD147 in human cells, we treated a human promonocytic cell line, U937, with subtilisin to strip cells of surface CD147, and we then monitored re-expression of CD147 on the cell surface in the presence or absence of CsA. These experiments demonstrated that CD147 surface expression was reconstituted to almost original levels 24 h after subtilisin treatment (*lightly shaded peaks* in Fig. 3*A*); longer incubation led to only a small increase in

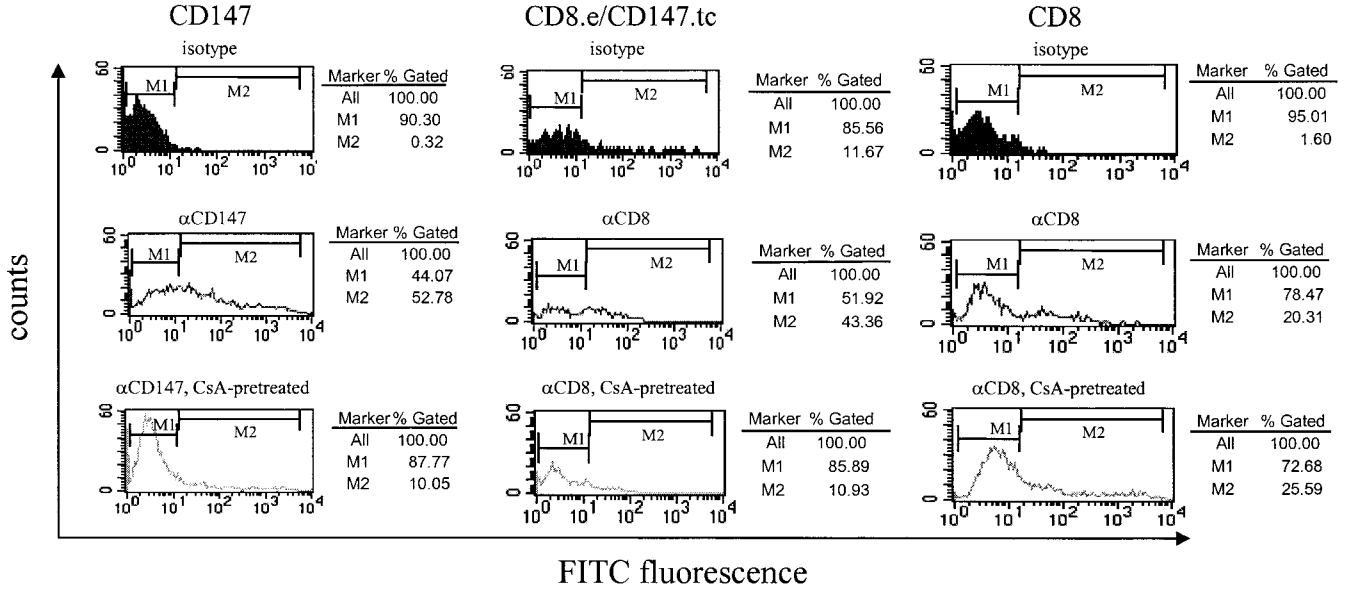

FIG. 1. **Cell surface expression of CD147 is inhibited by cyclosporin A.** CHO-K1 cells were treated or not with 5 μM CsA and transfected with a vector expressing human CD147, human CD8, or a hybrid protein in which the extracellular domain of CD8 (*CD8.e*) was fused to the transmembrane and cytoplasmic domains of CD147 (*CD147.tc*). 16 h after transfection, surface expression of CD147 was analyzed by flow cytometry after staining with FITC-conjugated anti-CD147. The gates were set up by measuring fluorescence after staining with FITC-labeled isotype, and the percentage of cells in each gate is shown at the *right*.

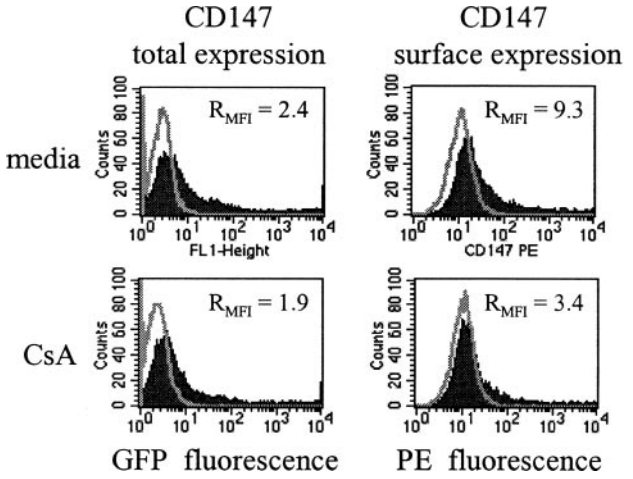

FIG. 2. **Cyclosporin A inhibits cell surface expression of CD147 without reducing its total expression.** Experiment was performed as in *A*, except that the construct expressing CD147 with GFP fused at the C terminus (CD147-GFP) was used instead of CD147. Cell surface expression of CD147 was measured by flow cytometry after staining with PE-labeled anti-CD147 antibody, whereas total CD147 was measured by GFP fluorescence. Untransfected cells were used as a negative control. R_{MFI} values show the ratio of mean fluorescence intensity of similarly treated transfected (*shaded peaks*) and untransfected (*unshaded peaks*) cells.

CD147 expression (not shown). Results presented in Fig. 3*A* show a consistent decrease in the re-expression of CD147 on the cell surface in the presence of CsA (Fig. 3*A*, *peaks* with *dark shading*). Most importantly, CsA did not affect re-expression of CD4 after subtilisin treatment (Fig. 3*A*, *right panels*), indicating that the observed effect was not due to toxicity of CsA and that it was specific for CD147.

By using the same approach, we studied the effect of CsA on CD147 surface expression in adherent human cells, HEK 293T. As shown in Fig. 3*B*, CD147 surface expression on HEK 293T cells was greatly reduced by subtilisin treatment (*peak* with *light shading*) but was almost completely restored after 12 h. Similar to the results with U937 cells (Fig. 3*A*), in the presence of CsA expression of CD147 12 h after subtilisin treatment was reduced by almost 2-fold as revealed by reduction of the mean fluorescence intensity in the CsA-treated culture (R_{MFI} = 1.9, Fig. 3*B*). Most importantly, no such reduction was observed with the transferrin receptor (CD71), another transmembrane protein, thus indicating the specificity of the CsA effect.

To visualize intracellular localization of CD147, we transfected HEK 293T cells with the CD147-GFP-expressing vector and analyzed CD147 localization in CsA-treated and untreated cells by confocal fluorescent microscopy. As shown in Fig. 3*C*, cell surface localization of CD147-GFP was reduced in CsA-treated cells, consistent with results in Fig. 3, *A* and *B*. Moreover, as expected for the transmembrane protein, CD147 partially co-localized with the Golgi marker (Golgi matrix protein GM130), and this co-localization substantially increased in CsA-treated cells (Fig. 3*C*, *yellow* staining in the merged image).

Taken together, results presented in this section suggest that transport of CD147 from the Golgi to the plasma membrane is regulated by a cyclophilin and may involve the active (PPIase) site of the cyclophilin molecule. Most importantly, this mechanism operates in both nonadherent (U937) and adherent (CHO and HEK 293T) human and non-human cells.

Cyclophilin-binding Site on CD147, the Critical Role of Pro^{211}—To gain further insight into the mechanisms of cyclophilin-mediated regulation of CD147 expression, we wanted to identify the domain of CD147 responsible for interaction with cyclophilins. Because CD147 interaction with cyclophilin appears to involve the PPIase domain (it is sensitive to CsA) and cyclophilins are highly homologous in their active site (26), we used readily available recombinant CypA for these studies. To identify the cyclophilin-binding region in CD147, we expressed truncated forms of CD147 composed of the extracellular domain alone (CD147.e), extracellular and transmembrane domains (CD147.et), or extracellular and cytoplasmic domains (CD147.ec) fused in-frame (Fig. 4*A*), and we performed binding analyses using immobilized CypA and these constructs. Specific binding was observed with the full-length CD147 (CD147.etc) and CD147.et constructs, which expressed the extracellular and transmembrane domains (Fig. 4*B*). No binding was detected with the CD147.e or CD147.ec constructs, which expressed the extracellular domain but did not include the

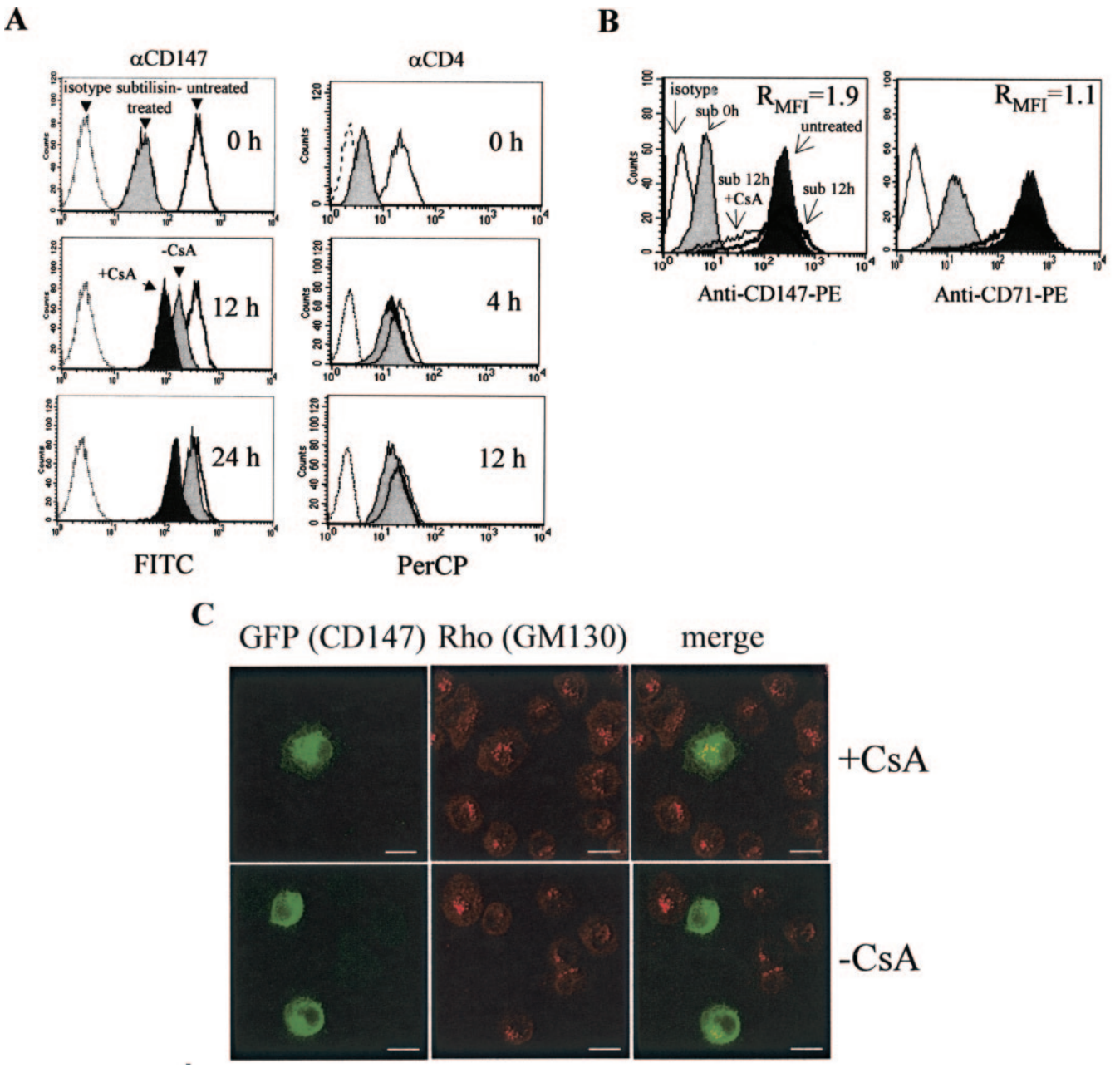

FIG. 3. **Cyclosporin A inhibits re-expression of endogenous CD147 after subtilisin treatment.** *A*, U937 cells were treated with subtilisin and cultured in the presence (*dark shading*) or absence (*light shading*) of CsA for the indicated time intervals. Surface expression of CD147 and CD4 was analyzed by flow cytometry after staining with FITC-conjugated anti-CD147 or PerCP-conjugated anti-CD4 antibody, respectively. Results are presented as an overlay of histograms obtained with subtilisin-treated and untreated cells. The experiment was performed two times with similar results. *B*, experiment was performed as in *A*, except that adherent HEK 293T cells were used instead of U937 cells. Because HEK cells do not express CD4, CD71 (transferrin receptor) was used as a specificity control. The *dark-shaded peaks* show surface expression of CD147 or CD71 in untreated cells, and the *light-shaded peaks* show surface expression immediately after subtilisin treatment. *Unshaded peaks* with a *thick line border* show surface expression of the receptors on subtilisin-treated cells after 12 h of incubation without CsA (*sub 12 h*), and *unshaded peaks* with a *thin line* border show expression on subtilisin-treated cells after 12 h of incubation with CsA (*sub 12 h + CsA*). R_{MFI} values show the ratio of mean fluorescence intensity of cells cultured in the absence and presence of CsA ($MFI_{sub\ 12h}/MFI_{sub\ 12h\ +\ CsA}$). *C*, HEK 293T cells were transfected with the CD147-GFP-encoding vector and cultured overnight in the presence (*upper panels*) or absence (*bottom panels*) of CsA (5 μM). Following permeabilization, the Golgi protein GM130 was stained using primary anti-GM130 mouse monoclonal antibody and rhodamine-labeled (*Rho*) anti-mouse secondary antibody. Images were acquired on a Bio-Rad confocal system with Z sections of 0.5 μm. Images were processed using Photoshop 7.0 and Bio-Rad plug-ins. *Scale bar* is 10 μm and relates to all images. Representative images are shown.

transmembrane domain of the protein. This result was unexpected, given that CD147 has been shown to function as a receptor for extracellular CypA (5). Similar results were obtained using CypB-coated beads (not shown), suggesting that interaction with the CD147 transmembrane domain may involve the active site common to all cyclophilins.

To test whether the transmembrane domain of CD147 was both necessary and sufficient to bind cyclophilin, we constructed a fusion protein composed of the extracellular domain of the CD8 α-chain (which does not bind CypA, Fig. 4*C*, *lane 5*) fused to the transmembrane and cytoplasmic domains of CD147 (CD8.e/CD147.tc). When expressed in CHO cells, this construct was susceptible to CsA inhibition, similar to CD147 (Fig. 1, *panels* in the *middle column*). A specific binding interaction was detected between this construct and CypA-coated beads (Fig. 4*C*, *lane 4*). To rule out the possibility of a nonspe-

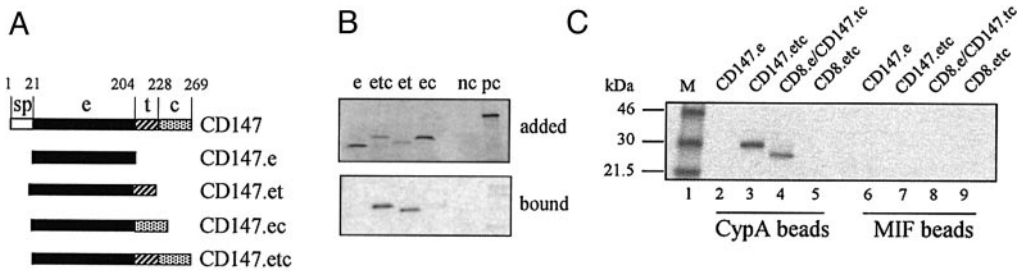

FIG. 4. **Analysis of CD147 interaction with CypA in solution.** *A*, CD147-derived constructs containing the extracellular domain (*CD147.e*), extracellular and transmembrane domains (*CD147.et*), extracellular domain fused to the cytoplasmic domain (*CD147.ec*), or a full-length CD147 (*CD147.etc*) lacking only the signal peptide (*sp*) were used in this study. *B*, CD147-derived constructs were cloned into the pT7Blue-2 vector and expressed by using a TNT-coupled reticulocyte lysate system in the presence of [^{14}C]leucine and [^{35}S]methionine (*upper panel*). Negative (*nc*) and positive (*pc*) controls provided with the TNT kit were also expressed. Binding reactions were performed with Sepharose beads coated with recombinant CypA, and agarose-bound proteins were loaded on SDS-PAGE and revealed by fluorography of the gels (*bottom panel*). *C*, binding was analyzed as in *B*. In addition to CD147.e and CD147.etc constructs used in *B*, two additional constructs containing the extracellular, transmembrane, and cytoplasmic domains of the CD8 α-chain (*CD8.etc*) or the extracellular domain of CD8 fused to the transmembrane and cytoplasmic domains of CD147 (*CD8.e/CD147.tc*) were expressed. Binding reactions were performed with Sepharose beads coated with recombinant CypA (*lanes 2–5*) or MIF (*lanes 6–9*).

cific binding to the beads, we performed a similar analysis with beads coated with the macrophage MIF, a cytokine with molecular mass similar to that of CypA (28). No binding was detected (Fig. 4*C*, *lanes 6–9*), supporting the specificity of the interaction between CD147 and CypA. Taken together, these results suggest that the transmembrane region of CD147 is both necessary and sufficient for the binding interaction with CypA in solution.

To map precisely the CypA-interacting region within the CD147 transmembrane domain, we synthesized three peptides (P1–P3) covering contiguous areas of the domain (Fig. 5*A*). Because these peptides were very hydrophobic and could be dissolved only in Me_2SO, we prepared water-soluble forms of the peptides by adding two lysine residues at the amino terminus (P4–P6). We also synthesized peptides with mutations in potential CypA-binding sites; peptide P7 had a Pro to Ala substitution in the position corresponding to Pro^{211} of CD147, whereas in peptide P8 Leu was replaced by Ala, thus disrupting a potential leucine zipper.

To analyze binding of peptides to CypA, we used mass spectral analysis. Peptides were incubated with CypA overnight at 4 °C, and unbound peptides were removed by centrifugation through the gel filtration column. Material that passed through the column was analyzed on the Kratos Kompact SEQ machine using α-cyano-4-hydroxycinnamic acid as a matrix. This analysis demonstrated that peptides P1, P4, and P8 bound CypA, whereas P2, P3, P5, P6, and P7 did not. A typical example of binding and nonbinding interactions is shown in Fig. 5*B* for peptides 4 and 6, respectively.

To compare peptide-CypA binding in a quantitative manner, we relied on HPLC analysis. Peptides were incubated with CypA as described above. The reaction mixture was run through a gel filtration column (to remove the unbound peptide), and CypA and bound peptide were fractionated by HPLC. All peptides used in this study separated well from each other and from CypA by elution time. The quantification of binding interactions is shown in Fig. 5*C*. The water-soluble forms of the investigated peptides (P4–P6) did not differ from their native analogs in CypA-binding properties. Consistent with results of mass spectrometry analysis, peptide 1 (and its water-soluble form peptide 4), but not peptides 2, 3, 5 or 6, bound CypA. The binding was due mainly to the Pro residue in the peptide sequence (corresponding to Pro^{211} of CD147), because it was almost completely abolished by a single point mutation Pro → Ala (compare P4 to P7). Disruption of the leucine zipper in P4 by the Leu → Ala point mutation did not reduce CypA binding (compare P8 to P4), indicating that the leucine zipper motif was not involved in this interaction.

Because the P211A mutation greatly reduced the interaction between the CD147-derived peptide and cyclophilin A, we tested CD147 surface expression in CHO cells transfected either with wild-type human CD147 with the GFP fused at the C terminus (CD147.wt-GFP) or human CD147.P211A-GFP carrying a proline 211 to alanine substitution. In both CD147.wt-GFP and CD147.P211A-GFP-transfected cultures, ~35% of the cells expressed GFP. Surface CD147 expression was analyzed by flow cytometry after staining with PE-conjugated anti-CD147 antibody and gating on GFP-positive cells. As shown in Fig. 6, surface expression of CD147.P211A was reduced compared with wild-type CD147 (reduction of mean fluorescence from 427.5 to 233.4). If P211 is critical for the cyclophilin-regulated surface expression of CD147, one would expect the cell surface expression of CD147.P211A mutant to be independent of cyclophilin and thus resistant to CsA. Indeed, although the cell surface expression of the CD147.P211A mutant was reduced when compared with the wild-type protein, it was not affected by CsA treatment (Fig. 6), in contrast to the wild-type CD147.

DISCUSSION

The experiments presented in this report demonstrate that the cell surface expression of CD147 correlates with the capacity of its transmembrane domain to interact with cyclophilin. We also identified Pro^{211} as a critical determinant in this interaction. When CD147-cyclophilin interaction was blocked, either by CsA or by mutating the critical proline 211 residue, transport of CD147 to the cell surface was delayed. This effect is consistent with the proposed role of cyclophilins in assisting transport of the proteins from the endoplasmic reticulum to the plasma membrane (15, 19).

Mutation of Pro^{211} impaired the binding to cyclophilin (Fig. 5) and the cell surface expression of CD147 (Fig. 6), suggesting that these two effects are related. However, another possibility is that Pro^{211} mutation reduces CD147 cell surface expression by affecting its conformation and not cyclophilin binding. Two lines of evidence argue against this explanation. First, the effect of Pro^{211} mutation is very similar in magnitude to the effect of CsA (compare expression of CD147.P211A in untreated cells to CD147.wt expression in CsA-treated cells in Fig. 6), whereas a more dramatic change would be expected to occur if protein conformation were grossly affected. Second, and more important, CsA treatment did not further reduce the cell surface expression of CD147.P211A, indicating that the mutant is not regulated by cyclophilin. It remains possible that conformational change is responsible for impairment of inter-

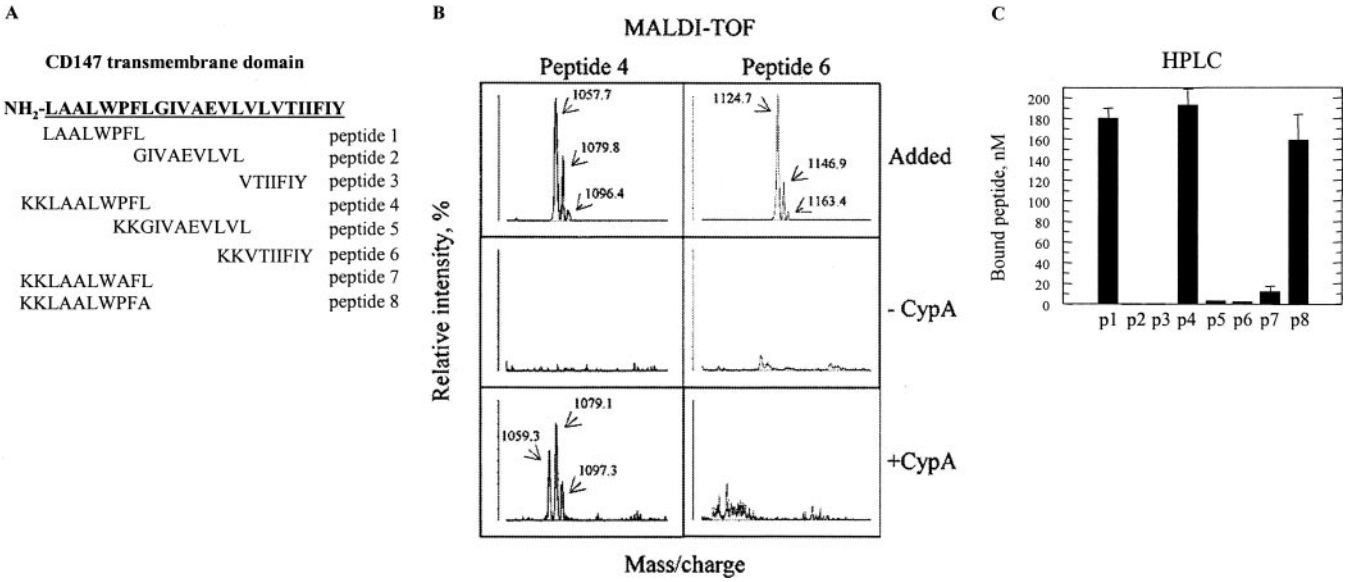

FIG. 5. **Proline 211 is critical for interaction between CD147 and CypA.** *A*, sequence of the CD147 transmembrane domain and peptides used in this study. *B*, analysis of peptide-CypA binding by MALDI-TOF. Results are shown for peptides 4 and 6. Analysis was performed for added peptides (*Added*), for the material that passed through the gel filtration column in the absence of CypA (−*CypA*), and for the material that passed through the column after incubation of the peptides with CypA (+*CypA*). Only the area of the spectrum corresponding to the low molecular mass (where peptides are found) is shown. *C*, analysis of peptide-CypA binding by HPLC. Following incubation with CypA, unbound peptides were removed by gel filtration, and material that passed through the gel filtration column (CypA-bound) was analyzed by HPLC. Quantification of binding was done by plotting absorbance at 214 nm for each peptide on a standard curve obtained by running various concentrations of this peptide on the same HPLC column. Three independent measurements were performed for each peptide, and results are presented as mean ± S.D.

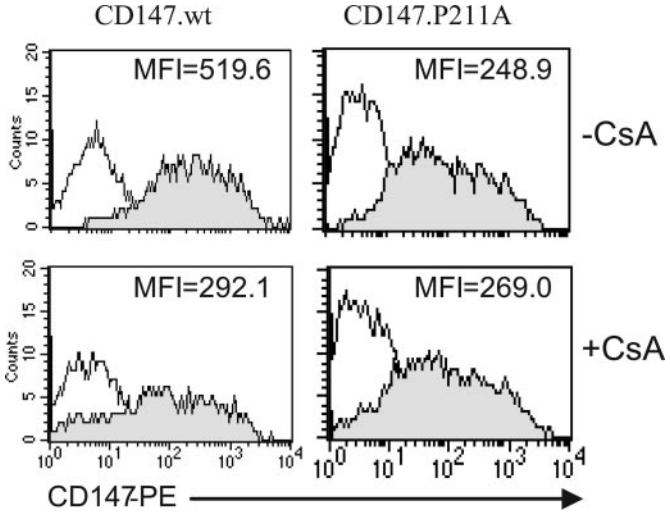

FIG. 6. **Cell surface expression of CD147 with mutation in Pro^{211} is not inhibited by CsA.** CHO cells were transfected with vectors encoding for GFP-fused wild-type CD147 (*CD147.wt-GFP*) or CD147 carrying alanine instead of proline in position 211 (*CD147.P211A-GFP*) and cultured in the presence or absence of CsA (5 μM). 16 h after transfection, expression of CD147 on the cell surface was analyzed by flow cytometry after staining with isotype control (*clear peaks*) or PE-conjugated anti-CD147 antibody (*shaded peaks*) and gating on the GFP-positive cells.

action with cyclophilin, and future studies will determine the effect of Pro^{211} mutation on CD147 conformation.

An unusual feature of CD147 is that this protein, which has been shown to function as a receptor for extracellular CypA and CypB (5, 6), can interact with cyclophilins as a mature protein expressed on the plasma membrane and as an immature form during its intracellular translocation. The first interaction involves the proline residue located in position 180, in the membrane-proximal region of the CD147 extracellular domain (5). It is conceivable that the Pro^{211}-containing region also takes part in this interaction with extracellular cyclophilins. Different computational techniques predict a slightly different position of Pro^{211} relative to the transmembrane region. According to TMpred (29, 30), the TM helix is located between residues 212 and 229, thus positioning Pro^{211} immediately prior to the TM region. Several other techniques predict Pro^{211} as a part of the TM region as follows: TOPPRED (31) (TM between residues 209 and 229), DAS (32) (residues 206–228), TMHMM (33) (residues 207–229), and PHDhtm (27) (residues 209–226). Although in all methods, except TMPred, the helix starts before residue 211, in most cases this proline residue is close to the helix end. The length of the predicted helix is in the range of 18–23 residues. The distance between the ends of such helices is 27–32 Å. Because the effective thickness of phospholipid membranes impenetrable for water is less than 25 Å, Pro^{211} would be accessible to solvent even if it is not the first residue in the TM helix. Therefore, it is likely that this proline residue is accessible for interaction with cyclophilin in the lumen of the Golgi vesicles.

The identity of the cyclophilin responsible for CD147 trafficking remains unknown. It appears unlikely that this cyclophilin is CypA, the most abundant cytoplasmic cyclophilin, as our preliminary experiments failed to identify CypA in CD147 immunoprecipitates.[2] The intracellular interaction of CD147 with cyclophilin likely occurs during transport of CD147 from the Golgi to the plasma membrane. Most importantly, Pro^{180} in CD147 extracellular domain, mutation of which has been shown to impair the receptor function of CD147 (5), is also accessible at this time and theoretically may interact with cyclophilin. However, this interaction is not involved in the regulation of CD147 intracellular trafficking, as substitution of Pro^{180} with Ala does not reduce the surface expression of CD147, while eliminating signaling response to extracellular CypA (5). Therefore, Pro^{211}, and not Pro^{180}, seems to define the binding site for the intracellular interaction between CD147 and cyclophilin. Because proline 211 is close to the extracellu-

[2] V. Yurchenko, T. Pushkarsky, J.-H. Li, W. W. Dai, B. Sherry, and M. Bukrinsky, unpublished results.

lar domain of CD147, the interacting cyclophilin must be on the luminal side of the Golgi membrane to access this site. This also argues against CypA as a regulator of CD147 expression, despite CypA binding to CD147 in solution; CypA is a cytosolic protein.

In summary, our results demonstrate that plasma membrane expression of CD147 is regulated by cyclophilin and identify Pro^{211} as the critical residue responsible for this regulation. The identity of the cyclophilin involved remains to be determined.

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