Caveolin-1 Regulates Matrix Metalloproteinases-1 Induction and CD147/EMMPRIN Cell Surface Clustering*

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Wei Tang and Martin E. Hemler‡
From the Dana-Farber Cancer Institute and Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115

CD147, a regulator of matrix metalloproteinase (MMP) production, showed highly specific association with caveolin-1 on the surface of multiple cell types. CD147-caveolin-1 complex formation was temperature and cholesterol dependent, reminiscent of associations seen within caveolae/lipid rafts. However, the subset of caveolin-1 associated with CD147 appeared exclusively within intermediate density sucrose gradient fractions, rather than in the low density fractions containing the bulk of caveolin-1. Mutagenesis experiments revealed that CD147 Ig domain 2 was required for caveolin-1 association. In contrast to CD147-caveolin-1 complexes, CD147-α2 integrin association was not disrupted upon cholesterol depletion, occurred in high density sucrose fractions, and did not involve CD147 Ig domain 2. Overexpression of caveolin-1 caused a specific decrease in clustering of cell surface CD147, as detected by “cluster specific” mAb M6/13. Conversely, a mutant CD147 deficient in caveolin-1 association showed enhanced spontaneous cell surface clustering (detected by mAb M6/13), and did not show decreased clustering in response to caveolin-1 overexpression. Furthermore, the same CD147 mutant yielded an elevated induction of MMP-1. In conclusion, caveolin-1 associates with CD147, in a complex distinct from CD147-α2 integrin complexes, thereby diminishing both CD147 clustering and CD147-dependent MMP-1-inducing activity.

Stromal fibroblasts secrete multiple matrix metalloproteinases (MMPs) that can promote tumor cell growth, survival, invasion, angiogenesis, and metastasis (1–3). A search for MMP-inducing tumor cell factors led to discovery and characterization of CD147/EMMPRIN (4, 5). This molecule, on the surface of carcinoma cells or in recombiant soluble form, stimulates production of MMP-1 (interstitial collagenase), MMP-2 (gelatinase A), and MMP-3 (stromelysin) but not TIMP-1 (4–7). CD147 associates with integrins in extracellular matrix degradation, and elevated tumor growth and metastasis in nude mice (15). In summary, CD147 on tumor cells stimulates MMP production by stromal cells and/or other tumor cells, thereby leading to extracellular matrix degradation, and elevated tumor growth and metastasis. The MMP-inducing functions of CD147 at least partly involve CD147 acting as a counter-receptor for itself (16).

Also, CD147 stimulates production of MMP-1 and MMP-3 in rheumatoid synovial tissue (17), and may facilitate erythrocyte circulation (18), and development of the thymus (19) and retina (20). Mice lacking the gene for CD147/EMMPRIN (called basigin in mice) showed defects in spermatogenesis and female fertilization (21, 22), an altered mixed lymphocyte reaction, and loss of an aversive response to a strong odor (23). A cytotoxic antibody to CD147 has shown promising results in the treatment of graft versus host disease, presumably by selectively targeting activated B and T lymphocytes (24). These diverse results emphasize the significance of CD147 and underscore the need for detailed understanding of its mechanism of action.

The variably glycosylated CD147 protein (32–60 kDa) contains two extracellular immunoglobulin domains, a transmembrane domain, and a 39-amino acid cytoplasmic domain (25). The homophilic counter-receptor binding activity of CD147 requires the first Ig domain (16). Inhibitors of CD147 homophilic interactions (bivalent CD147-Fc protein, monoclonal antibody) also inhibited MMP production and MMP-dependent invasion through Matrigel basement membrane (16).

A monoclonal antibody screen for αβ integrin-associated proteins revealed a robust and possibly direct association of the structurally similar αβ1 and αβ2 integrins (but not αβ3 or αβ5 integrins) with CD147/EMMPRIN (26). However, at present, there is no evidence to suggest that CD147 alters integrin function. CD147 also associates with the monocarboxylate transporters MCT1 and MCT4 (27), and facilitates their targeting to plasma membrane, where they act as lactic acid transporters. Studies using CD147 chimeras indicated that the cytoplasmic tail and/or transmembrane regions of CD147 may be particularly important for association with MCT1 (a protein that itself spans the membrane 12 times) (27).

Here we show that a population of CD147 (distinct from the integrin-associated population) co-immunoprecipitates with caveolin-1, with a resulting negative effect on CD147 cell surface clustering and function. The association of caveolin-1 with Ig superfamily proteins has generally not been observed. Instead, caveolin-1 is known primarily as an integral membrane protein, which oligomerizes and plays a central role in the formation of flask-shaped membrane structures known as caveolae (28). Caveolin-1 can associate either directly or indirectly...
rectly with a variety of signaling molecules (growth factor receptors, G proteins, Src family kinases, etc.), sometimes resulting in down-regulation of signaling function (28, 29). This down-regulation of critical signaling pathways is consistent with the oncosequossitive effects of caveolin-1 (29). In this regard, caveolin-1 can suppress cell proliferation, while being generally elevated on normal cells, but mutated or diminished on cancer cells (30). Caveolin-1 also can inhibit tumor cell invasion, soft agar growth, and MMP production (31). We suggest that caveolin-1 down-regulation of CD147 functions could contribute, at least in part, to the oncosequossitive effects of caveolin-1, and help to explain caveolin-1 effects on MMP production.

MATERIALS AND METHODS

Antibodies and Cell Lines—Antibodies to CD147 were rabbit polyclonal antiserum B10 and mAb 8G6 (16, 26), and mAb M6/13 and A3A6 (32, 33). Anti-caveolin-1 rabbit polyclonal was from BD Transduction Laboratories, San Diego, CA. Anti-GFP mAb 3E6 was from Obobiene, Carlsbad, CA, and anti-GFP rabbit polyclonal was from Clontech. Antibodies to integrins αm (mAb, IIE10), αv (mAb, A3X8; pAb, D23), and β1 (mAb, TS2/16) and to tetraspanins CD151 (mAb 5C11) and CD81 (mAb M38) were referenced previously (34). Human cell lines from embryonic kidney (HEK293), carcinoma (A431 and MDA231), and fibrosarcoma (HT1080) were grown in Dulbecco’s modified Eagle’s medium supplemented with antibiotics (penicillin and streptomycin) and 10% fetal calf serum. Transfected cell lines were maintained in Dulbecco’s modified Eagle’s medium with 0.5–1 mg/ml G418. Primary fibroblast cell line NHDF-AD was purchased from Cambrex, East Rutherford, NJ. Whereas A431 and HT1080 cells, with relatively high caveolin-1 levels, were used for endogenous studies; HEK293 cells, with relatively low levels, were used for caveolin-1 transfection studies. HT1080 cells, with relatively low levels of endogenous CD147, were preferred for studies of transfected wild type and mutant CD147. Using recombinant PCR, CD147 and caveolin-1 cDNAs were inserted into vector pEGFP-N1 (Clontech) to obtain CD147-GFP and caveolin-1-GFP, and CD147 cDNA was inserted into vector gDiRed1-N1 (Clontech) to obtain CD147-RFP. Immunoprecipitation and Immunoblotting—Cells (3 × 10^6) were lysed in 1.5 ml of immunoprecipitation buffer (1% Brij 96, 25 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM MgCl2, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin) for 2–4 h at 4°C. After centrifugation (12,000 rpm, 10 min), lysates were incubated with protein A-conjugated beads overnight to remove nonspecific binding material. After incubation with antibody for 1 h, Protein A-conjugated beads were added and further incubated at 4°C overnight, followed by four washes with lysis buffer. Immunocomplexes were eluted from beads with Laemmli sample buffer, resolved by SDS-PAGE under non-reduced conditions, and transferred to nitrocellulose membranes (BA58, 0.45 μm, Schleicher & Schuell). After blocking with PBST (PBS, 0.1% Tween 20) containing 5% powdered skim milk for 1 h, blots were incubated for 2 h or overnight with primary antibody in the same buffer as above, washed three times with PBST, and then incubated with secondary antibody. All bands were detected using an ECL detection kit (RPN 2106, Amersham Biosciences).

RESULTS

CD147-Caveolin-1 Association Is Specific, Reciprocally Demonstrated, and Occurs on the Cell Surface—While studying CD147-integrin associations, we uncovered surprising evidence that CD147 could associate with caveolin-1. As shown, we recovered caveolin-1 not only from whole cell lysates of A431 carcinoma cells (Fig. 1, lane h), and from caveolin-1 immunoprecipitates (Fig. 1, lanes f and j), but also from CD147 immunoprecipitates (Fig. 1, lanes e and i). Although tetraspanins CD81 and CD151, and integrin αv and integrin β1 subunits were expressed at high levels, we did not observe any caveolin-1 co-immunoprecipitated with those molecules (Fig. 1, lanes b–d and h). Likewise we did not recover caveolin-1 from immunoprecipitates of other highly expressed cell surface molecules including CD98, CD44, CD71 (transferrin receptor), or from other transmembrane Ig superfamily proteins (VCAM-1, EWI-2) (not shown). In a separate experiment, using HEK293 cells co-transfected with CD147-RFP and caveolin-1-GFP, immunoprecipitates of CD147 again yielded caveolin-1 (Fig. 1C, lower panel, lane b), and conversely, immunoprecipitates of caveolin-1 yielded CD147 (top panel, lane c). Thus, association can be demonstrated reciprocally. If cells were lysed in 1% Triton X-100 instead of 1% Brij 96, CD147-caveolin-1 association was not observed (not shown).

Next we determined that CD147-caveolin-1 complexes appear on the cell surface. The cell surface subset of CD147 was engaged on HT1080 cells, using mAb 8G6. Subsequent isolation of these CD147-containing immune complexes (top panel) revealed an abundance of associated caveolin-1 (Fig. 2, lower panel, lane b). After removal of cell surface CD147 complexes, CD147 remaining in the lysates (IN, intracellular) was isolated, with caveolin-1 associated at diminished levels (Fig. 2A, lower panel, lane c). In a separate experiment, cell surface CD147-GFP was engaged on HEK293 cells, using an anti-GFP mAb. Again, an abundance of associated caveolin-1 was observed (Fig. 2B, lower panel, lane f). In contrast, the intracellular pool of CD147 contained markedly less caveolin-1 (Fig. 2B, lower panel, lane g). In control experiments, incubation of intact cells with control Ig did not yield any associated caveolin-1 from the subsequently prepared Brij 96 lysate. Also, immunoprecipitation of total CD147 from lysate (T, total; i.e. without prior removal of the cell surface subset) yielded only a modest amount of associated caveolin-1 (Fig. 2B, lower panel, lane e). As seen from the top panels, CD147 was recovered at similar levels from surface and intracellular fractions, and at a higher level from total lysate. These results indicate (a) that caveolin-1 is indeed associated with cell surface CD147 in multiple cell types, and (b) that caveolin-1 recovery is more efficient when anti-CD147 antibodies are added to intact cells prior to lysis. In another experiment, immunoprecipitation of caveolin-1 from Brij 96 lysate of A431 cells yielded cell surface biotin-labeled CD147 (not shown), thus confirming the cell surface location of CD147-caveolin-1 complexes.

CD147 Associates with a Distinct Subset of Caveolin-1, in a Temperature- and Cholesterol-Dependent Manner—Several
proteins associate indirectly with caveolin-1 in the context of lipid-containing membrane microdomains, such that associations are stabilized at 4 °C, and dependent on cholesterol (29, 35–37). Indeed, association of CD147 with caveolin-1 was largely eliminated when immunoprecipitated CD147 samples were incubated at 37 °C (Fig. 3A, lane d) instead of 4 °C (lane c). Recovery of caveolin-1 was unaffected by temperature changes (lanes e and f), and no caveolin was associated with negative control α2 integrin (lanes a and b). In addition, association of CD147 with caveolin-1 was progressively lost upon preincubation of intact A431 cells with increasing doses of the cholesterol-depleting agent methyl-β-cyclodextrin (Fig. 3B). Whereas CD147 association with caveolin-1 was markedly diminished upon cholesterol depletion (Fig. 3, B, and C, lane d) CD147 association with α5 integrin was not (Fig. 3C, upper panel, compare lanes c and d). Likewise, recovery of α5 from A431 cell lysates was unaffected (upper panel, lanes e–g). No caveolin was associated with either α2 or α5 integrins (Fig. 3C, lower panel, lanes a, b, e, and f).

Complexes containing caveolin-1 typically appear in the low-density “light membrane” fractions of sucrose gradients (38). Indeed, from Brij 96 lysates of A431 cells, the bulk of caveolin-1 appeared in fractions (lanes 1–5) containing low density (5–35%) sucrose (Fig. 4C). In contrast, fractionated CD147 (Fig. 4A, lanes 4–8) was associated with a subset of caveolin-1 (Fig. 4B) that was mostly restricted to intermediate density fractions (lanes 6 and 7; ~35–40% sucrose). Thus, CD147-caveolin-1 complexes differ from the majority of other caveolin protein complexes, which are found in light membrane fractions (28). Upon depletion of cholesterol with MβCD, CD147 shifted from intermediate fractions toward the dense fractions in sucrose gradients (not shown). In a control experiment, CD71, representative of typical cell surface proteins, was largely confined to the dense fractions (Fig. 4D). In a separate experiment, CD147 was fractionated and associated α5 integrin was shown to be largely present in the dense fractions (Fig. 4F, lanes 8–11), and aligned more closely with total α5 integrin (Fig. 4G, lanes 8–11) than with CD147 (Fig. 4E, lanes 5–10). Results shown in Figs. 3 and 4 support the idea that CD147-caveolin association is indirect, and dependent on cholesterol, while occurring in the context of an atypical lipid-containing environment.

**CD147 Ig Domain 2 Plays a Critical Role in Caveolin-1 Association**—To map the site in CD147 required for caveolin-1

![Figure 2](https://example.com/figure2.jpg) **CD147-caveolin-1 association occurs on the cell surface.** A, intact HT1080 cells were incubated with mouse IgG or anti-CD147 mAb 8G6 at 4 °C for 1 h, washed, and lysed in 1% Brij 96. CD147 immuno- complexes were collected by binding to protein A-Sepharose (lanes a and b; S, cell surface), and then the remaining lysate was used for another immunoprecipitation (IP) of CD147 (lane c; IN, intracellular). B, in a separate experiment, HEK293 cells dual-transfected with CD147-GFP and caveolin-RFP were incubated with mouse IgG or anti-GFP mAb 3E6 at 4 °C for 1 h, cells were washed, lysed, and then immune complexes were analyzed (S, surface; lanes d and f). Following removal of cell surface CD147, remaining CD147 was immunoprecipitated (IN, intracellular; lane e). For one sample, anti-CD147 mAb 8G6 was added only after lysis (T, total; lane e). Immunoprecipitates were blotted for CD147 (polyclonal B10) and caveolin-1. Note that sizes of CD147 and caveolin-1 in panel B reflect the presence of RFP and GFP tags. IB, immunoblotted.

![Figure 3](https://example.com/figure3.jpg) **CD147-caveolin association is dependent on temperature and cholesterol.** A, integrin α5, CD147, and caveolin-1 were immunoprecipitated (IP) from lysates of A431 cells, and then protein A beads containing immobilized immune complexes were incubated at either 4 or 37 °C for 30 min, washed, and then caveolin-1 was analyzed by blotting (IB). Similar temperature effects have been seen in multiple experiments. B, intact A431 cells were treated with 0, 10, 20, and 40 mg/ml MβCD for 30 min at 37 °C, lysed using 1% Brij 96, and then CD147 was immunoprecipitated (mAb 8G6), and immune complexes were blotted for CD147 (upper panel) and caveolin-1 (lower panel). C, intact A431 cells were treated with or without 20 mg/ml MβCD for 30 min at 37 °C, lysed using 1% Brij 96, and then CD147 was immunoprecipitated (mAb 8G6), and immune complexes were blotted for CD147 (upper panel) and caveolin-1 (lower panel). IB, immunoblotted.

![Figure 4](https://example.com/figure4.jpg) **Sucrose density gradient analysis of CD147 protein complexes.** A431 cells (1–2 × 10⁶) were lysed in 2 ml of Mes-buffered saline (MBS; 25 mM Mes, pH 6.5, 0.15 M NaCl) containing 1% Brij 96 and protease inhibitor for 2–4 h, passed through a 22-gauge needle to shear DNA, and then Dounce homogenized for 10 strokes. The homogenate was adjusted to 40% sucrose by addition of 80% sucrose prepared in MBS and placed at the bottom of an ultracentrifuge tube, and then centrifuged at 45,000 rpm for 18–20 h in a SW41 rotor in a discontinuous 5, 35, and 45% sucrose density gradient (38). 12 fractions of 400 μl each were collected from the top of the gradient, and then CD147 complex was immunoprecipitated from each fraction, using mAb 8G6. A, samples were resolved by SDS-PAGE, and blotted using anti-CD147 antibody B10. B, the same samples were also blotted for caveolin-1. Similar results have been obtained in multiple experiments. C, samples from each lysate fraction were directly blotted with anti-caveolin-1 antibody, or D, with anti-CD71 antibody HEB21. E, in a separate experiment, sucrose gradient fractions were prepared, and CD147 was immunoprecipitated (IP) using mAb 8G6, and then blotted using polyclonal B10. In panels A and E, CD147 appears in both its high and low glycosylation form. F, CD147 immunoprecipitates were also analyzed by blotting for α5 integrin, using polyclonal D23. G, lysate fractions were directly analyzed for α5 using polyclonal D23.
to EWI-2 Ig domain 4, CD2 Ig domain 2, and VCAM-1 Ig domain 6, respectively. Mutants of CD147 were obtained using recombinant PCR, while deletion or replacement of CD147 domain 2 had no effect on association with caveolin-1. Deletion or replacement of the cytoplasmic tail did not. Deletion of the cytoplasmic domain alone (lane c) or deletion of Ig domain 1 (lane d) minimally affected caveolin-1 association. These results suggested that Ig domain 2 plays a critical role in the association. To test further the importance of CD147 Ig domain 2, it was replaced either with Ig domain 4 from EWI-2 (mutant D2c, lane f) or with Ig domain 2 from CD2 (mutant D2c, lane k) and in both cases caveolin-1 association was almost completely abolished. In addition, deletion of CD147 Ig domain 2 or Ig domains 1 and 2 abolished caveolin-1 association, whereas deletion of the cytoplasmic tail did not. Deletion or replacement of CD147 domain 2 had no effect on association with αvβ3, which recognizes clustered CD147 (32). Cells were also stained with control IgG and anti-CD151 mAb 5C11. Antibodies were detected using fluorescein isothiocyanate-conjugated secondary antibody (BIOSOURCE, Camarillo, CA), and quantitated using a FACSCalibur flow cytometer (BD Biosciences).

Possible Functional Relevance of Caveolin-1 Association—To begin to understand the functional importance of CD147-caveolin-1 association, we overexpressed caveolin-1 in HEK293 cells (about 8-fold over endogenous) and then analyzed its effect on cell surface clustering of CD147, using previously defined “cluster specific” monoclonal antibody M6/13 (32). As indicated, appearance of the M6/13 epitope on CD147 was markedly diminished (Fig. 6), whereas another epitope, defined by mAb 8G6, was only minimally affected. The M6/13/8G6 ratio, calculated from mean fluorescence intensities, was consistently reduced by ~45%. In control experiments, neither background IgG staining, nor staining of irrelevant protein CD151 were affected by caveolin-1 overexpression. We next hypothesized that if caveolin-1 association leads to decreased clustering, accompanied by decreased M6/13 epitope appearance, then loss of caveolin-1 association should lead to enhanced M6/13 epitope expression. Indeed, the M6/13 epitope was selectively enhanced upon expression of the CD147-D2c mutant (lacking caveolin-1 association) compared with wild type CD147 in HT1080 cells (Fig. 7, bottom panel). In contrast, mutant and wild type CD147 yielded similar levels of the epitope defined using mAb 8G6 (Fig. 7, third panel). The M6/13/8G6 ratio, calculated from mean fluorescence intensities, more than doubled (from 9.8 up to 20.8%). In control experiments, antibody binding to αv integrin and background IgG binding were unaffected by the CD147 mutant.
was utilized to analyze only the gated, GFP-positive HT1080 cells. F(ab')2 anti-mouse R-phycoerythrin-conjugated secondary antibody then analyzed as described in the legend to Fig. 6, except that goat anti-mouse R-phycoerythrin-conjugated secondary antibody was utilized to analyze only the gated, GFP-positive HT1080 cells.

lin-1 overexpression should have less effect on CD147-D2c, compared with wild type CD147. Indeed, when CD147-D2c was transiently expressed in 293 cells already overexpressing caveolin-1 or not overexpressing caveolin-1, the M6/13/8G6 ratio (~27%) was essentially the same (Table I). In contrast, transiently expressed wild type CD147 did show a significant decrease in the M6/13/8G6 ratio (from 41% down to 27%; Table I) because of the increased caveolin-1 expression.

Finally, we looked for the effects of altered caveolin-1 association and clustering on the MMP-1 inducing activity of CD147. Wild type and mutant CD147 were expressed on HT1080 cells at similar levels either 10-fold (Experiment 1) or 2-fold (Experiment 2) greater than endogenous CD147. Upon co-culture of these cells with primary human dermal fibroblasts, MMP-1 activity was substantially enhanced above that seen with either cell type alone (Fig. 8). Importantly, in both cases the level of MMP-1 activity was significantly increased when CD147-D2c was present. These results suggest that loss of caveolin-1 association not only enhances CD147 clustering, but also leads to elevated MMP inducing activity.

DISCUSSION

Characterization of the CD147–Caveolin-1 Complex—Here we demonstrate an association between an Ig superfamily protein, CD147, and caveolin-1, based on co-immunoprecipitation in three different cell types and reciprocal co-immunoprecipitation. The interaction was specific, as antibodies to tetraspanins (CD81 and CD151), integrins (α2, α3, and β1 subunits) and other cell surface proteins (CD98, CD44, and CD71) did not yield caveolin-1 under our 1% Brij 96 lysis conditions. Also, other Ig superfamily proteins (CD2, EWI-2, and VCAM-1) did not show caveolin-1 association. The CD147–caveolin-1 complex appeared on the cell surface, as seen in both cell surface antibody binding and cell surface labeling experiments. Also CD147–caveolin-1 association was temperature- and cholesterol-dependent, consistent with caveolin associations with many other proteins (29, 35–37). However, the CD147–caveolin-1 complex is atypical for two reasons. First, the CD147–caveolin-1 complex was retained in 1% Brij 96, but solubilized and disrupted in Triton X-100, whereas the majority of cell surface caveolin complexes were resistant to 1% Triton X-100 extraction. Second, the small subset of caveolin-1 associated with CD147 is not localized with the bulk of caveolin-1 in the detergent-insoluble light membrane fractions of sucrose gradients, but instead appears in intermediate density fractions. Differences in solubility between CD147–caveolin and other caveolin-1 complexes implies differences in the content and extent of ordered packing of their surrounding lipids. Differential solubilization of other lipid raft/caveolar proteins in Triton X-100 and Brij 96 has been noted (40). It was suggested that the most detergent-resistant proteins reside in highly ordered domains stabilized by cholesterol and sphingolipids, whereas other lipid raft/caveolar proteins may reside in semi-ordered lipid domains, possibly at the periphery of the highly ordered domains (40). It has not yet been determined whether CD147–caveolin-1 complexes reside at the periphery of caveolae. Whereas the bulk of plasma membrane-associated caveolin is typically resistant to non-ionic detergent solubilization, the presence of ganglioside GM3 can shift caveolin-1 to a more detergent soluble state (41, 42). It remains to be determined whether CD147–caveolin-1 complexes are enriched for GM3, thus perhaps helping to explain the atypical solubility of caveolin-1 that is associated with CD147.

Mapping of Critical CD147 Domains—Extensive CD147 mutagenesis experiments demonstrated that Ig domain 2 was required for caveolin-1 association, whereas other domains, including Ig domain 1, transmembrane, and the cytoplasmic tail were dispensable. Although CD147 Ig domain 2 contains two N-linked glycosylation sites, these are not required, because when those sites were mutated, caveolin-1 association was not eliminated (not shown). It is not yet known what specific structural feature of CD147 Ig domain 2 sets it apart from the membrane-proximal Ig domains of CD2, EWI-2, and VCAM-1, which do not support caveolin-1 association. What connects the extracellular Ig domain 2 of CD147 to caveolin-1, an integral membrane protein? One possibility is CD147-associated multimembrane spanning monocarboxylate transporters (MCT1 and MCT4 (27, 43). However, replacement of the transmembrane domain did not eliminate caveolin-1 association because of loss of a required membrane-embedded glutamic acid (44), but our replacements of the CD147 transmembrane domain did not eliminate caveolin-1 association. Instead we suspect that Ig domain 2 of CD147 may contain an unidentified caveolae/lipid raft targeting signal. In this regard, another transmembrane protein, EGFR, utilizes its membrane proximal extracellular domain for targeting to caveolin containing regions of the cell (45). Also, protein ectodomains of CD2 (46) and the PrP prion (47) target those proteins to lipid rafts. Hence, there is growing precedent for cell surface proteins using proteinaceous ectodomain signals for targeting to caveolae/rafts, but the recognition partners for these signals are not yet known.

CD147 association with caveolin-1 required Ig domain 2, was temperature- and cholesterol-dependent, and occurred within intermediate density fractions of a sucrose density gradient. In marked contrast, CD147 association with α2β1 integrin did not involve caveolin-1 and was unaffected by Ig domain 2 removal, depletion of cholesterol, or elevation of temperature to 37 °C, and occurred within distinct (high density) fractions of a sucrose gradient. The CD147–α2β1 interaction required CD147 Ig domain 1 (not shown) instead of domain 2. Hence, α2β1--CD147 complexes are quite distinct from CD147–caveolin-1 complexes,
CD147 Links to Caveolin-1

Table I

| Cell type            | IgG<sup>a</sup> | M6/13<sup>b</sup> | 8G6<sup>a</sup> | M13/8G6 ratio |
|----------------------|----------------|------------------|----------------|----------------|
| 293-wtCD147          | 2.7            | 186              | 442            | 41%            |
| 293-cav-wtCD147      | 9.2            | 124              | 429            | 26.7%          |
| 293-mCD147           | 2.7            | 85.1             | 1997           | 27.4%          |
| 293-cav-mCD147       | 8.5            | 99.1             | 328            | 27.0%          |

Numbers in these columns represent mean fluorescence intensity values. HEK293 cells stably expressing either wtCD147 or mCD147 (= CD147-D2c), and then populations of gated GFP-positive cells were analyzed for mAb M6/13 or 8G6 binding by flow cytometry as in Figs. 6 and 7. Ratios are determined after subtracting background IgG staining values.

Fig. 8. Loss of CD147 Ig domain 2 corresponds to increased MMP-1 production. HT1080 cells stably expressing either wild type CD147 or CD147-D2c mutant (domain 2 exchanged with CD2 domain 2) were co-cultured with primary human fibroblasts (2:3 ratio) for 12 h in 10% fetal calf serum, and then for 48 h in the absence of serum. Culture medium was collected, and analyzed in triplicate for MMP-1 activity in a colorimetric assay measured at OD<sub>405 nm</sub> (Biotrak kit, Amersham Biosciences). In experiments 1 and 2, wild type and mutant CD147 are each transiently transfected with either wtCD147 or mCD147 (= CD147-D2c), and then populations of gated GFP-positive cells were analyzed for mAb M6/13 or 8G6 binding by flow cytometry as in Figs. 6 and 7. Ratios are determined after subtracting background IgG staining values.

such that α<sub>iβ</sub> cannot provide a link between CD147 and caveolin or caveolae/rafts. Using antibodies to integrin β<sub>i</sub>, α<sub>iα</sub>, α<sub>i</sub>, and associated CD151 proteins we have consistently failed to co-immunoprecipitate any detectable caveolin from A431 cells or any other cell line, solubilized either in 1% Triton X-100 or 1% Brij 96 (e.g. see results here and elsewhere (48)). In contrast, other groups have reported co-immunoprecipitation of caveolin-1 with β<sub>i</sub> integrins from A431 cells and other cells (49–51). We suspect that engagement of intact cellular integrins with antibody-coated beads (49, 50) is likely to yield considerably larger complexes than we would obtain using soluble anti-integrin antibodies added to cell lysates. Also, we have found that incomplete solubilization, because of a low detergent/protein ratio, can yield many β<sub>i</sub> integrin-associated proteins including caveolin-1 (not shown).

Consequences of Caveolin-1 Association—Whereas increased caveolin-1 expression led to decreased CD147 clustering in HEK293 cells, expression of our “D2c” mutant that lacks caveolin-1 association showed increased spontaneous clustering (compared with wild type CD147) in HT1080 cells. Furthermore, CD147-D2c did not show diminished clustering in response to caveolin-1 expression in HEK293 cells. Hence caveolin-1 association and CD147 clustering are inversely related. For clustering experiments, we took advantage of previously defined cluster-specific anti-CD147 mAb tools (32). These antibodies have low affinity for CD147 (k = 2–6 × 10<sup>8</sup> M<sup>–1</sup> s<sup>–1</sup>), and hence depend on bivalent binding, which is most readily observed when CD147 is clustered (32).

For several reasons we suggest that negative regulation of CD147 clustering by caveolin-1 leads to decreased CD147-dependent MMP induction. First, our CD147-D2c mutant that lost caveolin-1 association and showed increased clustering also showed increased MMP-1 induction. Second, overexpression of caveolin-1 in HEK293 cells not only caused decreased clustering (this paper), but also decreased MMP-1 production by 35–40% in a co-culture assay with primary human fibroblasts (not shown), although we realize that such results are hard to interpret because caveolin-1 could conceivably have multiple other effects on MMP induction. Third, anti-CD147 cluster-specific mAb AAA6 (32) inhibited MMP production in cell co-culture assays (16), again linking clustering to MMP production. Fourth, our preliminary evidence<sup>2</sup> indicates that highly glycosylated CD147 does not associate with caveolin, but does trigger MMP production, and is more susceptible to covariant cross-linking, consistent with increased clustering. Fifth, additional data<sup>2</sup> shows that knockdown of caveolin-1 levels by RNAi leads to a shift in CD147 toward its more active, more highly glycosylated, clustered form. Sixth, for cell surface receptors in general, increased clustering is typically associated with receptor activation.

In conclusion, cell surface IgSF protein CD147 shows the unusual property of ectodomain-dependent association with caveolin-1, in a complex with atypical solubility and density characteristics. Furthermore, our evidence supports a model in which MMP induction is mediated by clustered CD147, with caveolin-1 exerting a negative regulatory role on both clustering and MMP induction. Such a mechanism may at least partially explain tumor suppressor effects of caveolin-1.

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Wei Tang and Martin E. Hemler

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