Metabolic Activation-related CD147-CD98 Complex* [S]

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Cell surface CD147 protein promotes production of matrix metalloproteinases and hyaluronan, associates with monocarboxylate transporters and integrins, and is involved in reproductive, neural, inflammatory, and tumor functions. Here we combined covalent cross-linking, mass spectrometric protein identification, and co-immunoprecipitation to show selective CD147 association with three major types of transporters (CD98 heavy chain (CD98hc)-L-type amino acid transporter, ASCT2, and monocarboxylate transporters) as well as a regulator of cell proliferation (epithelial cell adhesion molecule). In the assembly of these multicomponent complexes, CD147 and CD98hc play a central organizing role. RNA interference knock-down experiments established a strong connection between CD147 and CD98hc expression and a strong positive association of CD147 (and CD98hc) with cell proliferation. As the CD147-CD98hc complex and proliferation diminished, AMP-activated protein kinase (a cellular “fuel gauge”) became activated, indicating a disturbance of cellular energy metabolism. Our data point to a CD147-CD98 cell surface supercomplex that plays a critical role in energy metabolism, likely by coordinating transport of lactate and amino acids. Furthermore we showed how covalent cross-linking, together with mass spectrometry, can be used to identify closely associated transmembrane proteins. This approach should also be applicable to many other types of transmembrane proteins besides those associated with CD98hc and CD147.

Molecular & Cellular Proteomics 4:1061–1071, 2005.

CD147 (extracellular matrix metalloproteinase inducer (EMMPRIN), basigin, neurothelin, tumor cell-derived collagenase-stimulatory factor (TCSF), OX-47, 5A11, CE9, gp42, M6) is a cell surface protein with multiple glycosylated forms (1, 2). CD147 is ubiquitously expressed with highest levels on metabolically active cells, such as lymphoblasts (3), inflammatory cells (4), brown adipocytes (5), and malignant tumors (1). CD147 promotes production of matrix metalloproteinases (MMPs)\(^1\) (1) and hyaluronan (6) and is involved in reproduction, neural function, inflammation, tumor invasion, and human immunodeficiency virus infection (1, 7, 8). As a chaperone for monocarboxylate transporters (MCT1 and MCT4), CD147 enables insertion of MCT1 and MCT4 into cell membranes, which facilitates import and/or export of lactate and pyruvate (9, 10). CD147 also interacts with \(\beta1\) integrin (11), cyclophilin A (7), and caveolin-1 (12). Caveolin-1 association appears to restrict CD147 glycosylation and function (2). Consistent with the functional importance of CD147, CD147\(^{−/−}\) mice are sterile, have reduced body weight, and show impaired spermatogenesis, sensory, learning, and memory functions. Half of the surviving mice subsequently die of pneumonia (13). In some genetic backgrounds, the majority of mouse embryos lacking CD147 die at around the time of implantation (14).

Emerging proteomic technologies are helping to elucidate cellular protein-protein interaction networks (15, 16). However, elucidation of cell surface transmembrane protein interactions has lagged behind often due to issues involving detergent solubilization. To understand better the remarkably diverse functions of CD147, we sought to identify its major cell surface protein partners. First we used homobifunctional cross-linking agents to stabilize protein interaction networks on intact cells, and then we lysed cells using relatively harsh detergent conditions to disrupt non-cross-linked complexes. Next we immunisolated cross-linked CD147 complexes and used nanoscale LC-MS/MS to identify all transmembrane proteins in these complexes. Our data point to CD147 interacting not only with monocarboxylate transporters but also with amino acid transporters (CD98 heavy chain (CD98hc)-LAT1 and ASCT2) and a regulator of cell proliferation (EpCAM).

Like CD147, CD98hc (4F2, fusion-regulatory protein-1) also is a multifunctional glycoprotein with a single transmembrane domain, is highly expressed on proliferating cells, and functions as a chaperone for transporters (17, 18). Indeed CD98hc forms disulfide-bonded heterodimers with at least six different light chains (\(\gamma\)-type amino acid transporter 1 (LAT1), LAT2, y+LAT1, y+LAT2, Asc-1, and xCT) that serve as amino acid transporters; mAb, monoclonal antibody; pAb, polyclonal antibody; MHC, major histocompatibility complex; MCT, monocarboxylate transporter; mTOR, mammalian target of rapamycin; RNAi, RNA interference; SDFR1, stromal cell-derived factor receptor 1; GFP, green fluorescent protein; HEK, human embryonic kidney; IP, immunoprecipitation.

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Received, December 16, 2004, and in revised form, April 12, 2005

Published, MCP Papers in Press, May 18, 2005, DOI 10.1074/mcp.M400207-MCP200

\(^1\) The abbreviations used are: MMP, matrix metalloproteinase; AMPK, AMP-activated protein kinase; BS3, bis(sulfosuccinimidyl)suberate; hc, heavy chain; DSP, dithiobis(succinimidyl)propionate; hC, heavy chain; DSP, dithiobis(succinimidylpropionate); MHC, major histocompatibility complex; MCT, monocarboxylate transporter; mTOR, mammalian target of rapamycin; RNAi, RNA interference; SDFR1, stromal cell-derived factor receptor 1; GFP, green fluorescent protein; HEK, human embryonic kidney; IP, immunoprecipitation.

EpCAM, epithelial cell adhesion molecule; LAT, \(\gamma\)-type amino acid transporter; mAb, monoclonal antibody; pAb, polyclonal antibody; MHC, major histocompatibility complex; MCT, monocarboxylate transporter; mTOR, mammalian target of rapamycin; RNAi, RNA interference; SDFR1, stromal cell-derived factor receptor 1; GFP, green fluorescent protein; HEK, human embryonic kidney; IP, immunoprecipitation.
transporters (18). In addition, CD98hc may regulate cell fusion (19) and integrin-dependent adhesion functions (20, 21) while associating either indirectly (22) or directly (23) with β1 integrins. Our detailed analysis of CD98hc complexes confirmed CD98 association with CD147, monocarboxylate transporters, amino acid transporters (LAT1 and ASCT2), and epithelial cell adhesion molecule (EpCAM). Furthermore RNAi depletion of either CD147 or CD98hc diminished cell surface expression of both molecules and diminished cell proliferation. Together these results point to CD147 and CD98hc playing a central role within a "supercomplex" that is critical for cellular energy metabolism.

EXPERIMENTAL PROCEDURES

Antibodies—We used antibodies to CD147 (mAb 8G6; pAb B10 (11)), CD98hc (mAb 4F2 (24); pAb C-20, Santa Cruz Biotechnology), EpCAM (mAb KS1/4, BD Biosciences; pAb H-70, Santa Cruz Biotechnology), β1 integrin (mAb TS2/16), MHC class I (mAb W6/32), Na/K-ATPase α1 (mAb C464.4, Santa Cruz Biotechnology), AMP-activated protein kinase (AMPK) α and phospho-AMPKα (Cell Signaling Technology), MCT1 (pAb from Alpha Diagnostics), and β tubulin (mAb Tub2.1, Sigma). Anti-GFP (mAb 3E6, Qbiogene) was used for immunoprecipitation (IP), and anti-FLAG (mAb M2 and M2-agarose, Sigma) was used for IP and immunoblotting.

Cross-linking and LC-MS/MS—Intact cells were grown to 90% confluence in five 150-mm plates and then were washed three times with PBS. Cross-linkers DSP or BS3 (Pierce) were added to 1 mm (final concentration) in 20 ml Heps buffer, pH 7.5, 150 mM NaCl, and 5 mM MgCl₂ for 30 min at room temperature or for 1 h at 4 °C before termination with 25 mM glycine (pH 7.5). After washing with PBS, cells were lysed with lysis buffer (25 mM Hepes, pH 7.5, 150 mM NaCl, and 5 mM MgCl₂ supplemented with 1% Triton X-100 (Roche Applied Science) with proteinase inhibitor mixture (Roche Applied Science)) at 4 °C for 1 h. Lysate was centrifuged at 20,000 × g for 30 min, and the supernatant was precleared with 2 ml of protein A-agarose for 2 h at 4 °C. After centrifugation (6000 × g for 20 min), the supernatant was mixed with 1 ml of anti-FLAG M2-agarose (Sigma) or mAb 4F2-conjugated beads and shaken overnight at 4 °C. Beads were then washed six times with 30 ml of lysis buffer. Complexes were eluted with 2.5 ml of 100 mM glycine, pH 2.5, and then neutralized with 200 μl of 1× Tris–HCl, pH 7.5. Concentrated eluate (Centricon, Millipore) was resolved by SDS-PAGE using reducing conditions for BS3-cross-linked samples and non-reducing conditions for DSP-cross-linked samples. All Coomassie Blue-stained bands larger than 40 kDa were excised and sent to the Taplin Biological Mass Spectrometry Facility (Harvard Medical School). For protein identification, excised SDS-polyacrylamide gel bands were chopped into 1-mm³ pieces, and in-gel digestion with trypsin was performed as described previously (25). All data were acquired by nanoscale microcapillary liquid chromatography coupled to a linear ion trap mass spectrometer (ThermoElectron, San Jose, CA) as described previously (26). Briefly a gradient of increasing organic modifier eluted peptides into the mass spectrometer. The instrument was set to cycle between collecting one survey scan followed by five MS/MS scans on the five most abundant ions with dynamic exclusion of ions selected previously. MS/MS spectra were extracted using Bioworks 3.1 and searched with the Sequest (version 27) algorithm against the nonredundant human data base from NCBI, which contained 237,384 sequences. Typical ion trap parameters were used including a peptide tolerance of 2.0 Da, default fragment ion tolerance, variable modification of methionine (+16), no enzyme specificity, and up to three missed cleavages allowed. Peptide matches were deemed correct when two or more high scoring, fully tryptic peptides matched to a protein. Xcorr values of 1.8, 1.8, and 3.0 were used for 1+, 2+, and 3+ peptides, respectively, and no dCorr threshold was used. Peptide matches with three or fewer tryptic matches were manually verified. Also there was no smoothing of data, signal to noise criteria, charge state determination, or peak deisotoping utilized for these experiments.

Immunoprecipitation and Western Blotting—Cells grown in 100-mm plates were lysed with 1 ml of lysis buffer for 1 h at 4 °C. Centrifugation and preclearing were carried out as above. For each immunoprecipitation, 2–4 μg of antibody with 50 μl of protein A (or protein G)-agarose beads or 50 μl of FLAG-agarose beads were used with shaking at 4 °C overnight. Beads were then washed three times, and immune complexes were eluted using 60 μl of 1× Laemmli sample buffer (non-reducing) at 100 °C for 2 min. Sample aliquots (20 μl) were resolved using SDS-PAGE, and immunoblotting and flow cytometry were carried out as described previously (11). In some experiments, after blotting, relative band densities were assessed quantitatively using GeneTools version 3 (Syngene Laboratories, Frederick, MD).

Cell Lines—Human cell lines HT1080 (fibrosarcoma), SW480 (colorectal adenocarcinoma), and MCF7 (breast adenocarcinoma) from ATCC were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and (for MCF7 cells) an additional 10 μg/ml insulin. CD147 and stromal cell-derived factor receptor 1 (SDFR1) (GenBank™ accession number Gt:20552516, with 40% sequence identity with CD147) were C-terminally FLAG-tagged, and CD98hc and LAT1 were N-terminally FLAG-tagged and inserted into pcDNA3.1 (+) or pLXII vectors. Quantitation of immunoblotted CD147 was carried out using pAb B10. Ectopic CD147-FLAG expression was 1.4-fold higher than endogenous CD147 in HT1080 cells, 1.8-fold higher in HEK293 cells, and 2.1-fold higher in MCF7 cells. Similarly we blotted with anti-FLAG antibody to show that ectopic CD147-FLAG and SDFR1-FLAG were expressed at levels comparable to each other in HT1080, HEK293, and MCF7 cells. We also prepared C-terminal GFP-tagged CD147 and SDFR1 and N-terminal GFP-tagged LAT1 using pEGFP-C3 or pEGFP-N1 vectors (Clontech). Cells were either stably transfected using FuGENE 6 (Roche Applied Science) or infected and then selected using G418 or Zeocin (Invitrogen) for 4–6 weeks.

Oligonucleotides for CD147 and CD98hc RNAi—Sense and antisense oligonucleotides were cut with BamHI and HindIII, and purified fragments were ligated into RNAi plasmid pSilencer 3.1 (Ambion) and confirmed by DNA sequencing. The RNAi negative plasmid was from the same kit (Ambion). Cells were either stably transfected and SDFR1 and N-terminal GFP-tagged LAT1 using pEGFP-C3 or pEGFP-N1 vectors (Clontech). Cells were either stably transfected using FuGENE 6 (Roche Applied Science) or infected and then selected using G418 or Zeocin (Invitrogen) for 4–6 weeks.

Oligonucleotide sequences for CD147 and CD98hc RNAi were: sense, (BamHI) 5'GGGGACACCATGCTGGTCAAGATCTTATGAGACCATGATCTTCTTCGCAAGATCATGAGTGACATGATCTTCTTGGAAAGTTCCGGC-3' (Hind III); antisense, (Hind III) 5'GGGACACCATGCTGGTCAAGATCTTATGAGACCATGATCTTCTTGGAAAGTTCCGGC-3' (Hind III). For CD98hc RNAi oligonucleotide sequences, Pair 1: sense, (BamHI) 5'GGGGACACCATGCTGGTCAAGATCTTATGAGACCATGATCTTCTTCGCAAGATCATGAGTGACATGATCTTCTTGGAAAGTTCCGGC-3' (Hind III); antisense, (Hind III) 5'GGGACACCATGCTGGTCAAGATCTTATGAGACCATGATCTTCTTGGAAAGTTCCGGC-3' (Hind III). For CD98hc RNAi oligonucleotide sequences, Pair 1: sense, (BamHI) 5'GGGGACACCATGCTGGTCAAGATCTTATGAGACCATGATCTTCTTCGCAAGATCATGAGTGACATGATCTTCTTGGAAAGTTCCGGC-3' (Hind III); antisense, (Hind III) 5'GGGACACCATGCTGGTCAAGATCTTATGAGACCATGATCTTCTTCGCAAGATCATGAGTGACATGATCTTCTTGGAAAGTTCCGGC-3' (Hind III). For CD98hc RNAi oligonucleotide sequences, Pair 1: sense, (BamHI) 5'GGGGACACCATGCTGGTCAAGATCTTATGAGACCATGATCTTCTTCGCAAGATCATGAGTGACATGATCTTCTTGGAAAGTTCCGGC-3' (Hind III); antisense, (Hind III) 5'GGGACACCATGCTGGTCAAGATCTTATGAGACCATGATCTTCTTCGCAAGATCATGAGTGACATGATCTTCTTGGAAAGTTCCGGC-3' (Hind III).
**CD147-CD98 Protein Complex**

**Cellular Proliferation Assay and Statistical Analysis**—Cellular proliferation/survival was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Roche Applied Science) colorimetric method. Cell suspensions (1 × 10⁴ cells/100 µl) were plated into 96-well plates with four wells for each cell clone in complete medium. Control wells contained either mitomycin C (Sigma) (1, 2.5, or 5 µg/ml) or Dulbecco’s modified Eagle’s medium without serum. The plates were incubated for 3 or 4 days at 37 °C prior to the addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution and measurement of absorbance at 560 nm. Each experiment was repeated three times. The relative proliferation was calculated compared with mean of RNAi control. Data on CD147 and CD98hc expression levels were assessed by flow cytometry. Each experiment was repeated three times. The correlation between CD147 expression and relative proliferation was analyzed using VassarStats statistics (faculty.vassar.edu/lowry/VassarStats.html).

**RESULTS**

**CD147 Protein Partners Identified by LC-MS/MS—FLAG-tagged CD147 was expressed in HT1080 and MCF7 cells at levels only a little higher (1.4–2.1-fold) than endogenous CD147.** Intact HT1080 cells (Fig. 1, left lane) and MCF7 cells (Fig. 1, right lane) were then treated with chemical cross-linkers (non-reducible BS3 or reducible DSP, respectively), cells were lysed in Triton X-100, CD147 complexes were isolated, and then proteins within the indicated gel regions were identified using LC-MS/MS analysis. A summary of re-

**TABLE I**

| Name (GenBank™ GI no.) | Exp. I | Exp. II | Exp. III | Exp. IV | Exp. V | Exp. VI | Exp. VII |
|------------------------|-------|--------|----------|--------|--------|--------|---------|
| CD147 (34448)          | + (7)² | + (10) | + (8)    | + (8)  | −      | −      | −       |
| CD98hc (182864)        | + (13) | + (6)  | + (6)    | −      | + (23) | + (14) | + (20)  |
| ASC2 (1478280)         | + (5)  | + (3)  | −        | + (4)  | +      | +      | +       |
| LAT1 (639057)³         | + (3)  | −      | −        | −      | +      | +      | +       |
| EpCAM (186775)         | +      | + (3)  | + (2)    | −      | −      | −      | −       |
| MCT1 (561742)²         | +      | + (4)  | +        | −      | −      | −      | −       |
| MCT4 (2463683)²        | +      | + (4)  | +        | −      | −      | −      | −       |
| MCT8 (458253)          | +      | + (3)  | −        | −      | −      | −      | −       |
| CD29 (integrin β1) (31441)²  | + (3)  | −      | −        | −      | −      | −      | −       |

| Name (GenBank™ GI no.) | Exp. I | Exp. II | Exp. III | Exp. IV | Exp. V | Exp. VI | Exp. VII |
|------------------------|-------|--------|----------|--------|--------|--------|---------|
| Na/K-ATPase, α1 (219941)⁴ | + (2)  | + (5)  | −        | −      | +      | +      | +       |
| Na/K-ATPase, β3 (1522634)⁴ | −      | −      | −        | −      | +      | +      | +       |
| Glycoprotein Trop-2 (31590) | +      | −      | −        | −      | −      | −      | −       |
| I3 binding protein (17985370) | +      | −      | −        | −      | −      | −      | −       |
| CD71 (37432)³         | +      | + (2)  | −        | + (2)  | −      | −      | −       |
| Sel-1 homolog (6683457)³ | + (2)  | −      | −        | + (2)  | −      | −      | −       |
| CD166 (866257)        | −      | + (4)  | −        | −      | −      | −      | −       |
| CD44e (integrin α5) (31437)³ | −      | +      | −        | −      | −      | −      | −       |
| Leukemia virus receptor 1 (306769)³ | −      | −      | −        | −      | −      | −      | −       |
| HLA-A, A-25 (187794)³ | −      | −      | −        | −      | −      | −      | −       |

⁻ CD98 experiments were carried out using cells (MCF7) with little CD147 to allow assessment of CD98hc partners independently of CD147.

² + indicates that at least one peptide was obtained. − indicates no peptides obtained. Number in parentheses equals the number of peptides from LC-MS/MS if >1. The sequences of all peptides are listed in Supplemental Table S1.

³ CD98hc associations with LAT1 (27) and β1 integrin (22) have been noted previously.

⁴ CD147 associations with MCT1, MCT4 (9,10), and β1 integrin (11) have been noted previously.

* These results were not consistently observed in further biochemical experiments. All other results in the bottom part of the table were not tested in further biochemical experiments.

**Fig. 1. Representative samples of CD147-associated proteins.** HT1080 cells were treated with non-reducible cross-linker BS3, CD147-FLAG complexes were isolated from 1% Triton X-100 lysates, and then samples were resolved using reducing conditions (left lane). MCF7 cells were treated with reducible cross-linker DSP, CD147-FLAG complexes were isolated from 1% Triton X-100 lysates, and samples were resolved using non-reducing conditions (right lane). After staining with Coomassie Blue, the indicated regions were excised for LC-MS/MS analysis. R, reducing; Non-R, non-reducing; H, heavy; L, light.
CD147-CD98 Protein Complex

Fig. 2. Cross-linked CD147 complexes contain CD98hc. A, transfected MCF7 cells were treated with no cross-linker (lanes 1 and 4), DSP (lanes 2 and 5), or BS3 (lanes 3 and 6) and lysed using 1% Triton X-100. CD147 (lanes 1–3) and control SDFR1 (lanes 4–6) were immunoprecipitated using anti-FLAG antibody, and samples were blotted for endogenous CD98hc using pAb C-20. B, transfected HT1080 cells were treated with or without cross-linker (as in A), CD147-FLAG or negative control (SDFR1-FLAG) was immunoprecipitated, and samples were blotted for endogenous CD98hc. C, transfected HEK293 cells were treated with or without cross-linker (as indicated), CD147-FLAG or control SDFR1-FLAG was immunoprecipitated, and samples were blotted blot for endogenous CD98hc. For cells used in A–C, CD147-FLAG and SDFR1-FLAG were expressed at cleavable) cross-linking, immunoprecipitation of CD147 proteins was blocking access of the antibodies to CD147.

![Cross-linked CD147 complexes contain CD98hc.](image)

Confirmation of CD147 Association with CD98hc—Although suggestive, results in Table I are neither definitive nor quantitative. To firmly establish direct CD147-CD98hc association, we used covalent cross-linking and co-immunoprecipitation. Following either DSP (thiol cleavable) or BS3 (uncleavable) cross-linking, immunoprecipitation of CD147 yielded endogenous CD98hc from MCF7 (Fig. 2A, lanes 2 and 3), HT1080 (Fig. 2B, lanes 2 and 3), and HEK293 (Fig. 2C, lanes 4 and 6) cells. No CD98hc was obtained when cross-linker was omitted (Fig. 2, A and B, lanes 1 and 4, and C, lanes 1 and 2) or antibodies to control SDFR1 protein were utilized (Fig. 2, A and B, lanes 4–6, and C, lanes 3 and 5). Monomeric CD98hc appears as two bands (~85 and 92 kDa) likely due to variable glycosylation. The larger size of CD98hc (~140–230 kDa) captured in CD147 complexes from uncleavable BS3-cross-linked lanes is consistent with endogenous CD98hc being covalently cross-linked to CD147 (~50 kDa) plus additional components. One likely additional component is the known CD147 partner MCT1. As indicated in Fig. 2D (lane 3), MCT1 (45 kDa) cross-linked to CD147 (50 kDa) yielded a 95-kDa complex plus a larger complex (~140–230 kDa) similar in size to that containing CD98hc. In the absence of cross-linker, MCT1 (~45,000) associated with CD147 in Brij 97 lysate (Fig. 2E) but not in Triton X-100 (not shown).

![Further characterization of CD147-CD98hc complexes.](image)
and MCT1 was seen in mild detergents (1% Brij 99 and 6 firmed in Fig. 2, precipitation of CD98hc also yielded MCT1 (Fig. 4 being critical for CD98 association (not shown). Immunopre-

Fig. 4. CD98hc (and LAT1) co-precipitate CD147 (and MCT1). A, HEK293 cells expressing CD98hc-FLAG (lanes 1, 2, and 5–9) or FLAG vector alone (lanes 3 and 4) were lysed using Triton X-100 (lanes 1, 2, 4, 8, and 9), Brij 99 (lanes 3 and 5), CHAPS (lane 6), or Brij 97 (lane 7). A portion of cells (used for lanes 2 and 9) was treated with BS3 cross-linker. Following immunoprecipitation of CD98hc-FLAG or vector control FLAG using anti-FLAG antibody, samples were blotted for CD147. CD147(HG), high glycosylated form; CD147(LG), low glyco-
sylated form (2). Note that the membrane used in A was first used in C and then stripped and reprobed with anti-CD147 antibody B10. B, LAT1-FLAG was immunoprecipitated from HEK293 cells (lysed in Brij 99 or CHAPS), and samples were blotted for CD147. C, CD98hc immunoprecipitates (prepared as in A) were blotted for MCT1. neg.c-trl, negative control; ctrl, control.

Fig. 4. CD98hc (and LAT1) co-precipitate CD147 (and MCT1). A, HEK293 cells expressing CD98hc-FLAG (lanes 1, 2, and 5–9) or FLAG vector alone (lanes 3 and 4) were lysed using Triton X-100 (lanes 1, 2, 4, 8, and 9), Brij 99 (lanes 3 and 5), CHAPS (lane 6), or Brij 97 (lane 7). A portion of cells (used for lanes 2 and 9) was treated with BS3 cross-linker. Following immunoprecipitation of CD98hc-FLAG or vector control FLAG using anti-FLAG antibody, samples were blotted for CD147. CD147(HG), high glycosylated form; CD147(LG), low glyco-
sylated form (2). Note that the membrane used in A was first used in C and then stripped and reprobed with anti-CD147 antibody B10. B, LAT1-FLAG was immunoprecipitated from HEK293 cells (lysed in Brij 99 or CHAPS), and samples were blotted for CD147. C, CD98hc immunoprecipitates (prepared as in A) were blotted for MCT1. neg.c-trl, negative control; ctrl, control.

were almost completely disrupted in Triton X-100 (Fig. 3A). Immunoprecipitation of CD147 yielded CD98hc when the two molecules were co-expressed together in HEK293 cells (Fig. 3B, lane 8) but not when expressed separately and then the cells (Fig. 3B, lane 5) or lysates (lane 4) were mixed. Hence CD147 forms a cis-interacting complex with CD98hc. Negative control SDFR1 did not associate with CD98hc, whereas positive control LAT1 (an established CD98 light chain) did associate with CD98hc (Fig. 3B, lanes 7 and 6, respectively).

In a reciprocal experiment, CD98hc immunoprecipitation yielded a large complex (170–230 kDa) containing endoge-

ous CD147 when covalent cross-linker (BS3) was added to

HEK293 cells prior to Triton X-100 lysis (Fig. 4A, lane 9) but not when BS3 was omitted (lane 8). In mild Brij 99 or CHAPS detergent, CD147 was obtained even without cross-linking (Fig. 4A, lanes 5 and 6). Additional data, involving CD147 domain depletion and swapping, point to CD147 Ig domain 1 being critical for CD98 association (not shown). Immunopre-

cipitation of CD98hc also yielded MCT1 (Fig. 4C, lanes 5 and 6), which associates directly with CD147 (9), and as we con-

firmed in Fig. 2, D and E. The association between CD98hc and MCT1 was seen in mild detergents (1% Brij 99 and CHAPS; Fig. 4C, lanes 5 and 6) but not in more stringent detergent conditions (Brij 97; lane 7) under which MCT1 still associated with CD147 (Fig. 2E). Moreover MCT1 was only minimally cross-linked with CD98hc (Fig. 4C, lane 9). Rather most of the MCT1 (45 kDa) was directly cross-linked to CD147 (50 kDa) to yield a heterodimer of ~95 kDa (Fig. 4A, lane 2, and Fig. 2D, lane 3).

Associations Mediated through CD98hc—It seemed possible that some potential CD147 partners from Experiments I–IV of Table I (e.g. LAT1, EpCAM, and ASCT2) could be more proximal to CD98 than CD147. To explore this further, we identified CD98hc partners when CD147 expression was minimal. To achieve this, we used CD98hcFLAG-expressing MCF7 cells with low endogenous levels of CD147 as detected by flow cytometry and by Western blotting (not shown). After cross-linking of intact cells with either BS3 or DSP, cells were lysed in Triton X-100, and we isolated CD98hc-FLAG complexes (Table I, Experiments V and VII) or endogenous CD98hc complexes (Experiment VI) from MCF7 cells alone (Experiments V and VII) or after co-culture with HT1080 cells (Experiment VII). Again LC-MS/MS analyses revealed the presence of LAT1, EpCAM, and ASCT2 plus CD98hc itself. Despite elevated CD147 expression in HT1080 cells, HT1080 CD147 was not identified among the protein partners of MCF7 CD98hc (Experiment VII), consistent with CD98hc and CD147 not interacting in trans (as established in Fig. 3B). For some proteins listed in Table I (CD71 and Na/K-ATPase) we could not confirm CD98hc (or CD147) association in subsequent biochemical experiments (e.g. see Fig. 5, A–C). These and other proteins appearing twice or less in seven experiments (Table I, bottom) were not studied further.

LAT1, a Na+-independent transporter for large, neutral amino acids, naturally and directly associates with CD98hc via a disulfide bond (27). Although LAT1 immunoprecipitation did yield CD147 from mild Brij 99 and CHAPS lysates (Fig. 4B), LAT1 did not co-immunoprecipitate CD147 in more strin-

gent detergent conditions (Triton X-100 and Brij 97) either with or without cross-linking (not shown). These results are consistent with a CD147-CD98hc-LAT1 linkage. Confirming results in Table I (Experiments V–VII), CD98hc from SW480 cells also co-immunoprecipitated (Fig. 5A, lane 2) with EpCAM, an epithelial cell surface protein involved in tumor proliferation (28, 29). Conversely immunoprecipitation of endogenous CD98hc from SW480 cells yielded endogenous EpCAM (Fig. 5B, lane 2). EpCAM-CD98hc complexes were stable in 1% Brij 99 (Fig. 5, A and B) and CHAPS and Brij 97 (data not shown) but not in Triton X-100 (Fig. 5C, lane 4) unless captured using the covalent cross-linker DSP (Fig. 5C, lane 1). In each of these experiments, antibody (mAb 8G6) to endoge-

nous CD147 failed to yield much CD98 (Fig. 5, A, lane 1, and C, lane 2) or EpCAM (Fig. 5B, lane 3) at least partly because the 8G6 epitope (on extracellular Ig domain 1) is blocked by CD147-associated proteins (not shown). Although Na/K-

ATPase α1 was prominently expressed in SW480 cell lysates,
an antibody (mAb C464.4) to that protein yielded minimal CD98 or EpCAM and hence was used as a negative control in Fig. 5, A–C. As an additional negative control, anti-β1 integrin mAb TS2/16 also failed to yield EpCAM from SW480 cells.

Another protein in Table I, associating with both CD147 and CD98hc, is the neutral amino acid transporter ASCT2 (30). Confirming this result, CD98hc immunoprecipitation from HEK293 cells yielded ASCT2 in both Brij 99 and Brij 97 cell lysates (Fig. 5D, lanes 4 and 5). However, immunoprecipitation with anti-CD147 mAb 8G6 failed to yield ASCT2 (Fig. 5D, lanes 2 and 3) again because the 8G6 epitope is likely blocked. Treatment of HEK293 cells with DSP cross-linker prior to lysis allowed immunoprecipitation of endogenous CD98 and recovery of ASCT2 even in 1% Triton X-100 (not shown).

**Correlation between CD147, CD98hc Expression, and Cell Proliferation**—To explore further the CD147-CD98hc supercomplex, we used RNAi to knock down either CD147 or CD98hc. CD147 was reduced up to 80% in HEK293 cells as seen by immunoblotting (Fig. 6A, top panel) and flow cytometry (Fig. 6B, top panel). The same RNAi that diminished CD147 also reduced CD98hc expression (Fig. 6B, lower panel). Conversely knock-down of CD98hc caused a loss of CD147. Indeed cell surface expression data for 14 different CD147-depleted clones and 13 different CD98hc-depleted clones each showed strikingly parallel effects on both CD147 and CD98hc expression (Fig. 7A). Levels of other prominent cell surface proteins (β1 integrin and MHC class I) were unaffected by CD147 or CD98hc depletion (not shown).

Although there was no obvious morphological abnormality in CD147- and CD98hc-depleted HEK293 cells, they did show markedly decreased proliferation in proportion to diminished CD147 expression (Fig. 7B). Serum starvation and mitomycin C treatment likewise impaired HEK293 cell proliferation but without altering CD147 and CD98hc expression levels (not shown). Hence diminished CD147 and CD98hc levels are a cause rather than a consequence of diminished proliferation.

**DISCUSSION**

The CD147-CD98 Complex—Striking similarities between the MCT-CD147 and CD98hc-LAT1 complexes have previ-
viously been noted. Both CD147 and CD98hc 1) are cell surface glycoproteins with a single transmembrane domain, 2) are highly expressed on activated or proliferating cells, 3) associate directly with transporters containing several transmembrane domains, and 4) are required to bring those transporters (MCTs or LAT1 and related molecules) to the cell surface (9, 10, 18, 27). Now our results reveal the existence of a novel MCT-CD147-CD98hc-LAT1 transporter complex, which also includes another amino acid transporter (ASCT2) as well as a protein (EpCAM) previously linked to epithelial cell proliferation. At the core of this complex is the interaction between CD147 and CD98hc. This interaction is direct (as seen using two different covalent cross-linking agents), was reciprocally demonstrated, was captured on the surface of multiple intact cell lines, and was shown to occur in cis rather than in trans.

The close association between CD147 and MCT proteins (10) is confirmed by our covalent cross-linking of CD147 and MCT1 (Fig. 2D). However, although we did see association of MCT with CD98hc under mild detergent conditions (1% Brij 97 and CHAPS), we did not observe direct MCT-CD98hc cross-linking. CD98hc does associate directly, via a disulfide linkage, with LAT1 (31). However, although we did see association of LAT1 with CD147 under mild detergent conditions, we did not observe direct CD147-LAT1 cross-linking. Furthermore a CD98hc-C109S mutant, which lacks LAT1 light chain association (22, 32), retained CD147 association (not shown). These results support an MCT-CD147-CD98hc-LAT1 arrangement in which the MCT-CD147 and CD98-LAT1 heterodimers are linked via direct CD147-CD98hc contact (Fig. 8).

**Functions of CD147-CD98hc Complexes—** RNAi-mediated depletion of either CD147 or CD98hc resulted in parallel diminution of both components as well as activation of AMPK and decreased cell proliferation. We suggest that decreased cell proliferation is likely achieved through a regulatory pathway involving the mammalian target of rapamycin (mTOR) kinase (33, 34). Without CD147, cellular metabolism of lactate and pyruvate should be impaired because MCT1 and MCT4 do not insert into cell membranes properly (9, 10). Without

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**Fig. 7. Functional co-regulation of CD147 and CD98hc.** A, cell surface expression levels (in mean fluorescence intensity units) are plotted for CD98hc (y axis) and CD147 (x axis) for each clone of CD147 and CD98 RNAi-depleted cells. B, relative proliferation rates for CD147-depleted clones are plotted versus cell surface CD147 expression levels. For each result, n = 3, and S.E. ≤ 5% of mean proliferation rate. Relative proliferation rates are shown relative to RNAi negative control rates. C, CD147-depleted HEK293 clones were lysed in radioimmune precipitation assay buffer with protease inhibitor mixture supplemented with 2 mM PMSF, 1 mM Na3VO4, 1 mM NaF and then detected for expression of phosphorylated AMPKα and total AMPKα. Band densities were determined using GeneTools version 3 (Syngene Laboratories). Each band in the phospho-AMPK (P-AMPK) blot (upper panel) was normalized relative to total AMPK (lower panel). These values were then standardized relative to the negative control lane to yield the numbers shown below the lower panel. ctr, control; neg.ctrl, negative control.
CD147-CD98 Protein Complex

**Fig. 8. Model of the CD147-CD98hc supercomplex.** Results in Figs. 2 and 4 support a direct association between CD147 and CD98hc. Also CD147 associates directly with MCT1 and MCT4 (Ref. 10 and Fig. 2D), CD98hc associates directly with LAT1 (31), and EpCAM associates directly with CD98hc (see text). Although Experiment I (in Table 1) suggested that LAT1 and EpCAM could also directly contact CD147, this possibility was not confirmed in further experiments. Experiments in Table I suggested that ASCT2 could contact both CD147 and CD98. Results from subsequent experiments currently favor a CD98hc contact, but an ASCT2-CD147 contact remains a possibility.

CD98hc, LAT1 family transporters are mostly trapped inside of cells, and amino acid transport should be impaired (27, 35). Diminished intracellular levels of nutrients such as amino acids, glucose, pyruvate, and lactate should lead to impaired energy metabolism and a high AMP/ATP ratio, thereby triggering phosphorylation and activation of AMPK (36), which we did indeed observe. Decreased cellular nutrients and activated AMPK are both known to cause diminished activity of the serine/threonine kinase mTOR. This key regulatory “rheostat” in cells responds to environmental nutrient signals by regulating cell growth and proliferation at least in part by affecting protein biosynthesis (37).

Why are expression levels of CD147 and CD98hc regulated in parallel? One possibility is that expression of one component may facilitate expression of the other in the context of a MCT-CD147-CD98hc-LAT1 complex. For example, co-expression could boost the overall biosynthetic assembly of these complexes and/or could exert a stabilizing effect on each component once they reach the cell surface. Consistent with this “facilitated co-expression” idea, CD147 (1, 3–5), and CD98hc (38–41) are each up-regulated on tumor cell lines and other types of metabolically activated cells. Another possibility is that CD147 and CD98hc proteins are co-regulated at the transcriptional or translational level. In the preceding paragraph, we emphasized how decreased levels of CD147 and CD98hc could cause changes in AMPK and mTOR pathways, leading to diminished proliferation. However, diminished expression of CD147 and CD98hc could also be a consequence of altered AMPK and mTOR pathways. In this regard, down-regulation of mTOR activity can lead to decreased cell surface expression of CD98hc/4F2 and glucose transporter Glut1 (42), which is a molecule that can be co-regulated with MCT1 (43). Hence RNAi-dependent depletion of either CD147 or CD98hc could lead to decreased mTOR phosphorylation, resulting in decreased expression of both molecules. This regulation must be somewhat specific because levels of other highly expressed cell surface molecules (β1 integrins and MHC class I) were not affected by removal of either CD147 or CD98hc.

With the discovery of MCT-CD147-CD98hc-LAT1 complexes, we are now better able to understand a few observations that previously suggested a functional link between CD147 and CD98hc. In studies involving human immunodeficiency virus gp160-mediated cell fusion and monocytic cell fusion, antibodies to CD98hc and CD147 had cross-regulatory effects (44). In studies of T lymphocyte costimulation by U937 cells (45) and dendritic cells (46), antibodies to CD147 and CD98hc were among the few antibodies to have inhibitory effects. In another study involving U937 cells, anti-CD147 antibodies inhibited homotypic aggregation that was stimulated by an anti-CD98 antibody (47). Our results might now also help to explain how CD98hc/4F2hc could unexpectedly facilitate transport of pyruvate (48), which is typically a substrate for MCT1 and MCT4.

**Other Molecules Associated with CD147 and CD98hc—** Although EpCAM had not previously been shown to associate with either CD147 or CD98hc, we observed such an association in four separate mass spectrometry experiments and confirmed association with CD98hc in reciprocal co-immunoprecipitation and covalent cross-linking experiments. Like CD147 and CD98hc, EpCAM is also a cell surface glycoprotein that is highly expressed on most malignant epithelial cells. In this regard, EpCAM is being tested as a therapeutic target in antitumor clinical trials (28, 29). Another molecule, ASCT2 (ALC1A5, adipocyte amino acid transporter, hATB0), was also found to associate with CD147 and CD98hc as seen in five mass spectrometry experiments (Table I). ASCT2 association with CD98hc was confirmed by co-immunoprecipitation and covalent cross-linking experiments. ASCT2 contains eight transmembrane domains and acts as transporter for neutral amino acids (49). As seen for CD98hc (50, 51), ASCT2 also may contribute to cancer progression (52) consistent with tumors having an increased need for amino acid transport. Both EpCAM and ASCT2 showed preferential association with CD98hc over CD147 and thus are placed in contact with CD98hc in Fig. 8. However, it is possible that EpCAM and ASCT2 could also contact CD147 as suggested by results in Table I. Further experiments will be needed to resolve this issue.

Various β1 integrins have been shown previously to associate with CD147 (11) and with CD98 either directly (23) or indirectly (22). Indeed we did obtain evidence for β1 integrin association but in only one of seven mass spectrometry experiments. Also RNAi-mediated knock-down of CD147 and CD98hc did not decrease the levels of β1 integrins on the cell surface. Hence integrins were not emphasized in this study. Nonetheless it is possible that independently identified integrin-EpCAM (53), integrin-CD147 (11), and integrin-CD98hc complexes (22, 23) may be at least partially overlapping.
Association of CD147 with caveolin-1 has a negative effect on CD147 multimerization, glycosylation, and MMP induction. The smaller, less glycosylated form of CD147 preferentially associated with caveolin-1 with Ig domain 2 of CD147 being required (2, 12). In contrast, the larger, highly glycosylated form of CD147 associated with CD98hc with Ig domain 1 of CD147 being required. Hence the CD147-CD98hc complex is distinct from complexes containing CD147 and caveolin-1. It is notable that the highly glycosylated form of CD147, which associates with CD98hc, is also involved in induction of MMPs (54, 55). However, a functional link between CD98hc and MMP induction has yet to be established.

The appearance of MCT1 and MCT4 in Table I was not unexpected because both were previously shown to associate with CD147 (10). MCT8, a thyroid hormone transporter and another member of the MCT family, also appeared as a potential partner for CD147 in Table I. This result still needs further biochemical confirmation. However, if an MCT8-CD147-CD98hc-LAT1 connection does truly occur, it would be highly relevant toward the understanding of how thyroid hormone might be transported by both the CD98hc-LAT1 complex (56, 57) and the CD147-MCT8 complex (58, 59). Among the six known light chains that associate with CD98hc (18, 35), only the neutral amino acid transporter LAT1 appeared in Table I. It remains to be seen whether any of the other light chains might also appear in complexes with CD147.

Technical Considerations—Comprehensive proteomic technologies have produced a remarkable compilation of networks of potential protein-protein interactions (15, 16). However, within such networks, interactions among transmembrane proteins tend to be under-represented because it is difficult to recapitulate hydrophobic interaction conditions on a massive scale in vitro. Furthermore commonly used detergents such as Triton X-100 disrupt many functionally significant interactions. On the other hand, use of milder detergents may increase the recovery of functionally important complexes but at the same time can yield an unacceptable level of nonspecific, background interactions due to incomplete solubilization. To solve this problem, here we treated intact cells with covalent cross-linkers to capture existing interactions among transmembrane proteins. Thus we were able to use relatively stringent (1% Triton X-100) lysis and washing conditions to decrease levels of background proteins while retaining protein associations that otherwise would have been disrupted. In this way, we were able to discover several new interactions involving CD147 and CD98hc that had not been seen previously.

Antibodies to CD98hc and CD147 extracellular domains were of little use in isolating MCT-CD147-CD98hc-LAT1 complexes presumably because extracellular epitopes on these molecules were blocked by associated proteins. In this regard, the anti-CD147 mAb 8G6 epitope maps to the same region of CD147 (Ig domain 1) needed for CD98hc association. Instead we relied on antibodies to intracellular, C-terminal FLAG- and GFP-tagged forms of CD147 and CD98hc. One disadvantage to this approach is that anti-FLAG detection is extremely non-linear, thus making estimation of stoichiometry very imprecise. Nonetheless we did estimate (after correction for antibody detection efficiency) that at least 20–30% of CD147 and 20–30% of CD98 may be complexed with each other. In the case of EpCAM association, the epitope on CD98hc recognized by mAb 4F2 was not blocked, and we were able to recover ~30% of EpCAM in a complex with CD98hc.

Does our technical approach lead to the identification of proteins, captured by covalent cross-linking, simply because they are very abundant on the cell surface? Indeed we suspect that some highly abundant proteins listed at the bottom of Table I (e.g. transferrin receptor/CD71, CD44, and HLA class I) might appear because they were trapped among the others. Consistent with this interpretation, subsequent co-immunoprecipitation experiments did not support specific interactions with either CD147 or CD98hc. In contrast, interactions involving proteins listed at the top of Table I were confirmed in separate biochemical experiments. Furthermore to minimize concerns regarding high expression levels, ectopic CD147 was expressed at levels only 1.4–2.1-fold above endogenous CD147. Also many experiments utilized endogenous CD98hc, CD147, EpCAM, ASCT2, LAT1, and MCT proteins rather than ectopically overexpressed proteins. As further evidence of specificity, we carried out several additional mass spectrometry experiments aimed at isolating protein partners for other abundant cell surface proteins (laminin-binding integrins, EWI-2 protein, and tetraspanins). The dozens of potential protein partners identified in those experiments did not include CD147, MCT proteins, CD98hc, amino acid transporters, or EpCAM (not shown).

In conclusion, we have combined covalent cross-linking and mass spectrometry to discover novel physical associations among molecules involved in cell proliferation and transport. Functional co-regulation of cell surface CD98hc-LAT1 and CD147-MCT protein complexes is consistent with their physical association and demonstrates their joint role in cellular energy metabolism. The presence of additional transporter (ASCT2) and proliferation-related (EpCAM) components suggests the existence of a transmembrane protein supercomplex, which provides a novel physical framework for understanding functional connections among these diverse molecules.

Acknowledgments—We thank Dr. Tatiana Kolesnikova for antibodies and cDNAs for wild type and mutant CD98. We thank Dr. Wei Tang for CD147 cDNA and antibodies.

* This work was supported by National Institutes of Health Grant CA102034. 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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