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Colocalization of GP125/CD98 with Tropomyosin Isoforms at the Cell–Cell Adhesion Boundary[1]

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Two monoclonal antibodies designated as 1F6 and 4B10 were obtained on screening for reactivities to CD98-associated molecules by sandwich-type enzyme-linked immunosorbent assaying using hybridoma culture supernatants as the solid phase, cell lysates as an antigen source, and a mixture of biotinylated antibodies to CD98HC as a detector. Flow cytometric analysis with microspheres in combination with 1F6, 4B10, and anti-CD98HC also indicated the association of antibody-defined antigen(s) with CD98. 1F6 and 4B10, stained fibrillate components in fixed and permeated cells but were not reactive with unfixed live cells, suggesting that epitopes reside in the cytoskeleton-associated structure in the intracellular region. Two-color immunostaining followed by confocal microscopy revealed the colocalization of the antigen with CD98 at the cell–cell adhesion boundary of HeLa cells. 1F6 detected proteins with relative molecular masses of 33,000 to 43,000 on immunoblotting analysis involving cell lysates of human and rat cell lines. Analysis with a purified tropomyosin specimen from rabbit skeletal muscle demonstrated that 1F6 and 4B10 recognize tropomyosin. Two-dimensional gel electrophoresis followed by immunoblotting analysis revealed that 1F6 recognizes various tropomyosin isoforms. These results indicated that CD98 physically associates directly or indirectly with tropomyosin, and that this association is closely related to the cell–cell interaction.

Key words: cell–cell adhesion, cytoskeleton, GP125/CD98, monoclonal antibody, tropomyosin.

A glycoprotein with a relative molecular mass (M_r) of 125,000 (GP125/CD98) is a heterodimeric protein composed of an 85-kDa heavy chain (HC) and a 40-kDa light chain (LC), and was originally identified as a 4F2 cell-surface antigen associated with lymphocyte activation (*1–4*). GP125/CD98 is strongly expressed on a variety of tumors (*1*, *5–7*), suggesting its functional relevance to malignant transformation. In fact, monoclonal antibodies (mAb) against CD98 inhibited tumor cell growth (*5*, *7*), and CD98HC cDNA–transfected murine fibroblasts showed malignant phenotypes (*8*).

The injection of human CD98HC complementary RNA into *Xenopus laevis* oocytes induced system y^+–like amino acid transport activity (*9*, *10*). Its involvement in the system-L–like amino acid transport activity was also observed in rat cells (*11*). However, circumstantial evidence suggested that CD98 was strongly linked to amino acid transport but was not the transporter itself (*11*, *12*). Two light chains associated with CD98HC have been identified (*13–16*). They are TA1/E16 (*14*, *17*), also called LAT1 (*13*, *15*, *18*), and y^+LAT1 (*16*); the former mediates Na^+-independent large neutral amino acid transport (system L), and the latter mediates the transport of Na^+-dependent large neutral amino acids and Na^+-independent cationic amino acids (system y^+L). In addition, xCT, which mediates an Xc– amino acid transport system, has been identified as the third CD98LC (*19*), and we have reported another novel CD98LC (*20*).

In addition to amino acid transport, CD98 has been reported to be involved in a wide variety of cellular functions including cellular growth, cell survival and death (*21*), activation of β integrin (*22*), and Ca^{2+} influx (*23*). These cellular functions of CD98 cannot be simply explained by its role in amino acid transport. To understand the physiological roles of CD98 other than as a regulator of system L, y^+L, or Xc– amino acid transport, further analyses of CD98-associated molecules are required.

In the present study, we generated mAbs that recognize CD98-associated molecules, and using these mAbs we demonstrated the physical and functional association of tropomyosin (TM) isoform(s) with CD98.

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Abbreviations: F-actin, filamentous actin; FBS, fetal bovine serum; GP125, glycoprotein with relative molecular mass (M_r) of 125,000; GP125/CD98HC (LC), heavy (light) chain of GP125/CD98; mAb, monoclonal antibody; PBS, phosphate-buffered saline; s-ELISA, sandwich-type enzyme-linked immunosorbent assay; TM, tropomyosin; H(L)MW-TM, TMs of high (low) molecular weight.

MATERIALS AND METHODS

Cell Culture—Cell lines of human carcinomas from kidney (ACHN), urinary bladder (T24), and cervix (HeLa), human hepatoblastoma (HepG2), rat bladder carcinoma (BC47) and hepatoma (AH13), mouse hepatoma (Hepa1), myeloma (P3X63Ag8.653), and Balb3T3 fibroblasts were cultured in Dulbecco's modified Eagle's medium or RPMI medium supplemented with 7% heat-inactivated fetal bovine serum (FBS; ICN Biomedicals, Aurora, OH, USA) in a humidified CO_2 incubator.

Animals—Male and female Balb/c mice (6 to 8 weeks old) were purchased from Japan SLC (Hamamatsu), and housed in a controlled environment at 22°C.

Hybridomas—A female BALB/c mouse received three *i.v.* injections of ACHN human renal cancer cells (5×10^6) for each immunization at 3-week intervals. The immune spleen cells (1×10^8) were fused with P3X63Ag8.653 mouse myeloma cells (1×10^7) with 50% polyethylene glycol 1540 (Wako Pure Chem.). After cell fusion, hybridoma cells were selected in FBS-free Hymedium 606 (Kojin Bio., Saitama) supplemented with 3% Briclone (Dainihon Seiyaku, Osaka). The hybridomas were screened by examining the culture supernatants by means of a sandwich-type enzyme-linked immunosorbent assay (s-ELISA) as described below, and cloned by the limiting dilution method. Hybridoma clones (5×10^6) termed 1F6 and 4B10 were injected *i.p.* into male Balb/c mice pretreated with 2,6,10,14-tetramethylpentadecane (Wako). Ascites fluid was collected 7 to 14 days after cell inoculation, and then precipitated with 50%-saturated ammonium sulfate. Further purification was performed with Sepharose CL-6B (Pharmacia, Uppsala, Sweden), because both two mAbs were determined to be IgM, with the ascites samples by SDS-PAGE analysis.

Antibodies—Anti–CD98HC IgG mAbs, B3 (*1*), HBJ127 (*6*), 4F2 (*2*), 1-10, 5-21, an anti–CD98HC IgM mAb 4-26, an anti–CD98LC IgM mAb 3F2 (*20*), and a control anti–rat CD3 IgM mAb 1F4 (*24*) were used in this study.

S-ELISA—S-ELISA was performed with 1F6, 4B10, and anti-CD98HC mAbs HBJ127, 4F2, 1-10, and 5-21. Aliquots (50 µl) of undiluted hybridoma culture supernatants or purified antibodies (50 µg/ml) in Ca^{2+}, Mg^{2+}-free Dulbecco's phosphate-buffered saline (PBS, pH 7.4) were passively adsorbed in duplicate on the wells of 96-well polyvinyl chloride plates (Sumitomo Bakelite, Tokyo) overnight at 4°C. Each well was treated with Block Ace (Dainihon) 1:2 diluted with PBS for 3 h at 37°C to block nonspecific binding of the staining reagents. Fifty microliters of ACHN cell lysate (2×10^7 cells/ml) 1:5 diluted in PBS containing 0.05% Tween 20 (T-PBS) was supplemented with biotinylated mAbs (20 µg/ml), and then added to each well, followed by incubation overnight at 4°C. After washing extensively with T-PBS, 50 µl of 1:100 diluted avidin–biotin–peroxidase complex (ABC; Vector Laboratories, Burlingame, CA, USA) was added to each well, and then plates were incubated for 45 min at 37°C. Each well was washed extensively with T-PBS, and then supplemented with 0.1 M citric-acetate buffer (pH 6.0) containing 3,3′,5,5′-tetramethylbenzidine (0.1 mg/ml) and 0.01% H_2O_2 (75 µl in each well). Color development in the wells was stopped after 5 to 10 min by the addition of 0.5 M H_2SO_4 (75 µl in each well), and then the optical density of the solution at 450 nm was measured with a Model 550 Microplate Reader (Bio-Rad).

Western Blotting—Cells were extracted at a concentration of 2×10^7 cells/ml with lysis buffer [PBS containing 1% Nonidet P-40, protease inhibitors (1 nM pepstatin A, 100 nM leupeptin, 1 µM APMSF, 100 nM pephabloc SC, and 10 µM titriplex), and 20 U/ml benzonase] at 4°C for 3 h. After centrifugation at 10,000 ×*g* at 4°C for 30 min, the supernatants were boiled in Laemmli's sample buffer (*25*) for 5 min. The denatured proteins were separated under reducing or non-reducing conditions by 7.5 or 12% SDS-PAGE, respectively, and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Nonspecific binding sites on PVDF membranes were blocked with Block Ace for 2 h at 37°C, and then the membranes were incubated with purified antibodies (5 µg/ml) overnight at 4°C. The membranes were washed extensively with T-PBS and then incubated with rabbit anti-mouse immunoglobulin (Dako Japan, Kyoto) diluted 1:200 in T-PBS for 1 h at room temperature. After extensive washing with T-PBS, the membranes were incubated with horseradish peroxidase–conjugated protein A (Zymed, South San Francisco, CA, USA) diluted 1:10,000 in T-PBS for 45 min at room temperature. After extensive washing with T-PBS, the membranes were incubated with 0.05% 3,3′-diaminobenzidine (Wako) and 0.01% H_2O_2 in 0.1 M Tris-HCl (pH 7.4).

Two-Dimensional Gel Electrophoresis—Two-dimensional gel electrophoresis was performed using nonequilibrium pH gradient gels for the first dimension by O'Farrell's method (*26*), and then SDS–polyacrylamide gels for the second dimension by Laemmli's method (*25*). Samples of total cell lysates were prepared as follows. Cells (1×10^6) were dissolved in 50 µl of two-dimensional gel sample buffer [9.95 M urea, 2% Nonidet P-40, 2% Ampholine pH 5–7 (Pharmacia, Uppsala, Sweden), 0.5% Ampholine pH 3–10, and 100 mM 2-mercaptoethanol]. After concentration with Centrifugal Filter Devices (Millipore, Bedford, MA, USA) to 10 µl, cell lysates were loaded onto gels and electrophoresed in the first dimension.

Flow Cytometric Analysis of Cells—Cells (3×10^5 in each assay), which were unfixed or fixed with PBS containing 3% paraformaldehyde and 2% sucrose for 5 min and then permeated with Triton buffer (0.5% Triton X-100 in 20 mM HEPES, pH 7.4, 50 mM NaCl, 3 mM $MgCl_2$, 300 mM sucrose) for 5 min, were resuspended in 50 µl of PBS containing 1% bovine serum albumin (BSA), and then mixed with an equal volume of purified mAbs (5 µg/ml) for 1 h on ice. After washing with PBS, the cells were labeled with fluoresceine isothiocyanate (FITC)–conjugated rabbit anti-mouse immunoglobulin (Dako) for 30 min on ice. After washing of the cells with PBS, the immunofluorescence intensity of individual cells was determined with a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA, USA).

Flow Cytometric Analysis of Microspheres—The pellets obtained from 100 µl aliquots of Polybead suspensions (4.5 micron polystyrene microspheres; Polysciences, Warrington, PA, USA) obtained by centrifugation at 5,000 ×*g* for 10 min were resuspended in PBS containing anti-human CD98HC mAb HBJ127 $F(ab')_2$ (50 µg/ml) at 37°C for 1 h, washed three times with PBS by centrifugation, and then treated with Block Ace overnight at 4°C. The microspheres were mixed with 100 µl of HeLa cell lysate (2×10^7 cells/ml) overnight at 4°C, washed three times with T-PBS, and then

stored in 100 µl of T-PBS containing 1% BSA and 0.05% NaN_3. The microspheres (1 µl of suspension in each assay) were incubated with various IgM mAbs at 4°C for 1 h, washed three times with T-PBS, and then incubated with FITC-conjugated goat anti–mouse µ chain antibodies (Jackson ImmunoResearch, West Grove, PA, USA) diluted 1:100 in T-PBS containing 1% BSA. After three washes with T-PBS, the fluorescence intensities of microspheres were determined by FACScan.

Immunostaining of Frozen Sections—Frozen tissue sections (5 µm thick), prepared using a Leica CM1800 cryostat, were air-dried, fixed with ice-cold acetone for 5 min, treated with Block Ace for 1 h, and then incubated with undiluted culture supernatants or purified mAbs (5 µg/ml in 1% BSA-PBS) overnight at room temperature. After rinsing of the tissue sections with PBS, endogenous peroxidase activity was inhibited by immersing the sections in 3% H_2O_2-methanol for 5 min. After a PBS wash, the sections were incubated with biotinylated horse anti–mouse IgG (Vector) diluted 1:100 in PBS for 1 h. After washing three times with PBS, the sections were treated with ABC reagent (Vector) diluted 1:100 in PBS for 45 min. After washing three times with PBS, the sections were incubated with 0.05% 3,3′-diaminobenzidine (Wako) and 0.01% H_2O_2 in 0.1 M Tris-HCl (pH 7.4), and then counterstained with hematoxylin. Samples were then dehydrated in ethanol, cleared in xylene, and mounted with Permount (Fisher Sci-

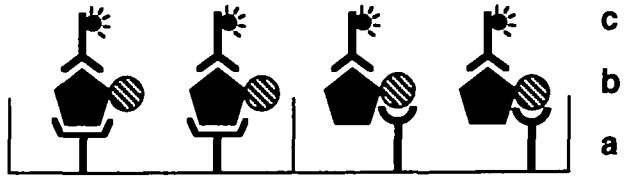

Fig. 1. **Scheme of s-ELISA for mAb screening.** (a) Coating of antibodies in FBS-free hybridoma culture supernatants on assay plates. (b) Addition of the ACHN cell lysate. (c) Detection of antibodies with biotinylated anti-CD98HC mAbs followed by ABC reagent. Left: Detection of antibodies (a) recognizing a different epitope on CD98HC (b: pentagons) defined with labeled antibodies (c). Right: Detection of antibodies (a) recognizing an epitope on the molecule (b: circles) associated with CD98HC defined with labeled antibodies (c).

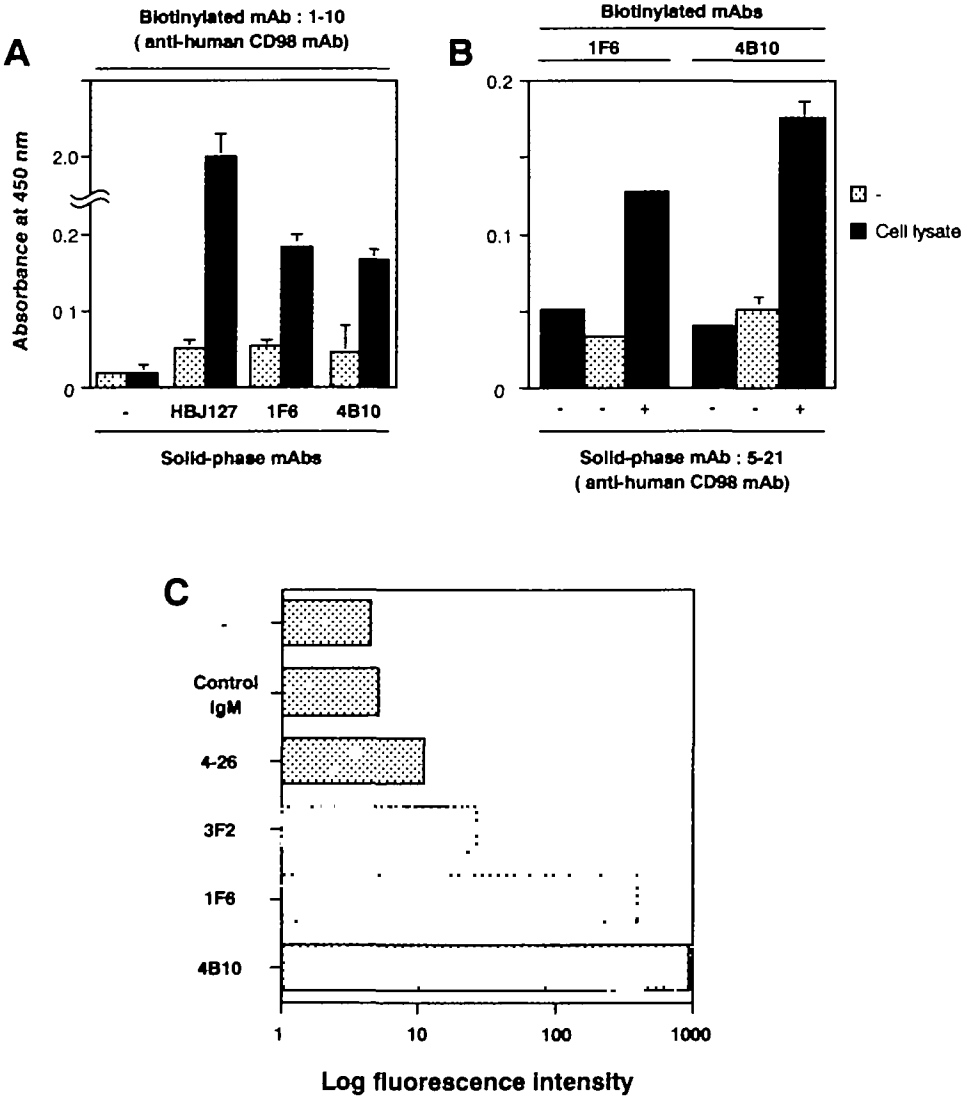

Fig. 2. **Determination of the association between CD98 and mAb-defined antigens by s-ELISA (A and B) and flow cytometry with microspheres (C).** In A and B, the association of antigens was detected with combinations of solid-phase mAbs and biotinylated mAbs. In C, microspheres coated with anti-CD98HC mAb (HBJ127) and preincubated with ACHN cell lysate were analyzed for immunoreactivity with the indicated IgM mAbs.

entific, Fair Lawn, NJ, USA). The localization of mAb-defined components was observed under a Zeiss Axiolab microscope.

One- or Two-Color Immunostaining of Cultured Cells—Cells grown on poly-L-lysine (Sigma, 10 μg/ml)–pretreated 8-chamber culture slides (Falcon, Franklin Lakes, NJ) were rinsed once with PBS, fixed with 3% paraformaldehyde–2% sucrose in PBS, and then permeabilized with Triton buffer (0.5% Triton X-100 in 20 mM HEPES, pH 7.4, 50 mM NaCl, 3 mM MgCl, and 300 mM sucrose). After a rinse with PBS, the cells in each well were overlaid with Block Ace for 1 h at 37°C, and then either used immediately or stored at 4°C for up to 1 week until use. For single-labeling, cells were successively treated with mouse mAb (undiluted culture supernatants or 5 μg/ml purified mAb), and FITC-conjugated rabbit anti–mouse immunoglobulin (Dako) diluted 1:200 with 1% BSA-PBS. For double-labeling, cells were simultaneously treated with 1F6 or 4B10 IgM and anti–CD98HC mAb HBJ127 IgG at a final concentration of 5 μg/ml each in PBS containing 1% BSA for 12 h at 4°C. After three washes with PBS, the cells were treated with Texas Red-conjugated goat anti–mouse μ chain antibodies and FITC-conjugated goat anti–mouse Fcγ chain antibodies (Jackson ImmunoResearch), which were diluted 1:100 in PBS containing 1% BSA for 2 h at 37°C. To study the colocalization of mAb-defined antigen and filamentous (F)-actin, cells were incubated with Alexa 594 phalloidin (Molecular Probes, Eugene, OR, USA) diluted to 50 nM in PBS for 15 min after FITC-labeling with mAbs. Nuclear DNA was stained with 4,6-diamidino-2-phenylindole (DAPI) at a concentration of 0.5 μg/ml for 20 min, and then samples were mounted in Permafluor (Immunon, Pittsburgh, PA, USA). The specificity of double-labeling was demonstrated by staining of cells with primary antibodies alone, followed by incubation with either anti-mouse μ chain or Fcγ chain secondary antibodies. Samples were examined under a fluorescence microscope (Leica DMLB) with a 100× objective. For colocalization experiments, confocal microscopy was performed with a Leica DMRB/E confocal microscope.

RESULTS AND DISCUSSION

Establishment of Hybridoma Clones That Secrete mAbs Recognizing CD98-Associated Molecules—Antibodies in serum-free hybridoma culture supernatants from 960 wells of hybridoma cultures were screened by s-ELISA with the lysate of an ACHN renal tumor and a mixture of biotinylated anti-CD98HC mAbs, since FBS in the medium may prevent the binding of a minor quantity of antibodies to the wells of assay plates (Fig. 1). Antibodies from two hybridoma clones designated as 1F6 and 4B10 showed positive reactions in this screening. To determine whether or not these mAbs recognize antigens associated with CD98, s-ELISA (Fig. 2, A and B) was performed with combinations of 1F6, 4B10, and anti-CD98 mAbs HBJ127, 1-10, and 5-21 using ACHN cell lysates as an antigen source. CD98 was efficiently detected with the combination of solid phase HBJ127 and biotinylated 1-10, which recognize different epitopes on CD98HC, demonstrating the validity of this assay system. Positive reactions were observed with the combination of 1F6 or 4B10 as the solid phase, and biotinylated anti-CD98 mAb 1-10 (Fig. 2A), and the reverse combinations; *i.e.* solid-phase anti-CD98 mAb 5-21 and biotinylated 1F6 or 4B10 (Fig. 2B), suggesting the association of CD98 with the antigens defined with 1F6 or 4B10. Flow cytometry with microspheres substantiated the association of CD98 with the mAb-defined antigen (Fig. 2C), *i.e.* microspheres coated with HBJ127, and bound CD98 and CD98-associated molecules from the cell lysate reacted with 1F6 and 4B10 as well as 4-26 and 3F2, which recognize epitopes on CD98 different from that recognized by HBJ127. The crossreactivity of 1F6 and 4B10 mAbs with CD98HC is implausible, since these mAbs were not reactive with the fusion protein between the CD98HC intracellular domain and glutathion *S*-transferase (data not shown).

Subcellular Localization of CD98-Associated Molecules—Next, we examined the reactivities of two mAbs with various tumor cell lines, and analyzed the subcellular localization of mAb-defined antigens. On flow cytometric analysis, 1F6 and 4B10 mAbs reacted with fixed and permeated tumor cell lines from various human, rat and mouse tissues, but did not react with unfixed live tumor cells, in contrast to the definite reactivity of an anti-CD98HC mAb HBJ127 with these cells, suggesting that mAbs recognize intracellular components common in various species including mouse (Fig. 3).

To determine the precise subcellular localization of mAb-defined antigens, fixed and permeated tumor cells were stained with 1F6 or 4B10, and then examined by fluorescent microscopy (Fig. 4). Both mAbs characteristically stained the fibrillate structure around the nuclei to inner cell membranes (Fig. 4, A and H), suggesting that the mAbs recognize cellular components associated with

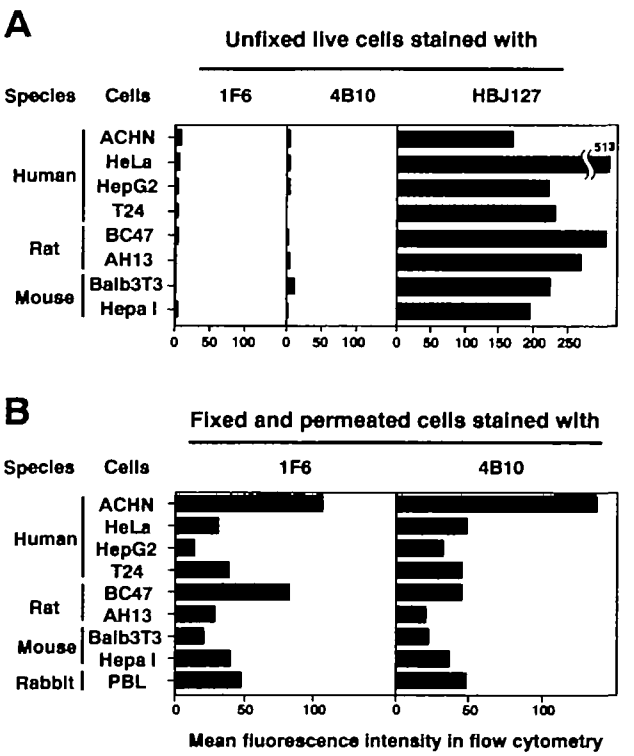

Fig. 3. **Reactivities of 1F6, 4B10, and anti-CD98 mAb with various cells on flow cytometry.** Unfixed live cells (A) or fixed and permeated cells (B) were labeled with 1F6, 4B10 or anti-CD98HC mAb (HBJ127), and then analyzed for fluorescence by FACScan.

cytoskeletal structures.

Confocal Analysis of the Distribution of CD98HC, F-Actin, and the Antigen(s) Defined with 1F6 and 4B10—To compare the subcellular localizations of mAb-defined antigens with those of CD98 and F-actin, HeLa cells were double-stained with 1F6 and HBJ127, or 4B10 and phalloidin, which specifically binds to F-actin, and then examined by confocal microscopy (Fig. 4). The localization of F-actin was identical with that of 4B10-defined antigen (Fig. 4, A, B, and C). Similar results were obtained on 2-color staining with 1F6 and phalloidin (data not shown). Although anti-CD98HC mAb HBJ127 mainly stained the cell surface (Fig. 4, D and G) and 1F6 showed intracellular staining (Fig. 4, E and H), colocalization was observed in restricted area of the inner cell membrane (Fig. 4I) at the cell–cell adhesion boundary in subconfluent or confluent HeLa cells. In contrast, colocalization was not observed in dispersed HeLa cells (Fig. 4F), suggesting a functional association between CD98 and mAb-defined antigens in cell–cell interaction.

Identification of Tropomyosin Isoforms as CD98-Associated Molecules—To identify the fibrillate components reacting with 1F6 or 4B10, immunoblotting analysis was carried out with ACHN human and AH13 rat cell lysates (Fig. 5A). 1F6 mAb detected three main bands corresponding to M_r of 33,000, 37,000, and 43,000 for ACHN cells, and ones corresponding to M_r of 33,000, 35,000, and 43,000 for AH13 cells. 4B10 mAb could not detect antigens in this assay (data not shown). Considering their M_r and subcellular localization, we examined the reactivities of both mAbs with a purified tropomyosin (TM) specimen derived from rabbit skeletal muscle by ELISA and immunoblotting analysis (Fig. 5, B and C). Both 1F6 and 4B10 mAbs reacted with the TM specimen in a dose-dependent manner on ELISA (Fig. 5B). In addition, 1F6 mAb was reactive with TM again in a

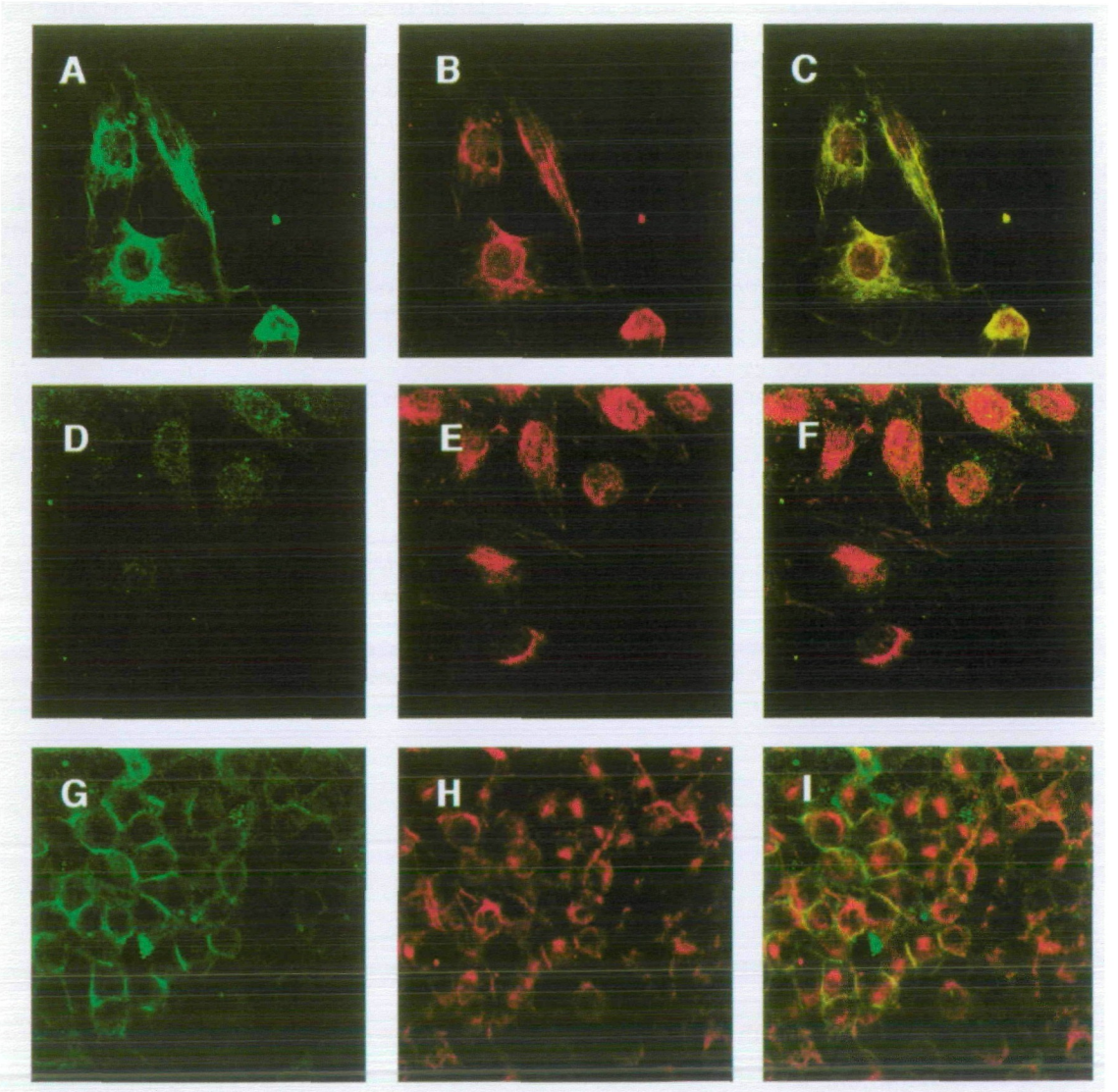

Fig. 4. **Subcellular localization of CD98 and mAb-defined antigens in HeLa cells on 2-color immunofluorescence staining followed by confocal microscopy.** HeLa cells grown on culture slides were fixed and permeated, and then stained with mAbs as described under "MATERIALS AND METHODS." A shows the staining with 4B10 followed by FITC-conjugated goat anti–mouse μ chain antibodies. D and G show the staining with anti-CD98HC mAb (HBJ127) followed by FITC-conjugated goat anti–mouse γ chain antibodies. B shows the staining with Alexa 594-phalloidin. E and H show the staining with 1F6 followed by Texas Red–conjugated goat anti–mouse μ chain antibodies. C, F, and I show the double-staining images of A and B, D and E, and G and H, respectively.

dose-dependent manner on immunoblotting analysis (Fig. 5C). Our present results strongly support a recent report of the possible association of CD98 with tropomyosin (*27*). The difference in molecular weight observed with 1F6 in ACHN and AH13 cells could be due to the difference in species or cell type.

TMs are a family of actin-binding proteins that stabilize actin microfilaments (*28*, *29*). In mammals, four TM genes corresponding to α-TM$_f$(*30*), β-TM (*31*), TM4 (*32*), and TM5 (*33*) have been identified, and through alternative splicing, they produce multiple TM isoforms in a highly tissue-specific manner (*29*). Non-muscle cells express multiple TM isoforms, most of which are alternatively spliced products of the same genes that produce muscle TM (*28*) through alternative exon use (*34*) conserved in many vertebrate species. This conserved splicing in fibroblasts produces two classes of TMs; ones of 284 amino acids and ones of 248 amino acids. Both high and low molecular weight TMs (HMW-TM and LMW-TM, respectively) are also found in cultured epithelial cells (*35–37*). 1F6 mAb detected 33 to 43-kDa proteins in rat and human cell lysates (Fig. 5A), indicating that they contained HMW-TM and LMW-TM isoforms.

To identify individual TM isoforms defined with 1F6 mAb, ACHN cell lysates were analyzed by two-dimensional gel electrophoresis followed by immunoblotting (Fig. 6). Many spots corresponding to high (indicated as a, b, and c) and low (indicated as d and e) molecular weight TMs were detected with 1F6 mAb, showing that 1F6 mAb recognizes various TM isoforms in ACHN cells.

TM isoforms have been implicated in various cellular functions including the regulation of cell cytokinesis (*38*), motility (*39*, *40*), morphogenesis (*41–44*), and transformation (*45–47*). Changes in the microfilament structure are highly related to both anchorage-independent growth and tumorigenicity (*48*). One or more HMW-TM isoforms are decreased or missing in transformed cells, while the levels of one or more LMW-TM isoforms are increased (*49–55*). Loss of the transformed phenotype is accompanied by restoration of the HMW-TM level (*49*, *52*, *56*), and overexpression of a transformation-sensitive HMW-TM isoform in ras-transformed cells can partially restore neoplastic growth and tumorigenicity, cause cell flattening and spreading, and increase the size and number of detectable stress fibers in a cell (*46*, *57*). Thus, it appears that the presence of HMW-TM is important for the integrity of actin filaments, and that the down-regulation of HMW-TM leads to the acquisition of malignant phenotypes through the destruction of the normal cytoskeletal organization.

Comparison of the Tissue Localizations of CD98HC and Tropomyosin—CD98 is expressed in restricted normal tissues, especially in tissues with growth potential, such as the small intestinal epithelium (*1*, *6*) and the basal layer of the squamous epithelium (*1*, *6*, *58*). To compare the distributions of mAb-defined TM isoforms and CD98, frozen sections of rat tongue were immunostained with mAbs (Fig. 7).

The basal layer of the squamous epithelium, which was characteristically stained with anti-rat CD98HC mAb B3, was also strongly stained with 1F6 and 4B10. In addition, TM isoforms defined with 1F6 and 4B10 were detected in the upper part of the epithelium and muscle tissues. This staining pattern suggested the existence of TM isoforms that are not associated with CD98. These observations suggested that the association of CD98 with tropomyosin isoforms is dependent on the tissue or cell type, and that various functions of CD98 could be affected by TM isoforms binding to CD98.

We recently demonstrated the emergence of various malignant phenotypes (*8*) when NIH3T3 cells are transfected with the normal coding sequence of CD98HC cDNA. The present results suggested a physical association between CD98 and TM isoforms, and its possible functional relevance in cell–cell adhesion. Interestingly, colocalization between CD98HC and cadherins was recently reported in murine cells (*18*), and the cell–cell adherens junction was shown to be a site for cadherin-mediated cell adhesion where actin filaments are densely associated with the plasma membrane (*59*). Further analysis of the association of CD98 and TM will lead to a better understanding of the reported multiple functions of GP125/CD98 other than in

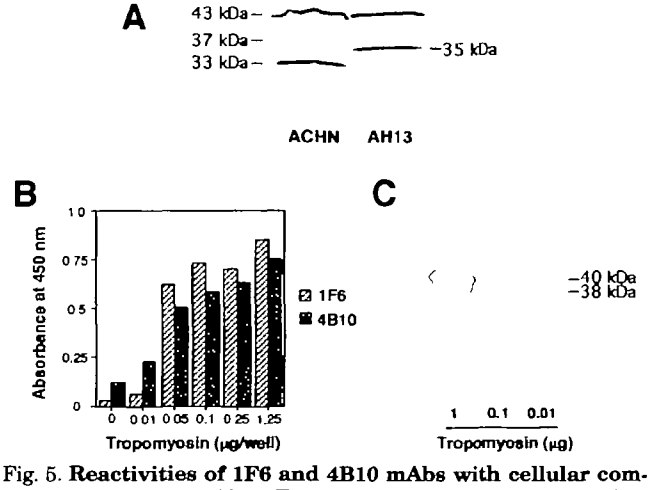

Fig. 5. **Reactivities of 1F6 and 4B10 mAbs with cellular components and a purified TM specimen on immunoblotting analysis and ELISA.** Cell lysates of ACHN and AH13 were blotted with 1F6 mAb after 15% SDS-PAGE under reducing conditions (A). The reactivities of 1F6 and 4B10 mAbs with the indicated amounts of the purified TM specimens derived from rabbit skeletal muscle were determined by ELISA (B). The indicated amounts of the purified TM specimen were blotted with 1F6 mAb after 15% SDS-PAGE under reducing conditions (C).

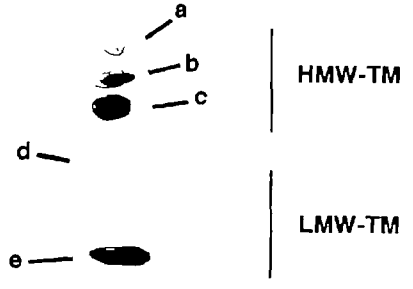

Fig. 6. **Two-dimensional gel electrophoresis followed by immunoblotting analysis of a total cell extract of ACHN cells defined with 1F6 mAb.** Electrophoresis was performed in the first dimension in gels containing pH 5–7 ampholytes, and in the second dimension in slab gels containing 15% polyacrylamide under reducing conditions. a–e correspond to TM isoforms TM-1 to TM-5 in Ref. *28*, respectively.

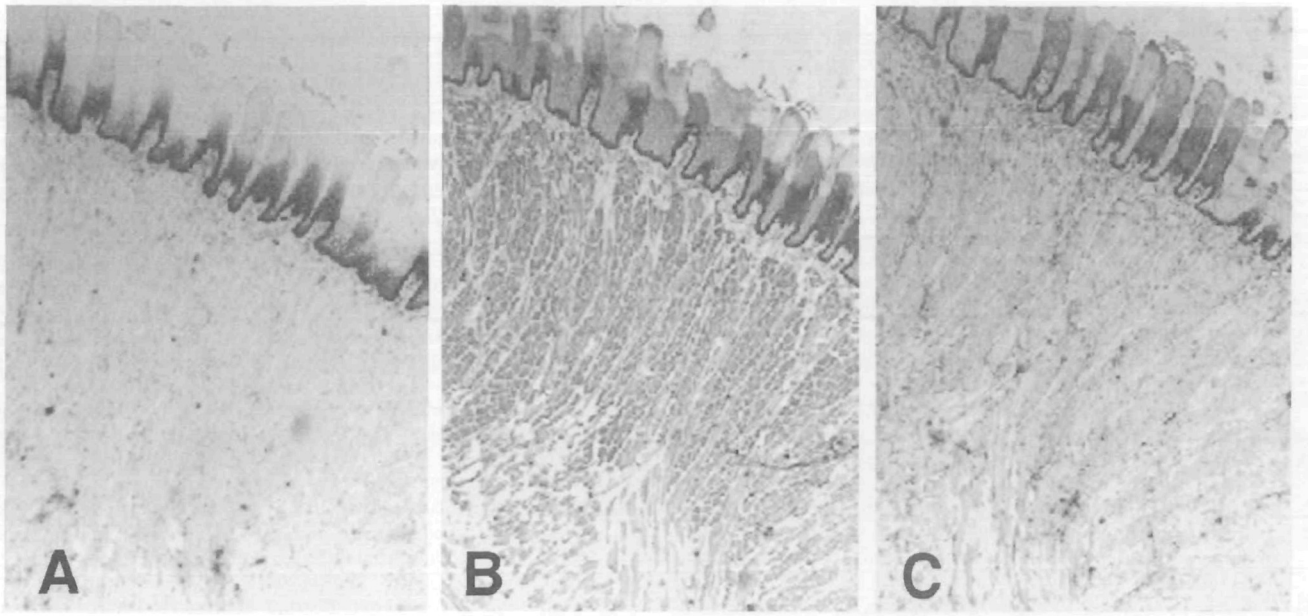

Fig. 7. **Immunostaining of rat tongue tissue with anti-rat CD98HC mAb B3 (A), 1F6 (B), and 4B10 (C).** In addition to the strong staining of the basal layers with all three mAbs, the upper layers were also stained with 1F6 and 4B10 mAbs.

amino acid transport, such as cell fusion (*60*), lymphocyte activation (*1–4*), cell proliferation (*1, 5, 6, 58*), and especially cell adhesion (*22*) and malignant transformation (*1, 6–8*).

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