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Identification and Immunological Characterization of a Novel 40-kDa Protein Linked to CD98 Antigen

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ABSTRACT. **Monoclonal antibodies (mAbs) were obtained from hybridoma clones established by cell fusion between mouse myeloma cells and spleen cells from a mouse immunized against an affinity-purified 40-kDa component of rat 125-kDa glycoprotein (GP125). Two mAbs designated as 3F2 and 6B4 detected a 40-kDa and a 125-kDa band under reducing and nonreducing conditions, respectively, in extracts prepared from rat, mouse and human tumor cells. Association of the 40-kDa protein with CD98 was revealed by sandwich-type enzyme-linked immunosorbent assay. The two mAbs were strongly reactive with various tumor cells and activated lymphocytes, but were only weakly reactive with resting lymphocytes. Confocal microscopy indicated colocalization of CD98 and the 40-kDa protein defined with 3F2 and 6B4 at the cell surface and perinuclear regions. On immunohistochemical analysis of frozen sections of rat tongue, the anti-rat CD98 mAb B3 selectively stained the basal layer and 3F2 stained the upper epithelial part in addition to the basal layer, indicating the existence of CD98-unlinked 40-kDa protein.**

Key words: CD98/GP125 heavy chain (HC)/GP125 light chain (LC)/monoclonal antibody (mAb)/amino-acid transporter/malignant transformation

GP125, a glycoprotein with a relative molecular mass (Mr) of 125,000, is a heterodimeric protein composed of an 85-kDa heavy chain (HC; CD98) and a 40-kDa light chain (LC). GP125 was originally identified as a cell-surface antigen associated with lymphocyte activation (6, 7, 21, 27), and is strongly expressed on the basal layer of squamous epithelium (6, 14, 19), intestinal epithelium (6, 14) and a variety of tumors (6, 8, 14, 29), suggesting its functional involvement in lymphocyte activation, cell proliferation and malignant transformation. In fact, monoclonal antibodies (mAbs) against CD98 inhibited lymphocyte activation (28) and cell proliferation (8, 29). In addition, CD98 cDNA-transfected NIH3T3 cells showed malignant phenotypes (5).

CD98 shows about 30% homology with the amino acid transporter NBAT/D2 (23), and injection of CD98 complementary RNA into *Xenopus laevis* oocytes induced a system y^+-like amino acid transport activity (1, 24). Its involvement in the system L-like amino acid transport activity was also observed in rat cells (2). However, circumstantial evidence suggested that CD98 was strongly linked to amino acid transport but was not the transporter itself (2, 11). Recently, two light chains associated with CD98 have been identified (10, 12, 13, 20); TA1/E16 (12, 25), also called LAT1 (10, 13, 17), and y^+LAT1 (20). 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 to amino acid transport, CD98 has been reported to be involved in a wide variety of cellular

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Abbreviations: GP125, glycoprotein of relative molecular mass (Mr) of 125,000; GP125-HC (LC), heavy (light) chain of GP125; mAb, monoclonal antibody; (y^+)LAT1, (y^+)L-type amino-acid transporter 1; EAAC1, excitatory amino-acid carrier 1; PBS, phosphate-buffered saline.

functions including cell growth, cell survival and death (22), activation of β integrin (4), and Ca^{2+} influx (3). 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 or y^+L amino acid transport, further analysis of CD98-associated molecules is indispensable.

We purified GP125 from rat hepatoma AH13 cells and determined a partial amino acid sequence of the 40-kDa component linked to CD98 (manuscript in preparation). The sequence of 13 amino-acid residues showed about 70% homology with human or rabbit excitatory amino-acid carrier 1 (EAAC1), which specifically transports glutamic acid (7, 12, 16), but showed no homology with LAT1 or y^+LAT1.

The 40-kDa protein is, therefore, the third light chain shown to be associated with CD98. To obtain insight into its function, we produced specific monoclonal antibodies to the 40-kDa protein and examined its localization and levels of expression in various cells and tissues using these monoclonal antibodies.

Materials and Methods

Cells

Cell lines of human cervix carcinoma (HeLa), human hepatoblastoma (HepG2), human bladder carcinoma (T24), rat hepatoma (AH13), rat bladder carcinoma (BC47), mouse hepatoma (Hepa I) and mouse bladder carcinoma (MBT-2) were cultured in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated fetal bovine serum (ICN Biomedicals, Aurora, OH, USA) in a humidified CO_2 incubator.

Animals

Donryu rats, F344 rats and Balb/c mice, 6–8 weeks old, were purchased from Kumagai Animal Farm (Hamamatsu, Japan), and housed in a controlled environment at 22°C.

Preparation of antigens

AH13 (5×10^9 cells) harvested from ascites of Donryu rats were solubilized with 10 mM Tris-HCl (pH 8.0) containing 0.5% Lubrol (Nakalai Tesque, Kyoto, Japan), 0.15 M NaCl, 1 mM iodoacetamide and protease inhibitors [1 nM pepstatin A (Wako Pure Chemical, Tokyo, Japan), 100 nM leupeptin (Wako), 1 μM p-(Amidinophenyl) methanesulfonyl fluoride hydrochloride (APMSF, Wako), 0.1 μM aprotinin (Merck, Darmstadt, Germany), 1 μM antipain dihydrochloride (Merck), 1.6 μM bestatin (Boehringer Mannheim, Tokyo, Japan)], and this cell extract was precleared by affinity chromatography with APH-8 (15) mAb (10 mg IgG in 2 ml Cellulofine; Seikagaku Kogyo, Tokyo, Japan), and subjected to affinity chromatography (10 mg IgG in 2 ml Cellulofine) with the anti-rat gp125-HC mAb B3 (6, 21, 29). GP125-HC and -LC were separated by SDS-PAGE of affinity column-bound proteins in the presence of 5% β-mercaptoethanol (β-ME).

Preparation of hybridomas secreting mAbs against a 40-kDa protein

LC proteins electro-separated using a Protean II xi cell (Bio-Rad, Hercules, CA, USA) in phosphate-buffered saline (PBS) were emulsified with an equal volume of Freund's incomplete adjuvant (Difco Laboratories, Detroit, MI, USA). A female BALB/c mouse received three injections of the emulsion equivalent to 2×10^8 cells in each immunization at 2-week intervals. The immune spleen cells (1×10^8) were fused with P3X63Ag8.653 mouse myeloma cells (1×10^7) by 50% polyethylene glycol 1540 (Wako). After cell fusion, hybridoma cells were selected in Hymedium 606 (26) (Kojin Co., Sakato, Japan). The hybridomas were screened by Western blotting analysis of the culture supernatants, and cloned by the limiting dilution method. Hybridoma clones (5×10^6), designated as 3F2 and 6B4, were injected i.p. into mice pretreated with 2, 6, 10, 14-tetramethylpentadecane (Wako). Ascites fluid was collected 10 to 14 days after inoculation, and precipitated with 50%-saturated ammonium sulfate. Further purification was performed by the gel filtration (Sepharose CL-6B, Pharmacia, Uppsala, Sweden), because SDS-PAGE analysis of two mAbs in the ascites samples were IgM.

Western blotting

Tumor cells were extracted at a concentration of 2×10^7 cells /ml by lysis buffer [PBS containing 1% NP-40, protease inhibitors (1 nM pepstatin A, 100 nM leupeptin, 1 μM APMSF, 100 nM pephabloc SC, 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 sample buffer. The denatured proteins were separated under reducing or non-reducing conditions by 7.5 or 12% SDS-PAGE, respectively, and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedfore, MA, USA). Nonspecific binding sites on PVDF membranes were treated with Block Ace (Dainihon Seiyaku, Osaka, Japan) for 2 h at 37°C, and the membranes were incubated with purified antibodies (10 μg/ml) overnight at 4°C. Membranes were washed extensively with PBS containing 0.05% Tween 20 (T-PBS) and incubated with rabbit anti-mouse immunoglobulin (Dako, Kyoto, Japan) diluted 1:200 in T-PBS for 1 h at room temperature. After extensive washing with T-PBS, 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 washing extensively with T-PBS, 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).

Sandwich-type enzyme-linked immunosorbent assay (s-ELISA)

S-ELISA was performed using 3F2, 6B4 and anti-CD98 mAbs, HBJ127 (14) and 1–10. Aliquots (50 μl) of antibodies (50 μg/ml) were passively adsorbed in duplicate on each well of 96-well plates (Sumitomo Bakelite, Tokyo, Japan) overnight at 4°C. Each well was treated with Block Ace for 3 h at 37°C to block nonspecific binding of the staining reagents. Fifty μl of T24 cell lysates (2×10^7 cells/ml) containing biotinylated mAbs (20 μg/ml) was added to each well and incubated overnight at 4°C. After washing extensively with T-PBS, 50 μl of 1:100 diluted ABC reagent (Vector Laboratories, Burlingame, CA, USA) was added to each well, and the plates were incubated for 45 min at 37°C. Each well was washed extensively with T-PBS and 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 minutes by addition of 0.5 M H_2SO_4 (75 μl in each well), and the optical density of the solution at 450 nm was measured with a Model 550 Microplate Reader (Bio-Rad).

Flow cytometric analysis of cells

Cells (3×10^5 in each tube), which were unfixed or fixed with PBS containing 3% paraformaldehyde-2% sucrose for 5 min and 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 suspended in 50 μl of PBS containing 1% bovine serum albumin (BSA), mixed with an equal volume of purified mAbs (10 μg/ml) for 1 h on ice. After washing with PBS, cells were labeled with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulin (Dako) for 30 min on ice. After washing of cells with PBS, immunofluorescence intensity of individual cells was determined using a FACScan flow cytometer (Becton-Dickinson, Sunnyvale, CA, USA).

Flow cytometric analysis for microspheres

The pellets from 100 μl of Polybead polystyrene 4.5 micron microsphere suspensions (Polysciences, Inc., Warrington, PA, USA) obtained by centrifugation at 5,000 g for 10 min were resuspended in anti-human CD98 mAb, HBJ127 (IgG, 100 μg/ml) at 37°C for 1 h, washed 3 times with PBS by centrifugation, and treated with Block Ace overnight at 4°C. Microspheres were mixed with 100 μl of HeLa cell lysate (2×10^7 cells/ml) overnight at 4°C, washed 3 times with T-PBS, and stored in 100 μl of PBS containing 1% BSA, 0.05% Tween 20 and 0.05% NaN_3. Microspheres (1 μl of suspension in each assay) were incubated with IgM mAbs (control IgM, anti-CD98; 4–26, 3F2 and 6B4) at 4°C for 1 h, washed 3 times with T-PBS, and incubated with FITC-conjugated goat anti-mouse μ chain antibodies (Jackson ImmunoResearch, West Grove, PA, USA) diluted 1:100 in PBS containing 1% BSA. After 3 washes with T-PBS, fluorescence intensities of microspheres were analyzed 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 incubated with hybridoma culture supernatants overnight at room temperature. After washing with PBS, endogenous peroxidase activity was inhibited by immersing sections in 3% H_2O_2-methanol for 5 min. After washing in PBS, sections were incubated with

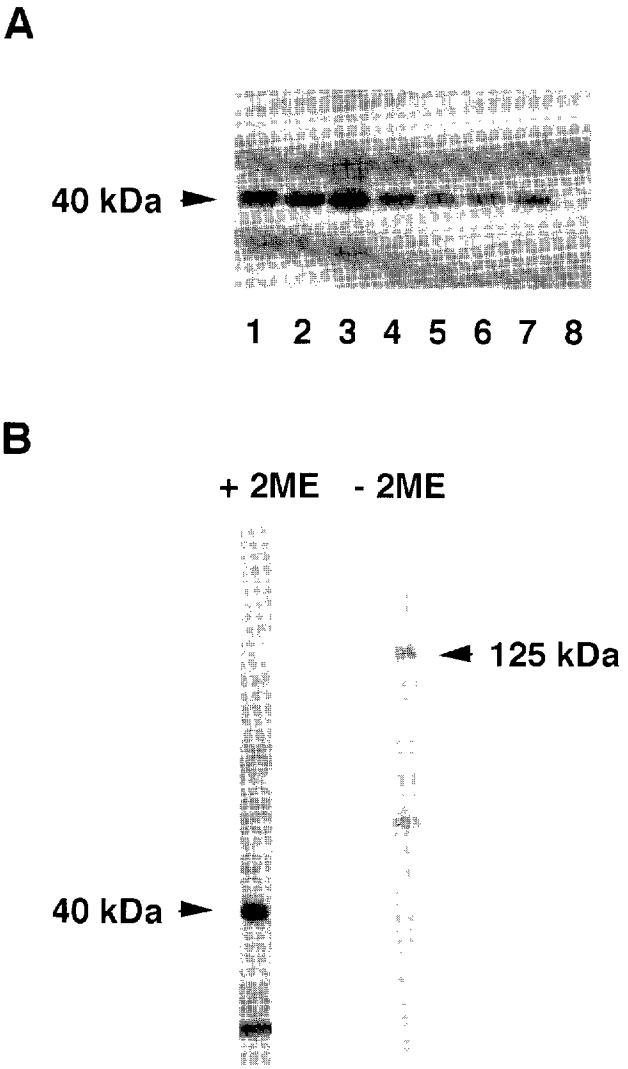

Fig. 1. Western blotting analysis of a 40-kDa protein disulfide-linked to CD98. (A): Cell lysate of AH13 was blotted with mAbs after 12% SDS-PAGE under reducing conditions with β-ME. Lane 1, 1G4; lane 2, 3F2; lane 3, 1A10; lane 4, 6B4; lane 5, 6B18; lane 6, 2B11; lane 7, 6B11; lane 8, medium. (B): Cell lysate of AH13 was blotted with 3F2 after 12% SDS-PAGE under reducing conditions, or after 7.5% SDS-PAGE under non-reducing conditions.

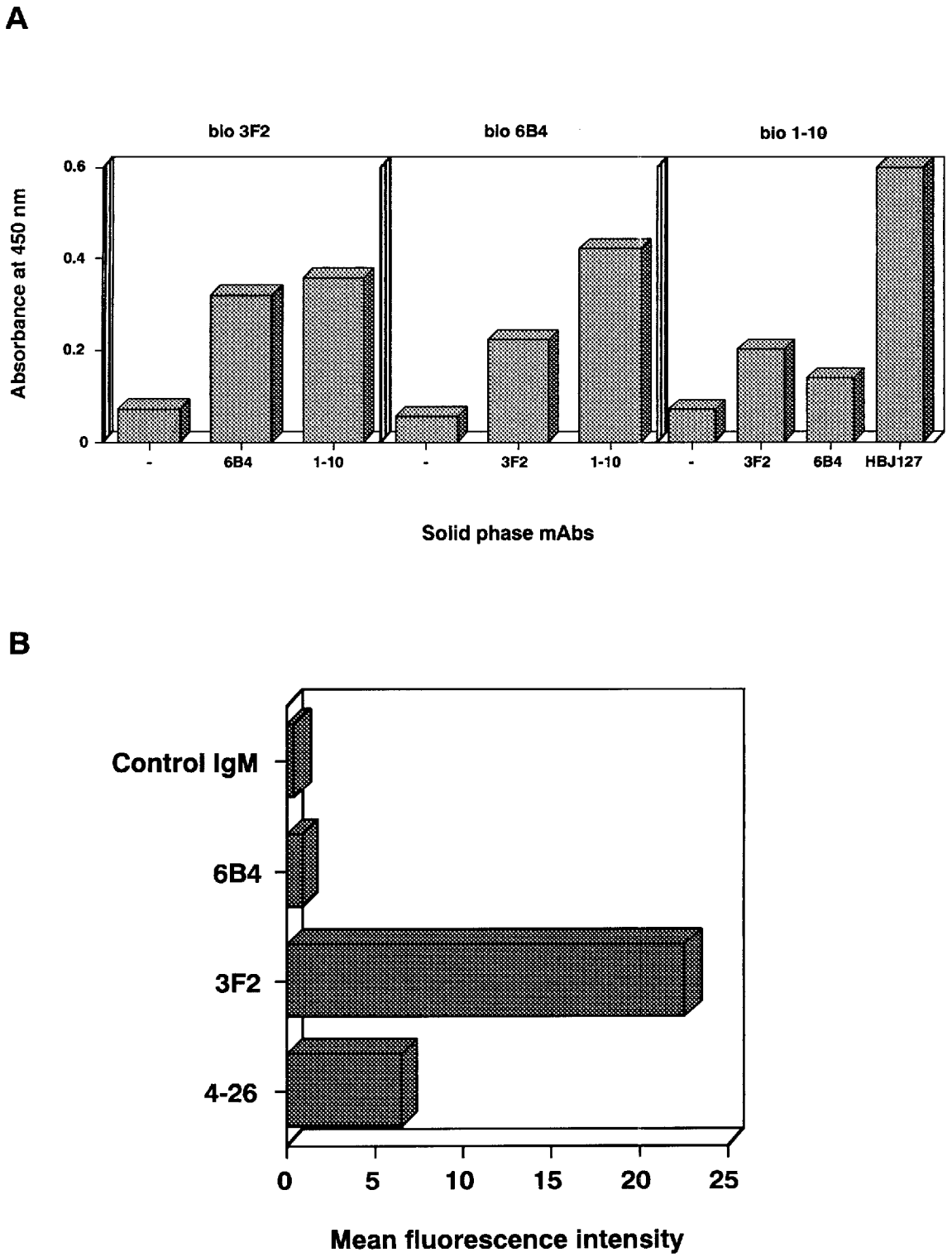

Fig. 2. Determination of the association of CD98 and a 40-kDa protein by sandwich-type ELISA (A) and flow cytometry with microspheres (B). In A, associations were detected with combinations with solid-phase mAbs and biotinylated mAbs. In B, microspheres, which were coated with anti-CD98 mAb (HBJ127) and reacted with HeLa cell lysate, were analyzed for immunoreactivity with the indicated IgM mAbs.

biotinylated horse anti-mouse IgG (Vector) diluted 1:100 in PBS for 1 h. After washing 3 times with PBS, the samples were treated with ABC reagent diluted 1:100 in PBS for 45 min. After washing 3 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 dehydrated with ethanol, cleared in xylene and mounted in Permount (Fisher Scientific, Fair Lawn, NJ). Localization of antibody-defined components was observed under a Zeiss Axiolab microscope and photographed.

Two-color immunostaining of cultured cells

Cells cultured in each well of 8-chamber slides (Falcon, Franklin Lakes, NJ, USA) were fixed with PBS containing 3% paraformaldehyde and 2% sucrose for 5 min, permeated with Triton buffer for 5 min, and treated sequentially with mAbs and 1:100 diluted FITC-conjugated goat anti-mouse γ chain and Texas Red-conjugated goat anti-mouse μ chain immunogloblins (Jackson). Between each step, cells were washed 3 times with PBS, and cell-bound immunofluorescence was observed under a Leica microscope, and analyzed using a MRC-1024 confocal imaging system (Bio-Rad).

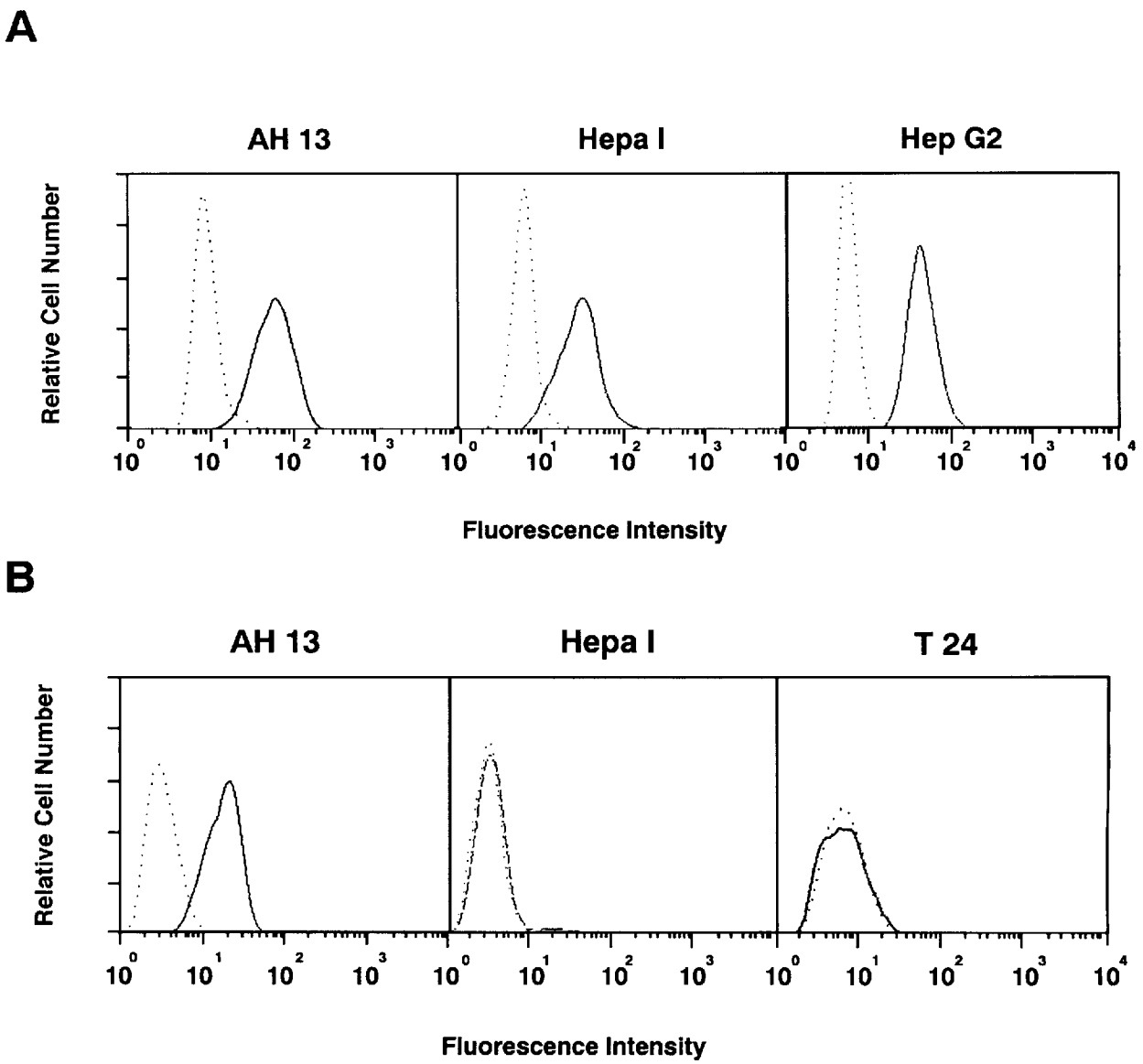

Fig. 3. Reactivities of mAbs with human and rodent tumor cells by flow cytometry. (A): Fixed and permeated cells were labeled with 3F2, and analyzed for the fluorescence by FACScan. (B): Unfixed cells were labeled with 6B4, and analyzed for cell-surface fluorescence by FACScan.

Results and Discussion

Preparation and characterization of mAbs which recognize a 40-kDa protein associated with CD98

A 40-kDa protein was purified from AH13 rat hepatoma cells by affinity chromatography using an anti-rat gp125-HC mAb column and subsequent SDS-PAGE in the presence of 5% β-ME. The purified protein was used as an antigen, and supernatants from about 300 wells of hybridoma cultures were screened by Western blotting analysis using AH13 cell lysates. Antibodies from seven hybridoma clones recognized 40-kDa proteins (Fig. 1A). In the following study, we used two mAbs designated as 3F2 and 6B4, which also recognized 40-kDa proteins in the lysate of human and mouse cells in addition to rat cells (data not shown). The mAb 3F2 detected a 125-kDa protein in the lysate of AH13 cells developed by SDS-PAGE under non-reducing conditions (Fig. 1B), suggesting that a 40-kDa protein recognized by this mAb is disulfide-linked to an 85-kDa protein (CD98) and forms a 125-kDa heterodimeric protein. Identical results were obtained with mAb 6B4 (data not shown).

To determine whether the 40-kDa protein recognized by the mAbs is associated with CD98, s-ELISA was performed using 3F2, 6B4 and the anti-CD98 mAbs HBJ127 and 1–10 (Fig. 2). Binding of 1–10 mAb was detected when HB127 mAb, which recognizes a different epitope on CD98, was adsorbed on the plates, demonstrating the validity of this assay system (Fig. 2A). Binding was also detected in s-ELISA with a combination of 3F2 and 6B4. In addition, similar results were obtained by combinations of 3F2 and 1–10 or 6B4 and 1–10, indicating the association of 40-kDa protein with CD98.

Flow cytometry of microspheres also demonstrated the association of CD98 and the antigen defined with 3F2 (Fig. 2B), i.e., microspheres bearing CD98 and CD98-associated molecules reacted more strongly with 3F2 than 4–26, which recognizes an epitope on CD98 different from that recognized by HBJ127. However, 6B4 gave a negative result in this assay, probably because the epitopes defined with HBJ127 and 6B4 lie close to each other. These observations indicated that 3F2 and 6B4 recognize different epitopes on the 40-

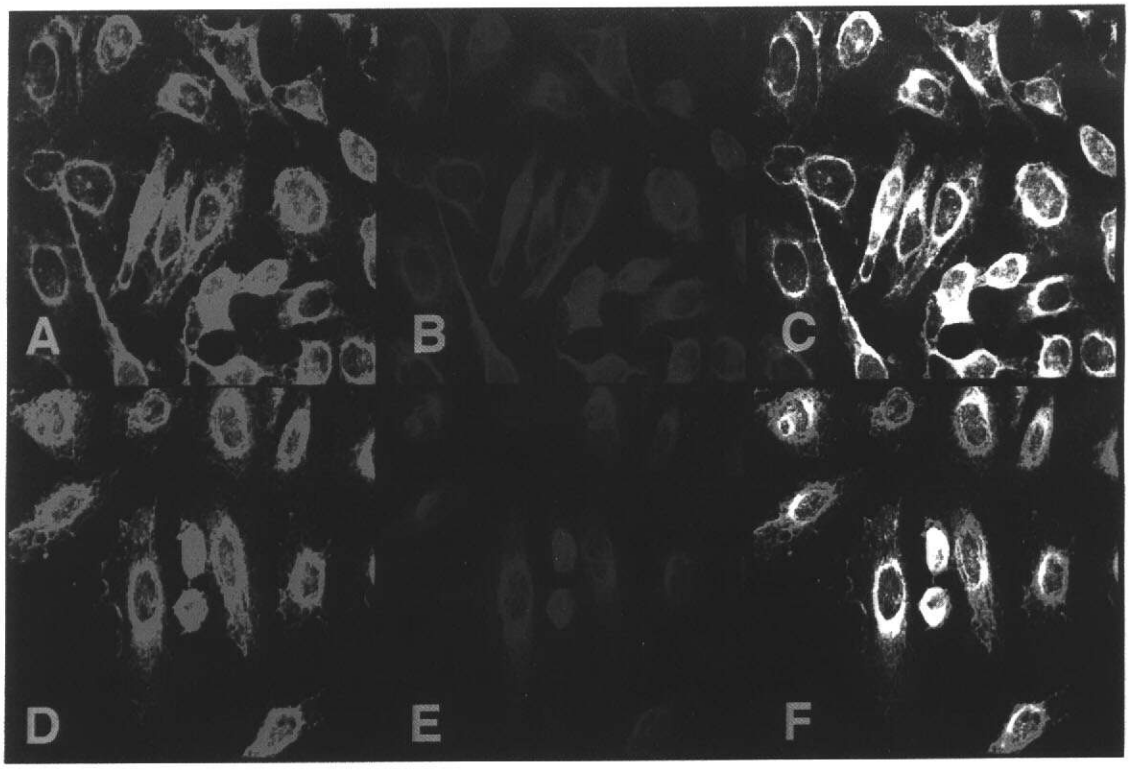

Fig. 4. Subcellular localization of CD98 and 40-kDa protein in HeLa cells by 2-color immunofluorescence staining followed by confocal microscopy. Fixed and permeated HeLa cells were stained with mAbs as described in *Materials and Methods*. A and D show staining by anti-CD98 mAb, HBJ127 followed by FITC-conjugated goat anti-mouse γ chain antibodies. B and E shows staining by 3F2 and 6B4, respectively, followed by Texas Red-conjugated goat anti-mouse μ chain antibodies. C and F shows the double-staining images of A and B, and D and E, respectively.

kDa protein and that the 40-kDa protein is a light chain of GP125.

Localization of the 40-kDa protein

We examined reactivities of mAbs 3F2 and 6B4 with various tumor cell lines by flow cytometry. The mAb 3F2 did not react with unfixed cells (data not shown), but reacted with fixed and permeabilized human, mouse and rat tumor cell lines, suggesting that this mAb recognizes a common epitope preserved among various mammalian species (Fig. 3A). In addition to the reactivity to fixed and permeabilized cells, 6B4 exceptionally reacted with an unfixed rat tumor cell line (Fig. 3B). These results indicated that the epitopes recognized by mAbs 3F2 and 6B4 are cryptic at the cell surface or reside mainly intracellularly, although the epitope of 6B4 mAb resides at least partly in the extracellular region in the case of the rat cells.

To compare the subcellular localizations of CD98 and the 40-kDa protein, HeLa cells were double-stained with HBJ127 and 3F2, or HBJ127 and 6B4, and analyzed by confocal microscopy (Fig. 4). Each mAb characteristically stained perinuclear regions in addition to the cell surface (Fig. 4A, B, D and E), and colocalization of CD98 and 40-kDa protein was observed mainly in perinuclear regions (Fig. 4C and F). In addition, 6B4 stained a more restricted cellular region compared with other mAbs, suggesting that the 6B4-defined epitope is cryptic under some conditions through the presence or absence of interaction with adjacent molecules.

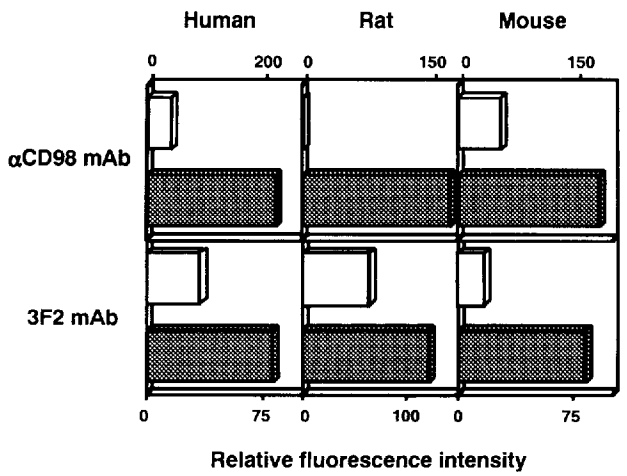

Fig. 5. Flow-cytometric analysis of CD98 and 3F2-defined 40-kDa protein in resting and activated T cells. Fresh and IL-2-activated T cells, which were fixed and permeated as described in *Materials and Methods*, were labeled with mAbs, and analyzed for cell-associated fluorescence by FACScan. Data represent mean fluorescence intensities.

Although the role of CD98 as a carrier of LAT1 from

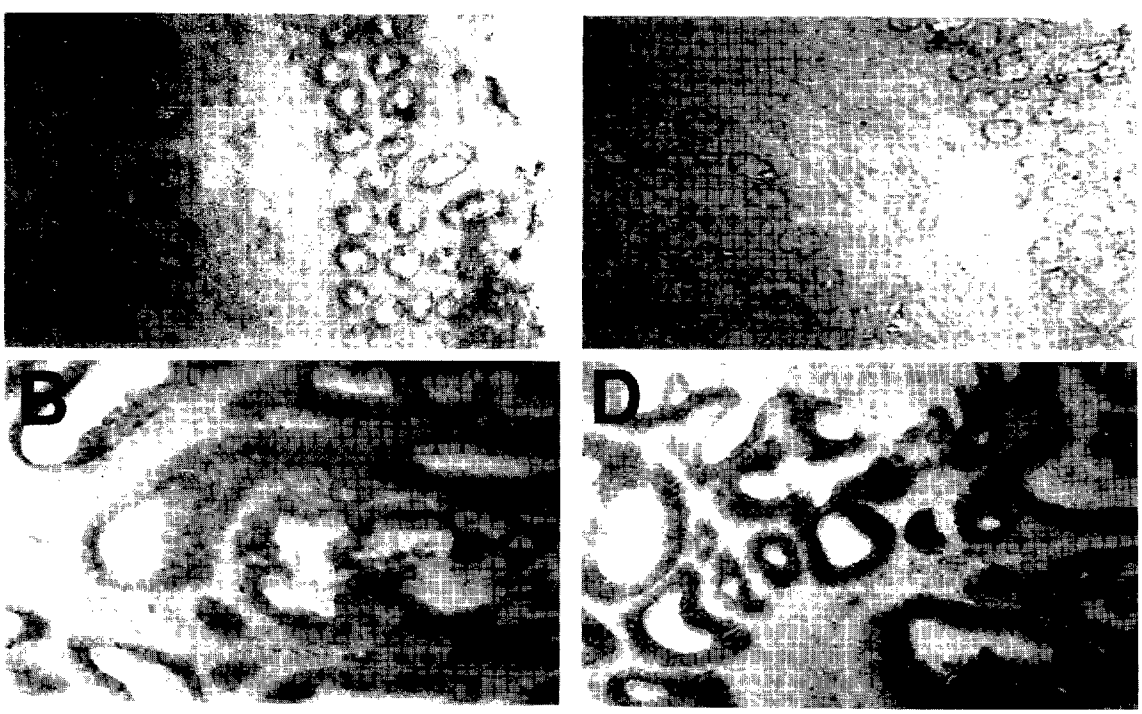

Fig. 6. Immunostaining of normal human colon (A and C) and colon cancer (B and D) with 3F2 (A and B) or 6B4 (C and D).

the intracellular region to the cell surface has been addressed (10, 13, 17), the colocalization of CD98 and the 40-kDa protein in the perinuclear region suggested a distinct role of CD98. Thus, it is conceivable that CD98 and the 40-kDa protein have roles in processes other than amino acid transport.

Expression of the 40-kDa protein in activated lymphocytes and malignant cells

Since CD98 is strongly expressed in activated lymphocytes (6, 7, 22, 27), we compared the expression of CD98 and the 40-kDa protein in resting and activated lymphocytes by flow cytometry using anti-CD98 mAbs (human, HBJ127; rat, B3; mouse, MB872) and 3F2. As in the case of CD98, the expression of the 40-kDa protein was high in interleukin 2-activated lymphocytes and low in resting lymphocytes (Fig. 5). An increase in the expression of the 40-kDa protein was also observed when lymphocytes were activated with concanavalin A (data not shown). These results suggested that in addition to CD98, expression of the 40-kDa protein is upregulated during the process of activation of lymphocytes.

To investigate the relevance of high expression of the 40-kDa protein to malignant transformation, frozen sections of human colon cancers and normal colon tissues were immunostained with mAbs 3F2 and 6B4. A typical staining pattern is shown in Figure 6. These mAbs heavily stained colon cancer cells, and only lightly stained normal colon epithelial cells, suggesting that high-level expression of the 40-kDa protein is linked to malignant transformation similarly to TA1/E16 (LAT1) (25). Preferential expression of the 40-kDa protein in cancer tissues versus normal tissues was also observed in the human esophagus and breast, and in human and rat urinary bladder (data not shown).

Expression of the 40-kDa protein and CD98 in normal tissues

CD98 is expressed in the restricted normal tissues, especially in those with growth potential such as the intestinal epithelium (6, 14) and the basal layer of squamous epithelia (6, 14, 19). To compare the distribution of CD98 and the 40-kDa protein in normal tissues, frozen sections of the rat tongue were immunostained with anti-rat CD98 mAb B3, and anti-40-kDa protein mAb 3F2 or 6B4 (Fig. 7). The mAbs B3 and 6B4 selectively stained the basal layer (Fig. 7A and 7C). In contrast, 3F2 mAb stained the upper epithelial part in addition to the basal layer (Fig. 7B). This staining pattern suggested the existence of 3F2-defined 40-kDa protein, which was not associated with CD98, and that 6B4-defined epitope is cryptic in this form of the 40-kDa protein. However, we cannot completely exclude the possibility that 3F2 and 6B4 recognize 40-kDa isoforms. In normal tissues, LAT1 is highly expressed in the brain, spleen, testis and placenta (10, 17), and y^+LAT1 is selectively expressed in the kidney and the intestine (20). On the other hand, the 40-kDa protein defined with 3F2 or 6B4 is expressed ubiquitously (data not shown). These observations suggested that the combination of

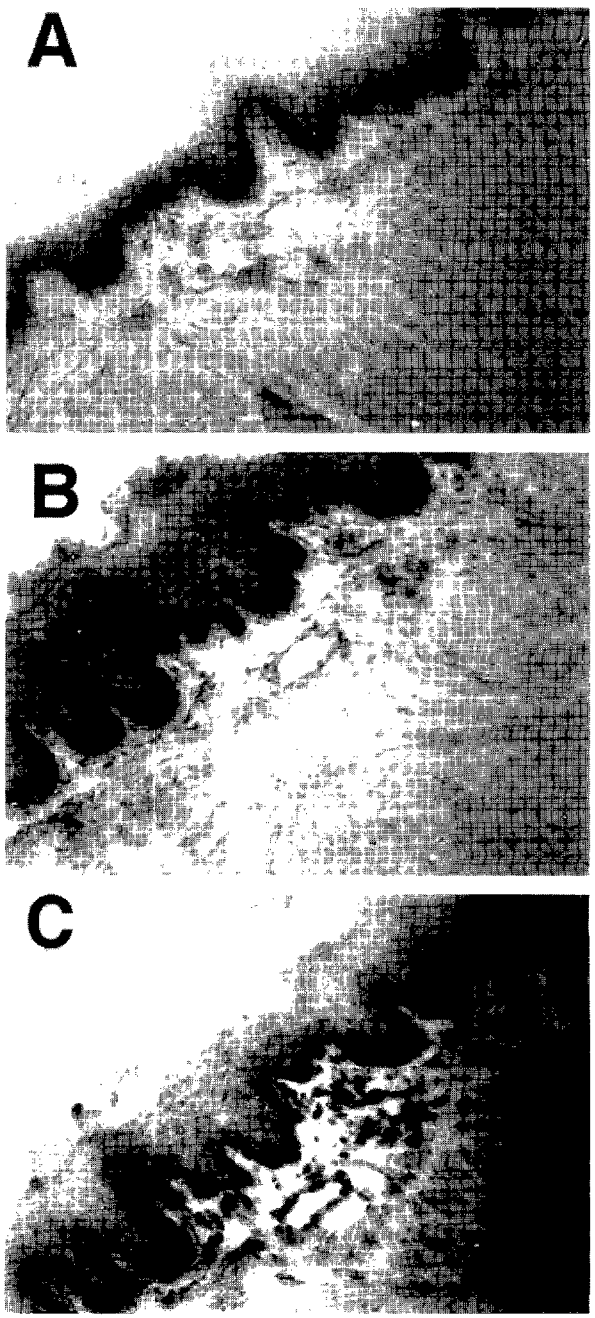

Fig. 7. Immunostaining of the normal rat tongue tissue with anti-CD98 mAb B3 (A), 3F2 (B), and 6B4 (C).

GP125-HC (CD98) and LC is determined by the tissue or cell type, and that GP125 could play various functions by changing its partners.

We detected a third light chain of GP125 and produced monoclonal antibodies specifically recognizing this protein. Analyses of functions of this new GP125-LC will lead to the understanding of some of the reported phenomena involving CD98 such as cell fusion (18), cell adhesion (4), lymphocyte activation (6, 7, 21, 27), cell proliferation (6, 8, 14, 19) and malignant transformation (5, 6, 14, 29). Cloning of the cDNA for the new GP125-LC is currently in progress.

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