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Expression of Ly-6D on the Surface of Normal and Neoplastic Mammary Epithelial Cells of the Mouse

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Rat were immunized with mouse mammary epithelial cells and monoclonal antibodies (MAbs) were obtained to identify antigens stably expressed on the surface of both normal and neoplastic mammary epithelial cells of the mouse. Examination of the reactivities of the MAbs by immunofluorescence staining of tissue sections showed that one of the antibodies, MAb 2A2, reacted with luminal epithelium of the mammary gland and spontaneous mammary carcinomas of SHN mice. Further examination of the tissue lysates by western blot analysis revealed that MAb 2A2 reacts with a 17-kDa antigen expressed in normal mammary epithelial cells and mammary carcinoma cells. The antigen recognized by MAb 2A2 was absent in the lysates of liver, lung, salivary gland, kidney, small intestine, ovary and uterus. After immunoaffinity purification of the MAb 2A2-recognized antigen and determination of its N-terminal amino acid sequence, we identified the antigen as Ly-6D, also known as ThB, which belongs to a family of glycosyl-phosphatidylinositol-anchored cell surface proteins. Northern blot analysis further demonstrated that Ly-6D mRNA is expressed in the mammary gland. Based on these observations we concluded that Ly-6D is stably expressed on the surface of both normal and neoplastic mammary epithelial cells of the mouse. Ly-6D will serve as a useful epithelial cell surface marker for the study of mammary gland development, as well as for breast cancer research.

Key words: Mammary epithelium — Mammary carcinoma — Monoclonal antibody — Ly-6D — Mouse

The mammary gland consists of different types of cells including ductal and alveolar epithelial cells lining the lumen of the mammary tree, myoepithelial cells surrounding these luminal epithelial cells, and fat and other stromal cells constituting the mammary stroma. Monoclonal antibodies (MAbs) have been extensively used to identify these different types of cells.[1–4] MAbs have also been used for the classification of breast cancer cells and for the detection of serum and tissue markers for diagnosis of breast cancer.[3–5] Of particular importance is the use of MAbs against cell surface antigens for the antibody immunotherapy of breast cancer.[6, 7] Antigens expressed on breast cancer cells, i.e., breast cancer-associated cell surface antigens, are likely to be the targets for the antibody immunotherapy. To date, a number of MAbs against various breast cancer-associated cell surface antigens have been developed and their antitumor effects have been evaluated in clinical studies. Among these are MAbs against HER2, EGF receptor and MUC-1.[6–9] The antigens recognized by these MAbs are expressed to varying degrees in different breast cancers. For example, HER2 has been reported to be overexpressed in 25 to 30% of breast cancers.[10] At present, only those antigens overexpressed in particular breast cancers are considered to be the target for antibody immunotherapy. Thus, to select the most effective MAbs for the antibody immunotherapy of a particular breast cancer, more breast cancer-associated surface antigens, preferably breast cancer-specific antigens or mammary epithelium-specific antigens, must be identified.

In this study, our objective was to find new antigens which are expressed stably on the surface of breast cancer cells. As a first step toward this end, we raised MAbs against normal mammary epithelial cells of the mouse and analyzed the target antigens of the obtained MAbs. Here, we report the identification of one of the antigens stably expressed on the surface of both normal and neoplastic mammary epithelial cells of the mouse.

MATERIALS AND METHODS

Animals BALB/c mice, C57BL/6 mice and Wistar rats were purchased from Charles River Japan (Kanagawa). SHN mice[11] kindly provided by Dr. H. Nagasawa, Faculty of Agriculture, Meiji University, and Dr. T. Mori, Department of Biological Sciences, University of Tokyo, were maintained in our laboratory.

Isolation and culture of mammary epithelial cells Mammary epithelial cells used for immunization were obtained by digesting the mammary gland of 10-week-old virgin female BALB/c mice with 0.05% collagenase (type I, 260 U/mg; Sigma, St. Louis, MO) in Hanks' solution

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for 2 h at 37°C. The resulting epithelial cell clumps were collected by centrifugation, pipetted to remove the stroma, and finally suspended in Hanks' solution.

Mammary epithelial cells used for cell culture were isolated as described previously.[12, 13] The cells were suspended in Dulbecco's modified Eagle's medium (DMEM; Nissui, Tokyo) containing 10% fetal bovine serum (FBS; GIBCO BRL, Grand Island, NY), 100 U/ml of penicillin (Sigma) and 100 μg/ml of streptomycin (Sigma). One milliliter of the cell suspension containing 2.5×10^5 cells was placed in each of the 2.2-cm wells (12-well microplate; Iwaki Glass, Chiba) which contained a glass coverslip. The cells were cultured at 37°C in a humidified 5% CO_2/95% air incubator.

Production of MAbs MAbs were obtained by a modification of the rat lymph node method.[14, 15] Prior to the immunization, 100 μl of an emulsion, made by mixing 8 volumes of Freund's complete adjuvant (DIFCO, Detroit, MI) and 5 volumes of water, was injected into each of the hind leg footpads of 14-week-old female Wistar rats to augment the immunological response.[16] On the next day, 1.5×10^6 mammary epithelial cells in 50 μl of Hanks' solution were injected into each of the footpads. This immunization was repeated on the next day, 6 days later and 27 days later. Seven days after the last injection, iliac lymph nodes were removed and the lymph node cells were fused with the mouse myeloma cells, Sp2/0-Ag14,[17] using 50% polyethylene glycol 4000 (Nacalai Tesque, Kyoto). Hybridomas were selected in an HAT medium consisting of GIT medium (Wako Pure Chemical, Osaka) supplemented with 10% FBS, 10% BM-condimed H1 (Boehringer Mannheim, Mannheim, Germany), 1% MEM non-essential amino acids (COSMO BIO, Tokyo), 1× HAT-media supplement (100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine; Boehringer Mannheim), 100 U/ml penicillin and 100 μg/ml streptomycin. Immunoglobulin was purified from the culture supernatant of hybridoma on a "HiTrap" Protein G column (Amersham Pharmacia Biotech, Buckinghamshire, UK). The immunoglobulin class/subclass was determined using a monoclonal antibody typing kit (The Binding Site, Birmingham, UK).

Immunofluorescence staining Fragments of the second thoracic mammary gland and the submaxillary salivary gland of female BALB/c mice were placed in O.C.T. compound (Miles, Inc., Elkhart, IN) and quickly frozen in liquid nitrogen. The frozen tissues were cut into 6-μm sections, fixed with 10% formalin in phosphate-buffered saline (PBS), pre-incubated with 10% normal goat serum (Vector Laboratories, Burlingame, CA) and subsequently incubated with 80 μl of either the hybridoma culture supernatant or MAb 2A2 (0.25 μg/ml of purified IgG in PBS) for 4 h at room temperature or overnight at 4°C. The sections incubated with normal rat IgG (0.25 μg/ml, ZYMED, South San Francisco, CA) served as negative controls. The sections were then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG (1:200 dilution in PBS; American Qualex, San Clemente, CA) for 2 h at room temperature. Cell nuclei were stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI, 0.5 μg/ml in PBS; Sigma).

Myoepithelial cells were stained with mouse anti-α-smooth muscle actin MAb (5 μg IgG/ml; Clone 1A4, Sigma) and subsequently with FITC-conjugated rabbit anti-mouse IgG (1:400 dilution in PBS; MBL, Nagoya). Normal mouse IgG (5 μg/ml; Intercell Technologies, Hopewell, NJ) was used as a negative control.

Mammary epithelial cells cultured on coverslips were first fixed with 10% formalin, then blocked with 10% normal goat serum, incubated with MAb 2A2 and subsequently with FITC-conjugated goat anti-rat IgG. Alternatively, the live mammary epithelial cells were first blocked with 10% normal goat serum, then incubated with MAb 2A2, fixed with 10% formalin and subsequently incubated with FITC-conjugated goat anti-rat IgG.

Western blot analysis Total cell lysates obtained by homogenizing the tissues in a sodium dodecylsulfate (SDS) buffer (2.3% SDS, 63 mM Tris-HCl, pH 6.8) were run on 13.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions. After electrophoresis, proteins were transferred onto a nitrocellulose filter (Hybond ECL, Amersham Pharmacia Biotech) using a semi-dry blotting apparatus. The filter was then soaked in a blocking buffer (PBS containing 0.1% Tween 20 and 2% bovine serum albumin) overnight at 4°C, incubated with MAb 2A2 (3.3 μg/ml) for 1 h at room temperature, and finally incubated with horseradish peroxidase-conjugated sheep anti-rat Ig (1:1000 dilution in PBS containing 0.1% Tween 20; $F(ab')_2$ fragment, Amersham Pharmacia Biotech) for 1 h at room temperature. An enhanced chemiluminescence detection system ("SuperSignal" West Pico Chemiluminescent Substrate, Pierce, Rockford, IL) was used to detect the signals on an X-ray film (Hyperfilm ECL; Amersham Pharmacia Biotech). Rainbow markers (Amersham Pharmacia Biotech) were used as size markers.

Purification of MAb 2A2-reactive antigen Spontaneous mammary carcinomas of female SHN mice were homogenized in 2 volumes of a lysis buffer [0.5% Triton X-100, 10 mM Tris-HCl (pH 7.5), 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM (p-amidinophenyl)methanesulfonyl fluoride hydrochloride (p-APMSF, Wako Pure Chemical), 5 μg/ml aprotinin (Wako Pure Chemical), 1 μg/ml pepstatin (Sigma), 2 μg/ml leupeptin (Nacalai Tesque)] and centrifuged at 20 000g for 20 min at 4°C. The pellet was quickly frozen in liquid nitrogen and stored at −80°C until use. The frozen material derived from a total of 85 g of mammary carcinomas was thawed and homogenized in 2 volumes of a modified SDS-free RIPA buffer[18] [1% NP-40 (Calbiochem, La

Jolla, CA), 0.25% deoxycholic acid (Sigma), 50 m*M* Tris-HCl (pH 7.4), 1 m*M* ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 150 m*M* NaCl, 10 m*M* sodium fluoride, 1 m*M* *p*-APMSF, 2.5 μg/ml aprotinin, 0.5 μg/ml pepstatin and 1 μg/ml leupeptin]. After centrifugaton of the homogenate at 20 000*g* for 20 min at 4°C, the MAb 2A2-reactive antigen was isolated from the supernatant by immunoabsorption as follows. The purified IgG of MAb 2A2 (150 μg of protein) was coupled to Protein G-conjugated agarose beads [400 μl (v/v), Protein G PLUS-AGAROSE, Calbiochem] by incubating in the SDS-free RIPA buffer for 10 min at room temperature and placed in a plastic column. The column was washed twice with the SDS-free RIPA buffer, the supernatant was placed on the column, and the column was washed with the SDS-free RIPA buffer. The immunoabsorbed material was then eluted with 260 μl of the SDS buffer, run on an SDS-PAGE gel under nonreducing conditions and transferred to a polyvinylidene difluoride (PVDF) membrane (Trans-Blot, Bio-Rad, Richmond, CA). After the transfer, the proteins on the membrane were stained with Ponceau-S. The stained protein of 17 kDa was excised and its N-terminal amino acid sequence was determined by a peptide sequencer (PSQ-1, Shimadzu, Kyoto). A portion of the eluted immunoabsorbed material was examined by silver staining (Ag-STAIN "DAIICHI," Daiichi Pure Chemicals, Tokyo).

Northern blot analysis Total RNA was extracted from BALB/c mouse tissues by the acid guanidinium thiocyanate-phenol-chloroform method.[19] The RNA (22.5 μg) was

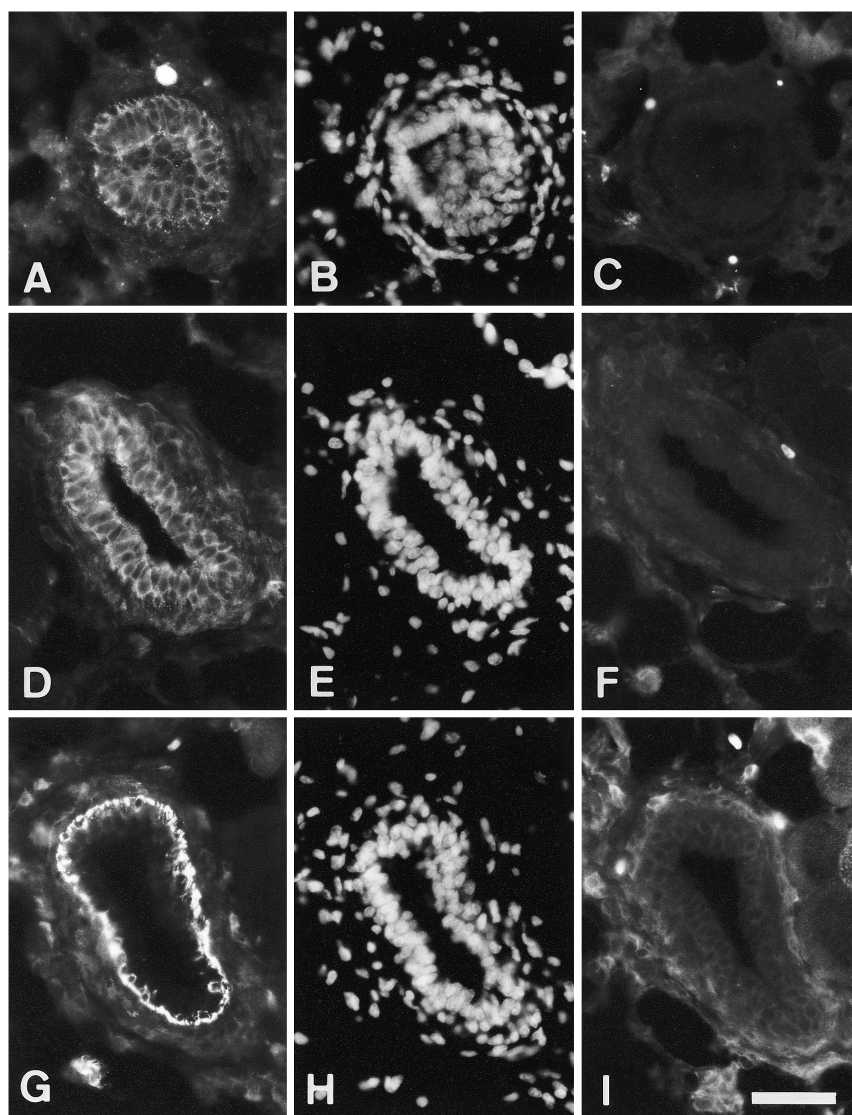

Fig. 1. Immunofluorescence staining of the mouse mammary gland. Frozen sections of the mammary gland of 11-week-old female BALB/c mice were fixed with formalin and incubated with MAb 2A2 (A, D), mouse anti-α-smooth muscle actin MAb (G), normal rat IgG (C, F) or normal mouse IgG (I). FITC-conjugated goat anti-rat IgG (A, C, D, F) or FITC-conjugated rabbit anti-mouse IgG (G, I) was used as a secondary antibody. The sections used for A, D and G were also stained for nuclei with DAPI (B, E, H). Serial sections were used for A and C, and for D, F, G and I. The scale bar in I indicates 50 μm.

electrophoresed on a 1.2% formaldehyde/agarose gel and blotted onto a Hybond-N+ membrane (Amersham Pharmacia Biotech). Hybridization was carried out for 2 h at 68°C in Rapid hybridization buffer (Amersham Pharmacia Biotech) with ^{32}P-labeled Ly-6D specific cDNA (0.7 kb) as a probe. The membrane was washed at 68°C in 2× SSC (standard sodium citrate)/0.1% SDS, 1× SSC/0.1% SDS and 0.2× SSC/0.1% SDS for each 20 min. The membrane was exposed to a Hyperfilm-MP (Amersham Pharmacia Biotech) for 24 h at −80°C.

RESULTS

Rat were immunized with epithelial cells freshly isolated from virgin mice to obtain MAbs which recognize antigens on the surface of mammary epithelial cells. The iliac lymph node cells of the immunized rats were then fused with mouse myeloma cells to obtain hybridomas.[14, 15] The hybridoma culture supernatants were screened by immunofluorescence methods for their differential reactivity with frozen sections of the salivary and mammary glands of the mouse. Out of 257 hybridoma culture supernatants screened, 198 reacted with the salivary gland, while 59 reacted only with mammary gland. Subsequent examination of these 59 mammary gland-positive supernatants for their reactivity with the mammary gland resulted in selection of 13 mammary epithelial cell-reactive MAbs. One of them, MAb 2A2 (Isotype: IgG2b) reacted with the periphery of the mammary epithelial cells present both at the terminal end of a mammary duct (Fig. 1A) and lining the lumen of a duct (Fig. 1D). The fluorescence was seen as a continuous line or occasionally as discontinuous dots around the periphery of the epithelial cells. The blood vessels and the stroma of the mammary gland were negative for staining with MAb 2A2. That the staining is specific to the luminal epithelial cells was verified by the staining of myoepithelial cells with an antibody against α-smooth muscle actin.[20, 21] The myoepithelial-specific fluorescence was present solely in the cells basal to the luminal epithelial cells, and absent in the MAb 2A2-positive luminal epithelial cells lining the ducts (Fig. 1G).

Mammary epithelial cells were cultured as monolayers and incubated with MAb 2A2 to examine whether MAb 2A2 binds to an antigen present on the outer surface of the cells. When live, unfixed cells were first incubated with MAb 2A2 and then fixed and incubated with FITC-conjugated secondary antiserum, fluorescence was seen as a uniform distribution of small dots on the epithelial cells (Fig. 2A). When the cells were first fixed with formalin and then incubated with MAb 2A2 followed by FITC-conjugated secondary antiserum, fluorescence was also seen, but it was localized around the periphery of the cells (Fig.

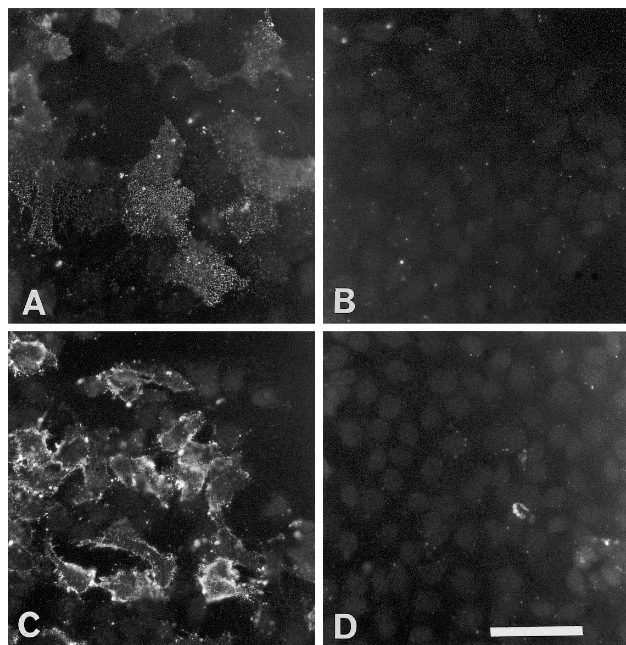

Fig. 2. Immunofluorescence staining of mouse mammary epithelial cells in monolayer culture. Mammary epithelial cells isolated from the 11-week-old BALB/c female mouse mammary gland were cultured for 2 days as described in "Materials and Methods." The cultured live cells were first incubated either with MAb 2A2 (A) or with normal rat IgG (B), then fixed with formalin and stained with FITC-conjugated goat anti-rat IgG. Alternatively, the cultured cells were first fixed with formalin and then incubated either with MAb 2A2 (C) or with normal rat IgG (D), and stained with FITC-conjugated goat anti-rat IgG. The scale bar in D indicates 50 μm.

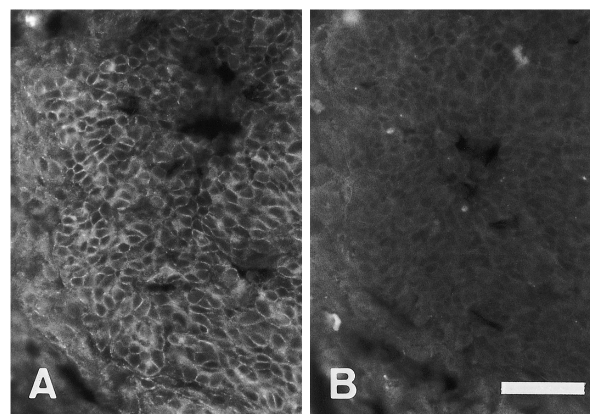

Fig. 3. Immunofluorescence staining of a mouse mammary carcinoma. Frozen sections were fixed with formalin and incubated with MAb 2A2 (A) or with normal rat IgG (B). FITC-conjugated goat anti-rat IgG was used as a secondary antibody. The scale bar in B indicates 50 μm.

2C). Although the patterns of distribution of the immunofluorescence were different, the presence of the MAb 2A2-reactive fluorescence in both fixed and unfixed mammary epithelial cells suggested that the epitope of the antigen recognized by MAb 2A2 is located on the outer surface of mammary epithelial cells.

We next examined whether the MAb 2A2-recognized antigen is also present on mammary carcinoma cells. After incubation of the frozen sections of spontaneous mammary carcinomas from SHN mice with MAb 2A2, a continuous line of immunofluorescence was seen around the periphery of the cancer cells (Fig. 3), suggesting that the distribution of the MAb 2A2-recognized antigen is similar in normal and neoplastic mammary epithelial cells.

In western blot analysis, MAb 2A2 clearly recognized a band of approximately 17 kDa in the mammary epithelial cell lysate. This band was virtually absent in other tissues examined, including the salivary gland, small intestine, spleen, kidney, lung, liver, ovary, uterus, prostate and testis (Fig. 4). As shown in Fig. 5, the MAb 2A2-recognized 17-kDa antigen was also detectable in all of the six spontaneous mammary carcinomas from SHN mice. This suggests that the same antigen is stably expressed in both normal and neoplastic mammary epithelial cells.

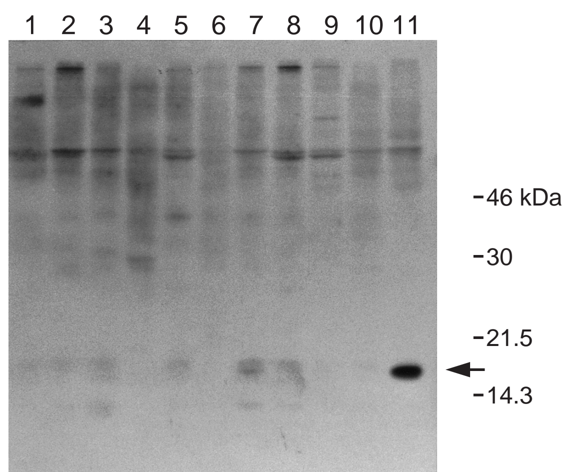

Fig. 4. Western blot analysis of the MAb 2A2-reactive antigen in mouse tissues. Total cell lysates were prepared from the salivary gland (lane 1), lung (lane 2), spleen (lane 3), liver (lane 4), small intestine (lane 5), kidney (lane 6), ovary (lane 7), uterus (lane 8) and mammary epithelial cells (lane 11) of 11- to 16-week-old female BALB/c mice, or from prostate (lane 9) and testis (lane 10) of 20-week-old male BALB/c mice. Twenty micrograms of each of the lysates was run on 13.5% SDS-PAGE under non-reducing conditions. The separated proteins were transferred onto a nitrocellulose membrane. The membrane was then incubated with MAb 2A2 and processed as described in "Materials and Methods." The arrow indicates the position of the MAb 2A2-reactive band. The positions of protein size markers are shown on the right.

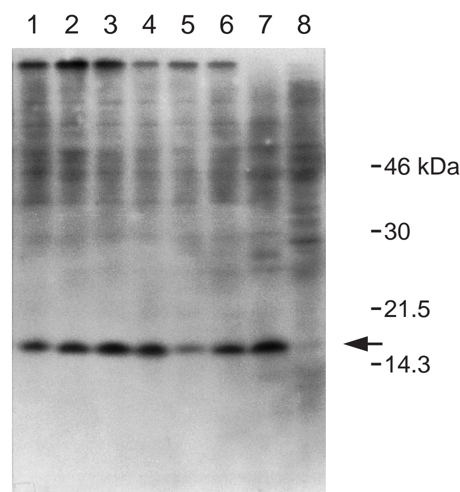

Fig. 5. Western blot analysis of the MAb 2A2-reactive antigen in spontaneous mammary carcinomas of the mouse. Total cell lysates were prepared from mammary carcinomas of SHN mice (lanes 1–6). Isolated mammary epithelial cell lysate (lane 7) and liver cell lysate (lane 8) were prepared from 16-week-old female SHN mice. Fifty micrograms of the total cell lysate was run on 13.5% SDS-PAGE under non-reducing conditions, and the separated proteins were transferred onto a nitrocellulose membrane. The membrane was then incubated with MAb 2A2 and processed as described in "Materials and Methods." The positions of protein size markers are shown on the right. The arrow indicates the position of the MAb 2A2-reactive band.

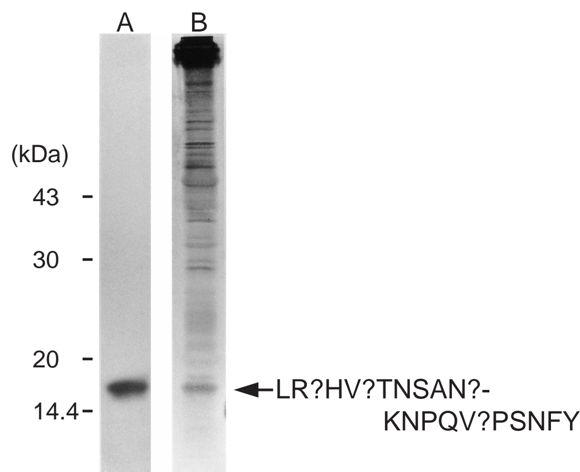

Fig. 6. Identification of the MAb 2A2-reactive antigen. The MAb 2A2-reactive antigen was partially purified from spontaneous mouse mammary carcinomas, run on 13.5% SDS-PAGE under non-reducing conditions, and blotted onto a PVDF membrane. The area of the membrane corresponding to the MAb 2A2-reactive antigen (A) was excised and processed for N-terminal amino acid sequence determination. The amino acid sequence determined is shown next to the silver-stained gel (B). The positions of protein size markers are shown on the left.

We purified the antigen from spontaneous mammary carcinomas of SHN mice by affinity chromatography on a column of MAb 2A2 followed by separation on SDS-PAGE to identify the MAb 2A2-recognized antigen (Fig. 6). N-Terminal amino acid sequence analysis of the 17-kDa band showed that the sequence is LR?HV?TNSAN?KNPQV?PSNFY. Positions marked as "?" are probably cysteines, since no signals were detectable in these cycles of Edman degradation. A search on the SWISS-PROT database revealed that the amino acid sequence matched with that of Ly-6D, also called ThB, a 15-kDa glycosyl-phosphatidylinositol- (GPI-) anchored cell surface protein that is expressed in thymus, spleen and skin of the mouse.[22–25]

It has been reported previously that Ly-6D is expressed at a very low level in the spleen of BALB/c mice and at a relatively high level in the spleen of C57BL/6 mice, and at high levels in the skin of both strains.[23, 24] We compared the expression of the MAb 2A2-recognized antigen in BALB/c mice and C57BL/6 mice to confirm that the MAb 2A2-recognized antigen is identical to Ly-6D, and to re-examine our previous observations (see Fig. 4). As shown in Fig. 7, the expression of the MAb 2A2-recognized antigen in the skin and mammary epithelial cells is high in both BALB/c and C57BL/6 mice. In contrast, its expression in the spleen is relatively low in C57BL/6 mice and extremely low in BALB/c mice, in accordance with the previous reports.[23, 24]

The isolation and sequencing of the MAb 2A2-reactive antigen and western blot analysis indicated that MAb 2A2 recognizes the surface antigen Ly-6D. Since the expression of Ly-6D mRNA in the mammary gland has not been reported, we next examined by northern blot analysis the Ly-6D mRNA expression in various mouse tissues including the mammary gland. As shown in Fig. 8, Ly-6D was highly expressed in the ear and bladder, with an intermediate level of expression in the thymus and the mammary gland. A low level of mRNA was detectable in the spleen. The Ly-6D expression was undetectable in the brain, lung, heart, liver, small intestine, kidney, striated muscle, ovary and uterus. Based on these observations, we conclude that the MAb 2A2-recognized antigen is identical to Ly-6D, and is expressed in both normal and neoplastic mammary epithelial cells of the mouse.

DISCUSSION

Our observations indicate that Ly-6D is expressed on the surface of normal and neoplastic mammary epithelial cells of the mouse. Ly-6D, also called mouse thymocyte B cell antigen (ThB), belongs to the murine Ly-6 antigen family.[26, 27] The Ly-6 family antigens are all membrane-

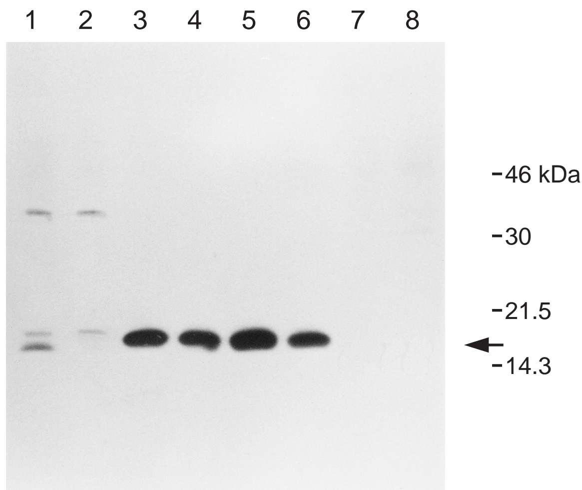

Fig. 7. Reactivity of MAb 2A2 in BALB/c and C57BL/6 mouse tissues. Total cell lysates were prepared from 17-week-old female C57BL/6 mice (lanes 1, 3, 5, 7) and 16-week-old female BALB/c mice (lanes 2, 4, 6, 8). Fifty micrograms of each of the total cell lysates from spleen (lanes 1, 2), ear (lanes 3, 4), isolated mammary epithelial cells (lanes 5, 6) and liver (lanes 7, 8) were run on SDS-PAGE under non-reducing conditions and electroblotted to a nitrocellulose membrane. The blot was then incubated with MAb 2A2 and processed as described in "Materials and Methods." The positions of protein size markers are shown on the right.

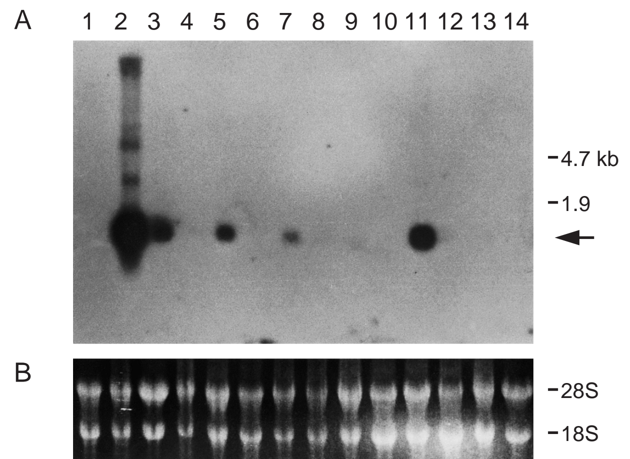

Fig. 8. Northern blot analysis of the expression of Ly-6D mRNA in mouse tissues. Total RNA was isolated from the 18-week-old female BALB/c mouse brain (lane 1), ear (lane 2), thymus (lane 3), lung (lane 4), mammary gland (lane 5), heart (lane 6), spleen (lane 7), liver (lane 8), small intestine (lane 9), kidney (lane 10), bladder (lane 11), muscle (lane 12), ovary (lane 13) and uterus (lane 14). The RNA (22.5 μg) was run on a 1.2% formaldehyde/agarose gel, blotted onto a nylon membrane, hybridized with a ^{32}P-labeled Ly-6D probe and exposed to an X-ray film (A). Ethidium bromide-stained gels are shown in B.

bound proteins expressed on the surface of lymphocytes. They are characterized by the presence of ten conserved cysteine residues in a protein having a molecular weight ranging from 12 kDa to 20 kDa, and are anchored to the plasma membrane with a GPI. Expression of several members of the Ly-6 family antigens has been reported in mouse mammary tumor cells. This includes MM antigen,[28] Ly-6A/E,[29] Ly-6C.1,[30] Ly-6E.1,[29, 30] and Ly-6G.1.[30] Katz *et al.*[29] reported that the expression of Ly-6A/E is associated with a highly malignant phenotype of mouse mammary tumor cells. In this study, we found that Ly-6D is expressed in both normal mammary epithelial cells and spontaneous mammary tumor cells of the mouse. Thus, no correlation was seen between malignancy and the expression of Ly-6D in mouse mammary epithelial cells.

The E48 antigen is the human homolog of mouse Ly-6D, and is expressed in squamous epithelia, transitional epithelia and their malignant counterparts.[24, 31] Thus, we examined whether or not MAb 2A2, the MAb against Ly-6D, recognizes the E48 antigen expressed on the surface of the human epidermoid carcinoma cell line, A431 cells.[31] The results of western blot analysis, however, suggested that MAb 2A2 did not cross-react with the E48 antigen (data not shown). The feasibility of radioimmunotherapy of head and neck squamous cell carcinoma (HNSCC) by using radiolabeled MAb E48 has been evaluated in preclinical and clinical studies.[32, 33] In preclinical studies, the ability of ^{131}I-labeled MAb E48 IgG to eradicate established HNSCC in nude mice was shown. In clinical studies, selective accumulation of MAb E48 IgG in HNSCC was shown. Quak *et al.*[31] reported that the E48 antigen was expressed in one of seven adenocarcinomas of the breast, but not expressed in the normal mammary gland. Since their report is the only publication describing the expression of the E48 antigen in the breast, we recently initiated screening for the expression of the E48 mRNA using a larger number of breast cancer samples.

In this study we have shown for the first time that Ly-6D is expressed on the surface of normal and neoplastic mammary epithelial cells of the mouse. The MAb against Ly-6D developed here will be useful as a marker for luminal epithelial cells for the study of mammary gland development, as well as for the study of mammary carcinogenesis. We are currently examining whether Ly-6D is also expressed in normal mammary epithelial cells during pregnancy, lactation and involution as well as in preneoplastic lesions and metastatic mammary carcinoma cells. Our preliminary observations suggested that the MAb against Ly-6D showed complement-dependent cytotoxicity on both normal and neoplastic mammary epithelial cells *in vitro* (data not shown). If killing of normal mammary epithelial cells by the MAb proves not to cause major adverse reactions *in vivo*, the MAb may find its usefulness in the treatment of breast cancer as long as it effectively kills cancer cells. This has to be clarified by further experiments *in vivo*.

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REFERENCES

1) Sonnenberg, A., Daams, H., van der Valk, M. A., Hilkens, J. and Hilgers, J. Development of mouse mammary gland: identification of stages in differentiation of luminal and myoepithelial cells using monoclonal antibodies and polyvalent antiserum against keratin. *J. Histochem. Cytochem.*, **34**, 1037–1046 (1986).
2) Taylor-Papadimitriou, J., Burchell, J., Chang, S., Bartek, J., Gendler, S. and Durban, E. M. Use of monoclonal antibodies to define the phenotype of cells cultured from the human mammary gland. *In* "Growth and Differentiation of Mammary Epithelial Cells in Culture," ed. J. Enami and R. G. Ham, pp. 1–37 (1987). Japan Scientific Societies Press, Tokyo.
3) Rønnov-Jessen, L., Petersen, O. W. and Bissell, M. J. Cellular changes involved in conversion of normal to malignant breast: importance of the stromal reaction. *Physiol. Rev.*, **76**, 69–125 (1996).
4) Rudland, P. S., Barraclough, R., Fernig, D. G. and Smith, J. A. Mammary stem cells in normal development and cancer. *In* "Stem Cells," ed. C. S. Potten, pp. 147–232 (1997). Academic Press, London.
5) Schwartz, G. F. and Schwarting, R. Clinical application of tissue and serum markers in breast cancer. *In* "Tumor Marker Protocols," ed. M. Hanausek and Z. Walaszek, pp. 61–91 (1998). Humana Press, Inc., Totowa, NJ.
6) Esteva, F. J. and Hayes, D. F. Monoclonal antibody-based therapy of breast cancer. *In* "Monoclonal Antibody-Based Therapy of Cancer," ed. M. L. Grossbard, pp. 309–338 (1998). Marcel Dekker, Inc., New York.
7) Maloney, D. G. Monoclonal antibodies. *In* "The Molecular Basis of Cancer, 2nd Ed.," ed. J. Mendelsohn, P. M. Howley, M. A. Israel and L. A. Liotta, pp. 407–501 (2001). W. B. Saunders, Philadelphia.
8) Albanell, J. and Baselga, J. The ErbB receptors as targets

for breast cancer therapy. *J. Mam. Gland Biol. Neoplasia*, **4**, 337–351 (1999).
9) Taylor-Papadimitriou, J., Burchell, J., Miles, D. W. and Dalziel, M. MUC1 and cancer. *Biochim. Biophys. Acta*, **1455**, 301–313 (1999).
10) Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A. and McGuire, W. L. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. *Science*, **235**, 177–182 (1987).
11) Nagasawa, H., Yanai, R., Taniguchi, H., Tokuzen, R. and Nakahara, W. Two-way selection of a stock of Swiss albino mice for mammary tumorigenesis: establishment of two new strains (SHN and SLN). *J. Natl. Cancer Inst.*, **57**, 425–430 (1976).
12) Enami, J., Enami, S., Kawamura, K., Kohmoto, K., Hata, M., Koezuka, M. and Koga, M. Growth of normal and neoplastic mammary epithelial cells of the mouse by mammary fibroblast-conditioned medium factor. *In* "Growth and Differentiation of Mammary Epithelial Cells in Culture," ed. J. Enami and R. G. Ham, pp. 125–153 (1987). Japan Scientific Societies Press, Tokyo.
13) Sasaki, M. and Enami, J. Mammary fibroblast-derived hepatocyte growth factor and mammogenic hormones stimulate the growth of mouse mammary epithelial cells in primary culture. *Endoc. J.*, **46**, 359–366 (1999).
14) Kishiro, Y., Kagawa, M., Naito, I. and Sado, Y. A novel method of preparing rat-monoclonal antibody-producing hybridomas by using rat medial iliac lymph node cells. *Cell Struct. Funct.*, **20**, 151–156 (1995).
15) Sado, Y. and Okigaki, T. A novel method for production of monoclonal antibodies. Evaluation and expectation of the rat lymph node method in cell and molecular biology. *Cell Biol. Int.*, **20**, 7–14 (1996).
16) Ohuchi, T., Katoh, N., Ueno, T. and Arai, T. Application of image processing to screening and characterizing of monoclonal antibodies recognizing PC12 cell surface antigens. *Bioimages*, **2**, 101–109 (1994).
17) Shulman, M., Wilde, C. D. and Köhler, G. A better cell line for making hybridomas secreting specific antibodies. *Nature*, **276**, 269–270 (1978).
18) Harlow, E. and Lane, D. "Antibodies: A Laboratory Manual," p. 447 (1988). Cold Spring Harbor Laboratory Press, New York.
19) Chomczynski, P. and Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, **162**, 156–159 (1987).
20) Skalli, O., Ropraz, P., Trzeciak, A., Benzonana, G., Gillessen, D. and Gabbiani, G. A monoclonal antibody against α-smooth muscle actin: a new probe for smooth muscle differentiation. *J. Cell Biol.*, **103**, 2787–2796 (1986).
21) Rønnov-Jessen, L. and Petersen, O. W. A function for filamentous alpha-smooth muscle actin: retardation of motility in fibroblasts. *J. Cell Biol.*, **134**, 67–80 (1996).
22) Yutoku, M., Grossberg, A. L., Stout, R., Herzenberg, L. A. and Pressman, D. Further studies on Th-B, a cell surface antigenic determinant present on mouse B cells, plasma cells and immature thymocytes. *Cell. Immunol.*, **23**, 140–157 (1976).
23) Eckhardt, L. A. and Herzenberg, L. A. Monoclonal antibodies to ThB detect close linkage of Ly-6 and a gene regulating ThB expression. *Immunogenetics*, **11**, 275–291 (1980).
24) Brakenhoff, R. H., Gerretsen, M., Knippels, E. M. C., van Dijk, M., van Essen, H., Olde Weghuis, D., Sinke, R. J., Snow, G. B. and van Dongen, G. A. M. S. The human E48 antigen, highly homologous to the murine Ly-6 antigen ThB, is a GPI-anchored molecule apparently involved in keratinocyte cell-cell adhesion. *J. Cell Biol.*, **129**, 1677–1689 (1995).
25) Gumley, T. P., McKenzie, I. F. C., Kozak, C. A. and Sandrin, M. S. Isolation and characterization of cDNA clones for the mouse thymocyte B cell antigen (ThB). *J. Immunol.*, **149**, 2615–2618 (1992).
26) Lai, L., Alaverdi, N., Maltais, L. and Morse, H. C., III Mouse cell surface antigens: nomenclature and immunophenotyping. *J. Immunol.*, **160**, 3861–3868 (1998).
27) Gumley, T. P., McKenzie, I. F. C. and Sandrin, M. S. Tissue expression, structure and function of the murine Ly-6 family of molecules. *Immunol. Cell Biol.*, **73**, 277–296 (1995).
28) Seto, M., Takahashi, T., Tanimoto, M. and Nishizuka, Y. Production of monoclonal antibodies against MM antigen: the serologic identification of MM antigen with Ly-6.2 alloantigen. *J. Immunol.*, **128**, 201–205 (1982).
29) Katz, B.-Z., Eshel, R., Sagi-Assif, O. and Witz, I. P. An association between high Ly-6A/E expression on tumor cells and a highly malignant phenotype. *Int. J. Cancer*, **59**, 684–691 (1994).
30) Treister, A., Sagi-Assif, O., Meer, M., Smorodinsky, N. I., Anavi, R., Golan, I., Meshel, T., Kahana, O., Eshel, R., Katz, B.-Z., Shevach, E. and Witz, I. P. Expression of Ly-6, a marker for highly malignant murine tumor cells, is regulated by growth conditions and stress. *Int. J. Cancer*, **77**, 306–313 (1998).
31) Quak, J. J., Balm, A. J. M., van Dongen, G. A. M. S., Brakkee, J. G. P., Scheper, R. J., Snow, G. B. and Meijer, C. J. L. M. A 22-kd surface antigen detected by monoclonal antibody E48 is exclusively expressed in stratified squamous and transitional epithelia. *Am. J. Pathol.*, **136**, 191–197 (1990).
32) Gerretsen, M., Quak, J. J., Brakenhoff, R. H., Snow, G. B. and van Dongen, G. A. M. S. The feasibility of radioimmunotherapy of head and neck cancer. *Oral Oncol., Eur. J. Cancer*, **30B**, 82–87 (1994).
33) de Bree, R., Roos, J. C., Quak, J. J., den Hollander, W., Wilhelm, A. J., van Lingen, A., Snow, G. B. and van Dongen, G. A. M. S. Biodistribution of radiolabeled monoclonal antibody E48 IgG and $F(ab')_2$ in patients with head and neck cancer. *Clin. Cancer Res.*, **1**, 277–286 (1995).