Anti-Tumor Antibody BR96 Blocks Cell Migration and Binds to A Lysosomal Membrane Glycoprotein on Cell Surface Microspikes and Ruffled Membranes

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Abstract. BR 96 is an internalizing antibody that binds to Lewis Y (Le^y), a carbohydrate determinant expressed at high levels on many human carcinomas (Hellström, I., H. J. Garrigues, U. Garrigues, and K. E. Hellström. 1990. *Cancer Res.* 50:2183–2190). Breast carcinoma cell lines grown to confluence bind less BR96 than subconfluent cultures (Garrigues, J., U. Garrigues, I. Hellström, and K. E. Hellström. 1993. *Am. J. Path.* 142:607–622). However, when the confluent cells are induced to migrate by scratch wounding, they again bind BR96 suggesting that antigens bearing the Le^y determinant may promote cell migration. In the present study, BR96 was found to be highly enriched on microspikes and ruffled membranes, cell surface structures involved in cell migration. In addition, BR96 was a potent inhibitor of cell migration in vitro. When stationary BR96 treated cells were exposed to fresh culture media, membrane ruffles and microspikes developed at the cell margin and migration resumed. Immunogold microscopy showed that BR96 antigens were enriched on these membrane protrusions.

BR96 cell surface immunoprecipitation analysis of ^{3}H-glucosamine labeled breast carcinoma cells identified antigens with approximate molecular weights of 135 kd (upper antigen) and 85 kd (lower antigen). A short amino terminal sequence (8 residues) of the upper antigen matched that of human lysosomal membrane glycoprotein 1 (LAMP-1). In addition, the upper antigen was detected on immunoblots probed with anti-LAMP-1, and within the intracellular compartment BR96 was found predominantly in endosomes and lysosomes.

A soluble LAMP-1/immunoglobulin fusion protein (LAMP-1/Ig) was transiently expressed in both BR96 binding and nonbinding cell lines. Immunoblot analysis of LAMP-1/Ig's from the various cell lines showed that (*a*) acquisition of the BR96 epitope is probably controlled at the level of polylactosamine modification (e.g., fucosylation) rather than LAMP-1 gene expression; (*b*) alternate forms of LAMP-1/Ig comigrate with the lower BR96 antigen raising the possibility that it may be a degradation product of the upper antigen; and (*c*) LAMP-1/Ig expressed in 3396 breast carcinoma cells has approximately 30-fold more BR96 epitopes than LAMP-1/Ig from non-tumorigenic mammary epithelial cells. Together these data indicate that a major BR96 antigen, LAMP-1, is present on unique cell surface domains involved in cell locomotion as well as membranes of the endocytic compartment. Altered glycosylation of LAMP-1 expressed in transformed cells may contribute to their ability to disseminate.

MANY ligands have been identified that can regulate cell locomotion (Turley, 1992). Some studies indicate that altered cell migration is associated with aberrant glycosylation. In a number of instances, highly metastatic tumor cells were found to express more polylactosamine oligosaccharides (Galβ1-4GlcNAcβ1-3)$_n$ than normal or poorly metastatic ones (Hubbard, 1987; Pierce and Arango, 1986; Yamashita et al., 1984; Yousefi et al., 1991). Poly N-acetyllactosamine chains are often modified with fucose and sialic acid to give rise to unique terminal structures such as sialyl Lewis X (S-Le^x).[1] Recently, S-Le^x has been identified as a ligand for the selectin family of adhesion receptors which are important in leukocyte and endothelial cell interactions in inflammation and possibly tumor cell metastasis (Bevilacqua et al., 1993). Other studies however indicate that polylactosamines may promote cell migration through their ability to destabilize adhesion to the extracellular matrix (Laferte and Dennis, 1988). There is lit-

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1. *Abbreviations used in this paper*: mw, molecular weight; S-Le^x, sialyl Lewis X; TR, anti-transferrin receptor.

tle information however on the distribution of polylactosamine containing molecules relative to cell/substratum contact points, or other membrane domains such as ruffled membranes or microspikes which are known to be involved in migration (Trinkaus, 1985).

BR96 is a mAb selected on the basis of its enhanced binding to carcinomas relative to most normal tissues (Hellström et al., 1990). Assays using defined oligosaccharides have shown that Lewis Y (Fuc α1,2 Gal β1,4 [Fuc α1,3] GlcNAc β1,3...), a unique carbohydrate structure at the termini of polylactosamine oligosaccharides, is a critical structure recognized by BR96 (Hellström et al., 1990). We have also shown that BR96 can inhibit tumor cell growth, and when bound to adherent cells, is internalized via coated pits to endosomes, multivesicular bodies, and finally, degraded in lysosomes (Garrigues et al., 1993). The fact that BR96 can effectively target the intracellular compartment has proved useful in therapy experiments where animals bearing established human carcinomas were cured after treatment with BR96 doxorubicin immunoconjugates or Pseudomonas PE40 exotoxin fusion protein (Trail et al., 1993; Friedman et al., 1993).

Studies looking at Le^y expression and cell growth showed that carcinoma cells grown to confluence bind significantly less BR96 than subconfluent cultures (Garrigues et al., 1993). Furthermore, when contact inhibition is broken by scratch wounding, cells growing out of the confluent region reexpress the BR96 antigen (Garrigues et al., 1993). These experiments suggest that either cell contact causes an inhibition of antigen expression or that the BR96 antigen is upregulated on migrating cells.

In the present study, we have investigated further the role of the BR96 defined Le^y antigen in cell migration. We show that BR96 specifically localizes to microspikes and ruffled membranes at the cell surface, and, under conditions favoring mAb internalization, BR96 alters cell morphology and completely blocks cell migration. The BR96 antigen was also isolated and its amino terminal sequence matched that of LAMP-1 (for review see Fukuda, 1991). cDNA expression studies confirm that BR96 can bind to LAMP-1, and reveal that LAMP-1 derived from carcinoma cells has significantly more BR96 epitopes than LAMP-1 expressed in non tumorigenic mammary epithelial cells. These data show that the potential functions of LAMP-1 are not limited to the biogenesis of the lysosome. Instead, cell surface LAMP-1 participates in cell migration, possibly by facilitating microspike formation or the development of cell/substratum contacts.

Materials and Methods

Antibodies and Cells

The human breast carcinoma line 3396 and mAbs BR96, anti-transferrin receptor (TR), and control anti-*Pseudomonas aeruginosa* flagellum (IIG5) have been described before (Garrigues et al., 1993) as has the human mammary epithelial line 184 AIN-TH (Clark et al., 1988). mAb H4A3 is specific for human LAMP-1 (Mane et al., 1989).

Immunocytochemistry

For cell surface labeling studies, cells were plated as described previously (Garrigues et al., 1993), fixed with 2% glutaraldehyde in PBS for 2 min, and then 8% paraformaldehyde for 30 min at 25°C, washed, and treated with freshly prepared 0.1% $NaBH_4$ for 30 min. Nonspecific binding sites were blocked with PBS containing 5% BSA, 3% normal human serum, 1% normal goat serum, and 0.5% gelatin (blocking buffer) for 1 h at 25°C. BR96 and TR were directly conjugated to 15-nm and 10-nm colloidal gold, respectively (Larsson, 1988), and were diluted in diluent (blocking buffer/PBS 1:4) to an approximate protein concentration of 0.1 μg/ml. Gold-labeled mAbs (BR96/gold, TR/gold) were incubated for 16 h at 4°C, washed, fixed as above, washed in dH_2O, treated with fish skin gelatin (0.5% in dH_2O) for 30 min, and silver enhanced (Goldmark Biologicals, Phillipsburg, NJ) for 4 min at 25°C. The slides were then washed extensively in distilled H_2O and viewed by darkfield microscopy.

For SEM, cells were treated as above except silver enhancement was for 8 min. The preparation was then air dried and coated with carbon. Images were detected either as secondary electron emissions or backscatter electron emissions at 10 kV using a JSM 6300 F scanning electron microscope (JEOL Ltd., Tokyo, Japan).

For intracellular localization studies, fixed cells were permeabilized with 0.5% Triton X-100 for 15 min and washed before the blocking step as described above. Instead of gold labeled mAbs, BR96/FITC (25 μg/ml) was used instead. The labeled preparation was mounted in Slowfade (Molecular Probes, Eugene, OR).

Internalization Assays

Cells were plated as described above, pulsed with TR/gold for 2 h at 37°C, briefly "squirt" washed, and chased at 37°C in Iscove's modified Dulbecco's media containing 15% FBS (15% IMDM) for 3 h. The cells were fixed, permeabilized, silver enhanced, blocked, and prepared for BR96/FITC immunofluorescence as described above. Gold and FITC-labeled probes were localized using simultaneous darkfield and epifluorescence. The objective was equipped with an iris. The darkfield light source was set at 4.5 V. The epifluorescence filters were exciter BP 485/520, dichromatic FT 510, and barrier LP 520 (Carl Zeiss, Inc., Thornwood, NY). Images were recorded on Fuji P1600 film (ASA 1600) using 63× and 100× objectives.

For ultrastructural studies cells were pulsed and chased with TR/gold as described above, and then fixed in 2% glutaraldehyde for 30 min. They were subsequently embedded in Medcast resin and thin sections were collected on copper grids coated with formvar.

For the transferrin receptor degradation studies, cells (5×10^5/35-mm dish) were pulsed and chased with ^{125}I-TR (25 μg/ml) as described above. At the end of the chase period, the media were collected, the dishes were extensively washed and the cells lysed with TNEN (20 mM Tris-HCl buffer, pH 8.0, containing 100 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1% aprotinin, and 0.5% NP-40) for 20 min at 4°C. Aliquots from the media and cell fractions were counted in a gamma counter and analyzed on nonreducing 10% tricine gels (Novex, San Diego, CA). A phosphoimager (Molecular Dynamics, Sunnyvale, CA) was used to quantitate relative intensity of individual bands on gels.

Migration Assays

3396 cells were plated on plastic slides (10^5 cells/slide) as described (Garrigues et al., 1993). After culturing for 24 h, the monolayers were scratched and multiple images ($n = 6$) along the wound were recorded ($t = 0$ h) using the Image I image analysis software (Universal Imaging Corp., West Chester, PA) and a Nikon microscope equipped with a 4× objective. The cultures were immediately treated with various concentrations of BR96 or IIG5 control mAb diluted in 15% IMDM. After an overnight incubation, the cells were cultured for an additional 48 h with mAbs which were replaced twice daily with fresh mAbs. The cells were fixed for 2 h with 2% glutaraldehyde in PBS and stained with Coomassie Blue. Multiple images along the wound at positions used for $t = 0$ h were recorded ($t = 65$ h). The images at $t = 0$ h and $t = 65$ h were used to quantitate the area within the wound, lacking cells. For each position the area difference (i.e., the area at $t = 0$ h minus the area at $t = 65$ h) was determined. The data is presented as the percent migration by comparing area differences for BR96 (or IIG5) treatment to area differences for 15% IMDM treatment.

Antigen Isolation and Characterization

3396 cells were lysed with TNEN as above. The lysate was chromatographed on a *Ulex europeus I* lectin column (2 ml bed vol) equilibrated with PBS containing 0.5% NP-40 (PBS/NP-40). The column was washed and eluted with PBS/NP-40 containing 10% fucose. The second fraction (1 ml each) was iodinated (2 mCi) with iodobeads (Pierce, Rockford, IL) for 15 min at 25°C. Labeled proteins eluting in the void volume of a Sephadex

G25/PBS column were collected and reabsorbed with a control mAb affinity column (mAb IIG5, 20 mg/2 ml column). After a 1-h incubation at (4°C), unbound proteins were collected and added to a mAb BR96 affinity column (20 mg/2 ml gel). The column was incubated at 4°C for 1 h, washed with TNEN, and eluted with triethylamine pH 11.5. Eluates were immediately neutralized with 1 M Tris/HCl pH 4.15 and analyzed by SDS-PAGE on 7.5–15% gradient gels.

For cell surface immunoprecipitation experiments, 3396 cells were labeled for 36 h with 400 μCi [^{3}H]glucosamine diluted in 15% IMDM. The cells were washed with ice cold media and treated with BR96 (50 μg/ml) or control mAb for 1 h at 4°C. Unbound mAb was removed by washing (4°C) and the cells lysed as described above. The lysate was cleared by centrifugation (400 *g*). Rabbit anti-mouse IgG (50 μg) and *Staphylococcus aureus* (150-μl packed volume) were added to the lysate. The mixture was rotated for 1 h at 4°C. The *Staphylococcus aureus* were washed in TNEN and boiled in reducing sample buffer to dissociate the immune precipitates. The samples were analyzed by SDS-PAGE using 10% tricine gels.

Proteins corresponding to potential BR96-defined antigens were identified by comparing protein bands stained with Coomassie blue from 3396 lysates that comigrated with [^{3}H]glucosamine labeled, BR96 cell surface immunoprecipitated antigens. The BR96 antigens were purified by preparative SDS-PAGE and electroelution using 6% tris glycine gels (Novex, San Diego, CA). The gels were stained for 15 min with 0.5% Coomassie blue diluted in H_2O. Protein bands migrating at 140, 135, 85, and 80 kd were excised and cut into 1-mm cubes. The gel pieces were soaked in tris glycine running buffer for 1 h and electroeluted at 200 V for 2 h using an apparatus from Amicon (Beverly, MA). Protein bands were also labeled with ^{125}I using the chloramine T method (McConahey and Dixon, 1980). Labeled proteins were electrophoresed on 10% tricine gels and either analyzed by autoradiography to assess their purity or immunoblotting. In the latter case, proteins were transferred to Problot (Applied Biosystems, Foster City, CA) according to Hirano et al. (1990). The transfers were blocked in blotto for 1 h and incubated with control mAb, BR96, or anti-LAMP-1 for 16 h at 4°C. The immunoblots were washed and incubated with alkaline phosphatase-labeled goat anti-mouse IgG (Tago, Burlingame, CA) diluted (1:1,000) in blotto for 2 h. The transfers were washed and developed using Western Blue, Promega, (Madison, WI).

Amino terminal sequence data was obtained for the 135-kd protein isolated from 24 preparative gels as described above or by BR96 affinity chromatography as follows. mAb IIG5 (2 mg) and BR96 (20 mg) were coupled to 1 ml and 7 ml, respectively, of AminoLink coupling gel as specified by the manufacturer (Pierce, Rockford, IL). Fifty ml of packed 3396 cells were solubilized in TNEN buffer (see above) and passed over the IIG5 column. The nonbinding cell lysate was mixed with the BR96 affinity gel and rotated for 16 h at 4°C. The gel was collected in a 10-ml column fitted with a bed support. The BR96 affinity column was washed with 50 ml TNEN and eluted with 10 ml of triethylamine pH 11.5. The eluate was neutralized with 1 M Tris HCl pH 4.15 and concentrated to 3 ml by speedvac. The eluate was electrophoresed on four 10% tricine gels. The region of the gel from 100 kd to 180 kd was excised and electroeluted as described above. The eluate was concentrated to 40 μl, electrophoresed and transferred to Problot as above. A small piece of the blot was subjected to BR96 immunoblotting, the remainder was stained with Coomassie blue. The 135-kd band which comigrated with the BR96 immunoreactive band was sequenced by G. M. Hathaway, Ph.D., Biotechnology Instrumentation Facility, University of California, Riverside, CA.

Plasmid Construction

RNA was isolated from 3396 cells using reagents from Stratagene (La Jolla, CA). cDNA was synthesized using a Gene Amp RNA PCR kit (Perkin Elmer, Norwalk, CT) using a primer (primer 1) starting at nucleotide 1296 (*) of LAMP-1 (Fukuda et al., 1988) and having the sequence GTTCTCGTC TGATCACACTCCT CCACAGAGC* and containing a Bcl I site (underlined). Nucleotide 1312 was removed to allow reading alignment of LAMP-1 with Ig in the fusion construct (see below). Reactions contained 1 μg of RNA, 10 pmol of primer, 1 U RNase inhibitor, 10 mM dNTP's, 5 mM $MgCl_2$, and 1× PCR II buffer. The samples were heated to 94°C for 2 min and cooled to 41°C. Reverse transcription was carried out for 45 min at 41°C using 2.5 U M-MLV reverse transcriptase. A LAMP-1 cDNA was amplified by PCR using primer 1 as the reverse primer and the oligonucleotide TGGA AAGCTTGATATCA*CCGTACCCGGCCGCCTCG (the Hind III site is underlined) corresponding to nucleotide 170 (*) of LAMP-1 as the forward primer (primer 2). PCR reactions contained 50 pmol of primers 1 and 2 and 2.5 U of Taq polymerase. The thermocycler was run at 72°C for 15 min, and then for 33 cycles as follows: 1 min 96°C, 2 min 60°C, and 2 min 72°C. Products of the PCR reaction were cleaved with restriction endonucleases (Hind III and Bcl I) at sites introduced in the PCR primers and gel purified.

The immunoglobulin (Ig) segment of the fusion construct corresponding to the human IgCγ1 was isolated from a pUC B7/Ig construct using restriction endonucleases Bcl I and Xba I as described (Linsley et al., 1991). The fusion construct was assembled by ligating Hind III/Bcl I cleaved Lamp-1 fragment to the Bcl I/Xba I cleaved fragment containing IgCγ1 sequences into the Hind III/Xba I cleaved πLN mammalian expression vector (provided by A. Aruffo). Ligation products were transformed into DH10B/p3 cells (GIBCO BRL, Gaithersburg, MD), and putative transformants were analyzed by restriction endonuclease digestion. Clones were sequenced using Sequenase version 2.0 (U.S. Biochemicals, Cleveland, OH).

Transfection

3396, 184AIN-TH, 3719 cells were transfected using the procedure of Seed and Aruffo (1987). Cells were plated at 2.5 × 10^6 per 150-mm dish and cultured for 24–48 h. Plasmid DNA (25 μg/dish) was added in 10 ml of 15% IMDM containing 0.1 mM chloroquin and 0.5 mg/ml of DEAE Dextran. The cells were incubated for 2.5–3 h at 37°C, shocked for 2 min with 10% dimethyl sulfoxide in PBS, washed, and incubated in IMDM for 24 h. The culture media was replaced with low serum (0.1%) IMDM which was collected 2–3 times at four day intervals.

Purification of Ig Fusion Protein

Ig fusion proteins were purified on immobilized protein A (Repligen Corp., Cambridge, MA). The affinity matrix was incubated for 1 h in blotto to block nonspecific protein adsorption, washed extensively with PBS, eluted with 0.1 M sodium citrate buffer, pH 2.2, and reequilibrated with PBS. The immobilized protein A (0.1 ml) was added to culture media (20 ml) collected from the transfected cells, rotated for 16 h at 4°C and poured into a disposable column. The column was washed with PBS (5 × 1 ml) and eluted as described above. Fractions (200 μl) were immediately neutralized with one third volume of 1 M Tris, pH 11.0. Protein concentrations were determined by ELISA using chimeric L6 (Fell et al., 1992) as a standard. In some cases, the LAMP/Ig fusion proteins were concentrated on a Ultrafree MC membrane (Millipore, Bedford, MA). Purified fusion proteins were analyzed by immunoblotting as described above and the sequence of the first eleven amino terminal residues was determined to verify proper signal peptide cleavage.

BR96 epitope density on LAMP-1/Ig's expressed in different cell lines was determined by ELISA. Immulon plates (Dynatech Laboratories, Chantilly, VA) were coated with various concentrations of LAMP-1/Ig, blocked with blotto, and incubated with either BR96, IIG5, or mouse anti-human IgG1 (Caltag, San Francisco, CA) at 5 μg/ml for 16 h at 4°C. The plates were washed 5× (PBS/0.05% Tween) and incubated with a horseradish peroxidase-labeled goat anti-mouse IgG/M (Tago) diluted 1:1,000 for 2 h. The plates were washed as above. Diluent buffer and chromogen substrate came from Genetic Systems Corp. (Redmond, WA).

Results

BR96 Localizes to Distinct Cell Surface Domains: Microspikes and Membrane Ruffles

3396 cells were fixed and surface labeled with BR96/gold. The preparation was viewed by darkfield microscopy (Fig. 1). Labeled cells appear pink/red similar to the color of the BR96/gold complex (Fig. 1). Areas of membrane not labeled are dark green/blue (Fig. 1 *a*, *open arrow*). Large accumulations of BR96 appear as bright spots (Fig. 1, *arrowhead*). Note that on individual cells, distinct membrane domains are visible based on color differences generated by BR96 binding. For example, at the cell margin, areas of ruffled membrane are pink/red (Fig. 1, *a* and *b arrow*) and express more BR96 antigen than the adjacent smooth membrane which is green/blue. The perinuclear distribution of the bright spots representing focal concentrations of gold suggests that BR96 is associated preferentially with microspikes (Fig. 1, *a* and

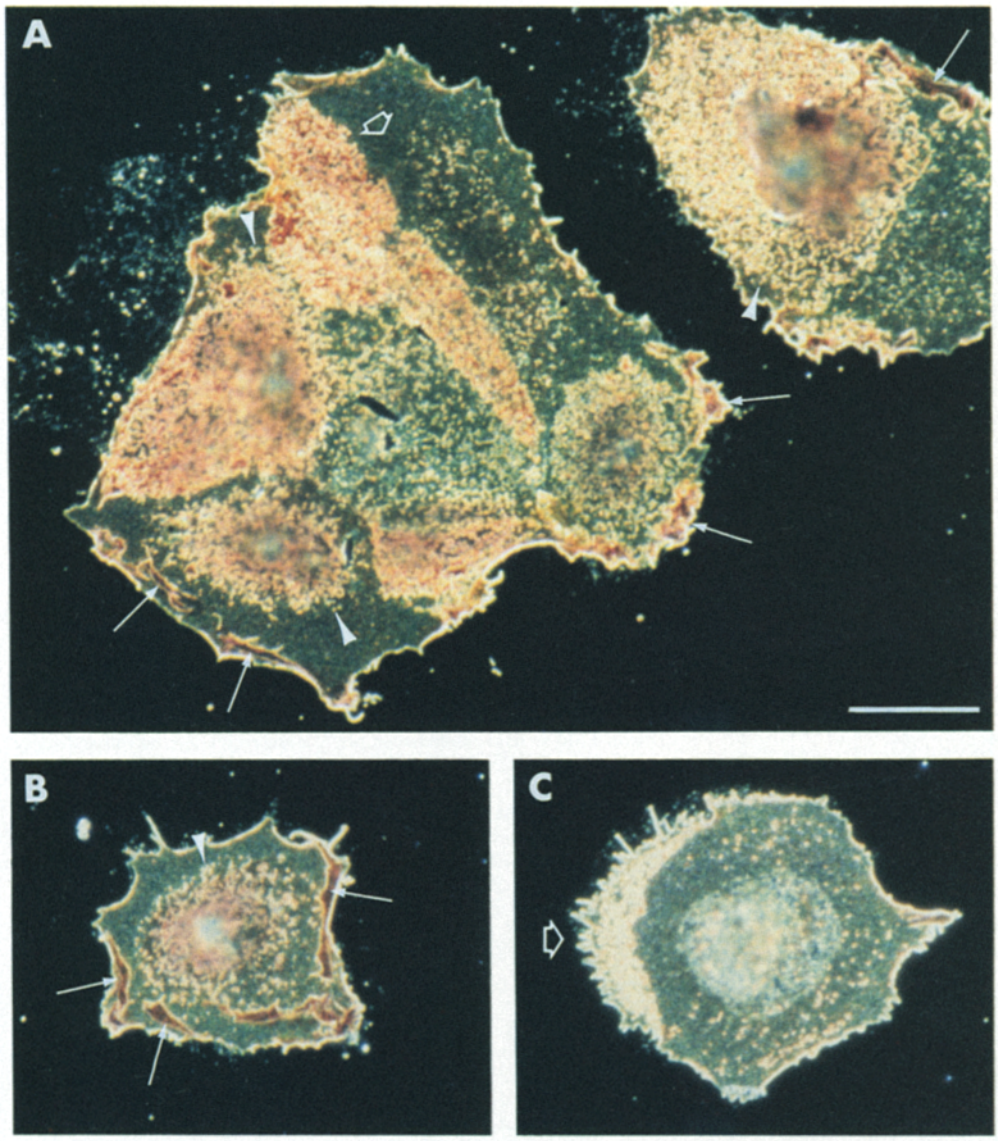

Figure 1. BR96 cell surface localization. Fixed 3396 cells were stained with BR96/gold and viewed by darkfield microscopy. Bright spots represent focal accumulations of BR96 (*arrowheads*). Unlabeled membrane regions are dark green/blue (Fig. 1 *a*, *open arrow*). At the cell margin, BR96 labeled ruffled membranes are pink/red (*arrows*). BR96 is expressed at the leading lamella of migrating cells (Fig. 1 *c*, *open arrow*). Bar, 25 μm.

b, *arrowheads*). Occasionally single migrating cells were observed (Fig. 1 *c*). In this case BR96 showed a polarized distribution being associated with the leading lamella (Fig. 1 *c*, *open arrow*) while other membrane areas were labeled much less.

To investigate the cell surface in more detail, BR96/gold-labeled cells were examined by SEM. Fig. 2 *a* shows the surface detail of a number of gold-labeled cells as well as one antigen negative cell (see Garrigues et al., 1993) which is distinguished by having poorly resolved surface structures (Fig. 2 *a*, *arrow*). Note that on the BR96 labeled cells the ruffled membrane at the cell margin (Fig. 2 *a*, *open arrow*) and the prominent microspikes (Fig. 2, *a* and *b*, *arrowheads*) are distinct against a dark background due to the signal generated by the gold-labeled BR96. When using backscatter (Fig. 2 *c*) to preferentially detect higher energy electrons being emitted by the gold probe, it is clear that the punctate apical cell surface labeling seen by light microscopy is due to BR96 binding to microspikes. Surface labeling studies, therefore, show that BR96 is localized to both apical microspikes and ruffled membranes at the cell margin which represent distinct membrane domains. The polarized expression on migrating cells suggests that the BR96 antigen may play a role in cell migration.

Intracellular Localization: The BR96 Antigen Is a Component of the Endosome/Lysosome Compartment

Mammary epithelial cells 184 AIN-TH (184) express BR96 antigens on the same cell surface protrusions as 3396 cells (see below), and were selected for intracellular antigen localization studies because their flat morphology resulted in higher resolution of BR96 within cytoplasmic compartments as compared to 3396. The 184 cells were fixed, permeabilized, and labeled with BR96/FITC. As with 3396 cells, BR96 stained ruffled membranes at the cell margin (Fig. 3, *open arrow*) and microspikes (Fig. 3, *arrowheads*) at the surface of 184 cells. In addition, BR96/FITC labeled many vesicles which were distributed from the cell margin to the perinuclear region (Fig. 3, *arrow*).

To determine if these vesicles were part of the endocytic pathway, pulse/chase studies using ^{125}I TR and double label

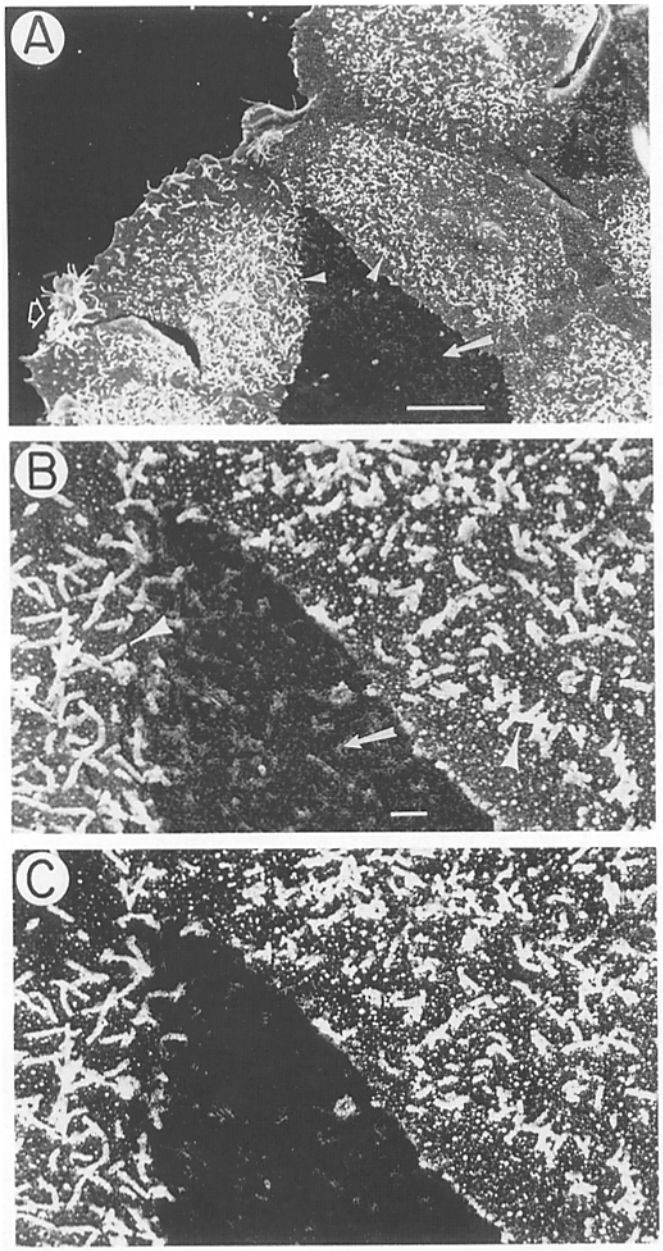

Figure 2. BR96 immunoscanning electron microscopy. Fixed 3396 cells were labeled with BR96/gold, dried, coated with carbon, and viewed by scanning electron microscopy detecting either secondary (*a* and *b*) or backscattered (*c*) electron emissions. The ruffled membranes at the cell margin (Fig. 2 *a*, *open arrow*) and the prominent microspikes (*arrowheads*) are very distinct due to electron emissions from the gold probe. The microspikes of a BR96 antigen negative cell (*arrow*) lacking the gold probe however are poorly resolved. Bar, 10 μm (*a*) and 1 μm (*b* and *c*).

(TR/gold, BR96/FITC) immunochemistry were performed. 184 cells were pulsed for 2 h with ^{125}I TR at 37°C, washed, and chased for 3 h at 37°C. The media fraction was collected, and the cells solubilized with detergent. Approximately 52% of the radiolabel was found in the media fraction (Fig. 4 *a*). Analysis by SDS-PAGE (Fig. 4 *b*) showed that a significant proportion of the TR had undergone degradation (18 kd, 71%) relative to the untreated TR (180 kd). Similarly a portion of the cell associated TR had also been degraded (15 kd, 24%). In addition, ultrastructural immunogold microscopy showed that the TR was predominantly localized to multivesicular bodies and lysosomes (Fig. 4 *c*, *arrows*).

To see if BR96 labeled the same vesicles as TR, 184 cells were pulsed and chased with TR/gold as described above, fixed, permeabilized, and stained with BR96/FITC. The cells were viewed using either epifluorescence alone (Fig. 5 *a*) or with a combination of epifluorescence and darkfield microscopy (Fig. 5 *b*). A significant number of the BR96 labeled vesicles (Fig. 5 *a*, *arrows*) also contained colloidal gold (Fig. 5 *b*, *arrowheads*). Therefore, within cells, BR96 recognizes an abundant protein associated with vesicles identified in pulse/chase studies as endosomes and lysosomes.

BR96 Inhibits Cell Migration

Localization of BR96 to microspikes and ruffled membranes combined with our previous observation that BR96 binds more to cells growing out of a scratch wounded culture (Garrigues et al., 1993) suggests that the BR96 defined antigen is involved in cell migration. To gain insight into antigen function, cells were cultured with BR96 under conditions which favor mAb internalization as a means of decreasing cell surface antigen concentrations (Drebin et al., 1985). Cultures of 3396 cells were scratch wounded and grown for ~65 h in the presence of various concentrations of BR96 or IIG5 control mAb. Cell migration was quantitated by measuring cell free regions of the scratch before and after mAb treatment and the distance migrated expressed as a percentage relative to the media control. The morphology of cultures treated with 10 μg of BR96 (a) or IIG5 (b) and stained with Dapi is shown in Fig. 6. The white line (t = 0 h) is a tracing of the wound edge just before mAb treatment. The migration of BR96 treated cells was completely blocked. Note at the wound edge in (a) many nuclei are above the image focal plane (arrow) indicating cell aggregation. In contrast, control mAb-treated cells (b) have migrated to fill in the wound and clearly were not aggregated. A quantitative assay (Fig. 6 *c*) shows the dose dependence of BR96 induced inhibition of migration. Cell locomotion was completely blocked using 5–20 μg of BR96. Other internalizing mAbs (BR110, BR111) which bind to 3396 cells but do not recognize Le^y, had no effect on cell migration (data not shown). In addition BR96 was not cytotoxic since cell migration resumed when BR96 was replaced with fresh culture media. Within 24 h, the cells at the wound edge had extended numerous microspikes onto the substratum. BR96/gold immunochemistry showed that these structures in many instances contained more antigen than adjacent cell bodies (Fig. 6 *d*). The data shows, therefore, that when BR96 is internalized, cell morphology at the wound edge is altered and migration ceases. Upon BR96 removal, new antigen positive microspikes appear at the margin and cell locomotion resumes. The BR96 antigen, therefore, may contribute directly to cell migration or it may be essential for maintaining a morphology which is conducive to cell migration.

BR96 Recognizes a Lysosomal Membrane Glycoprotein Expressed at the Cell Surface

Previously we showed that fucose is a critical residue in the

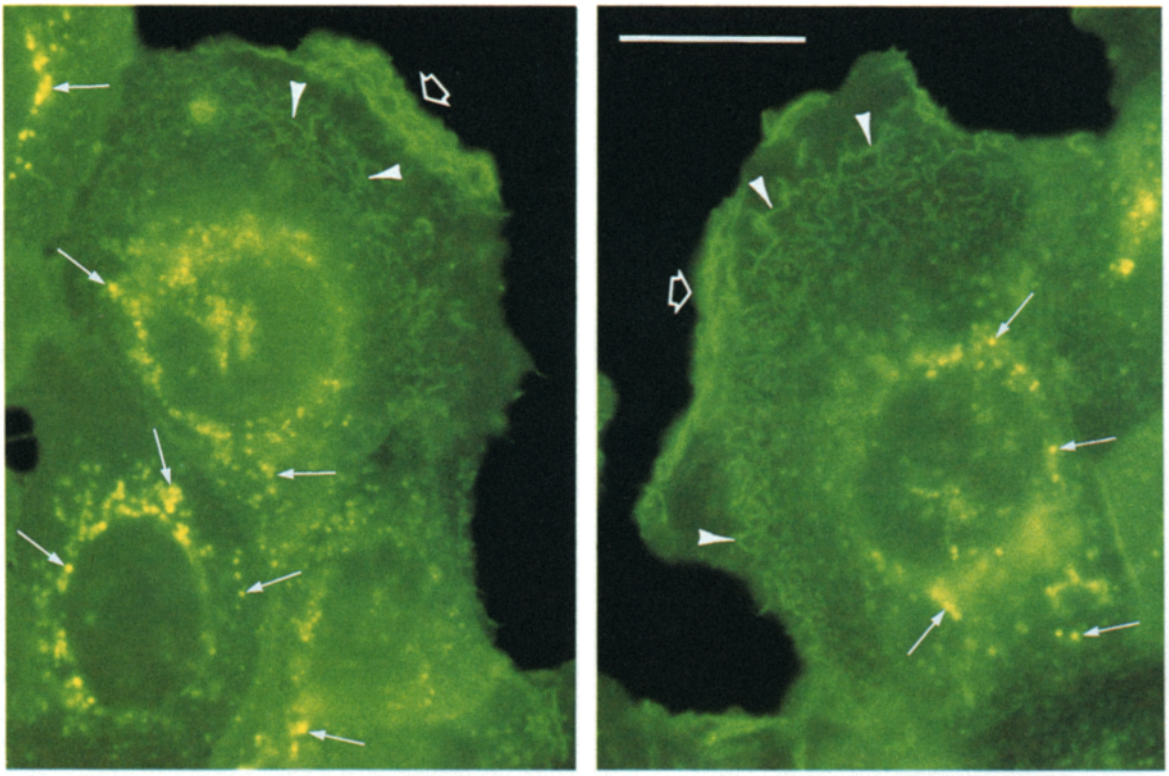

Figure 3. BR96 localization using permeabilized cells. 184 mammary epithelial cells were fixed, permeabilized with Triton X-100, and stained with BR96/FITC. BR96 localized to ruffled membranes at the cell margin (*open arrow*), microspikes (*arrowheads*), and to vesicles (*arrows*) found throughout the cytoplasm especially in the perinuclear region. Bar, 20 μm.

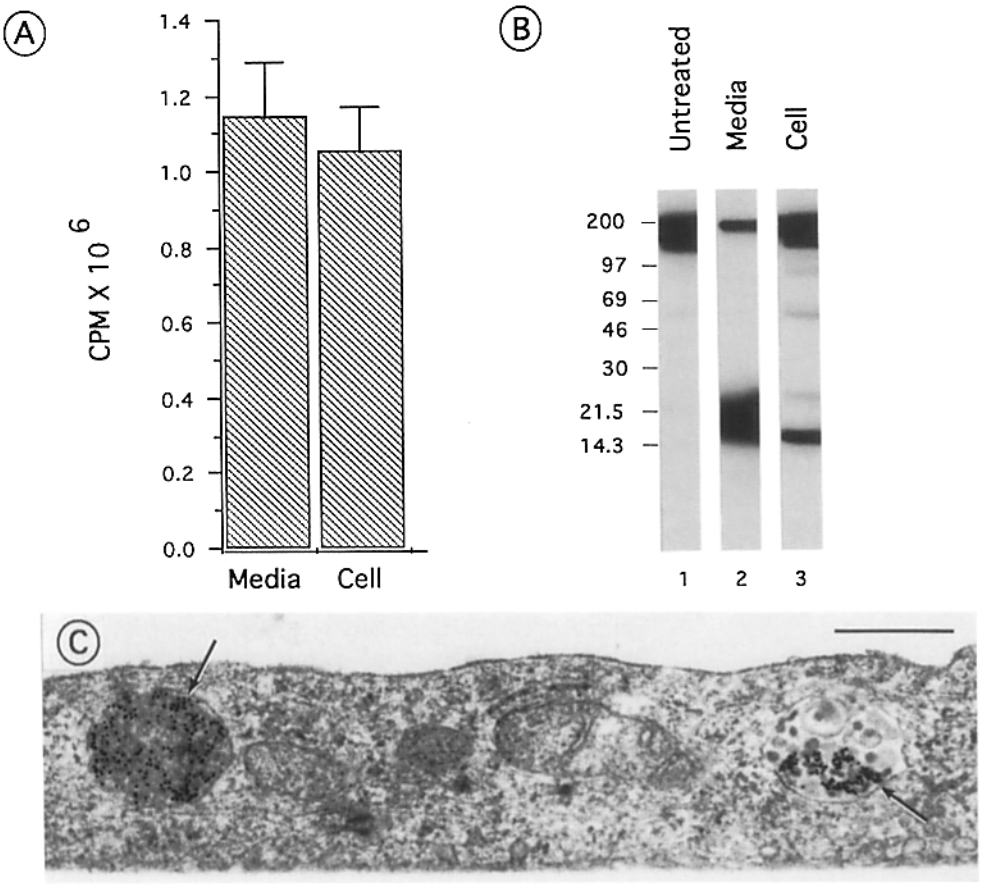

Figure 4. Internalization of an anti-transferrin receptor mAb. 184 mammary epithelial cells were pulsed with ^{125}I TR for 2 h, washed, and chased for 3 h at 37°C. Aliquots from the culture supernatants (media) and the cell layer (cell) were collected and quantitated by gamma counting (*a*) or analyzed by SDS-PAGE to monitor degradation (*b*). Similar pulse/chase experiments with TR/gold were prepared for electron microscopy (*c*). Note the TR/gold accumulated within multivesicular bodies (Fig. 4 *c*, *right arrow*) or electron dense lysosomes (Fig. 4 *c*, *left arrow*). Bar *C*, 0.2 μm.

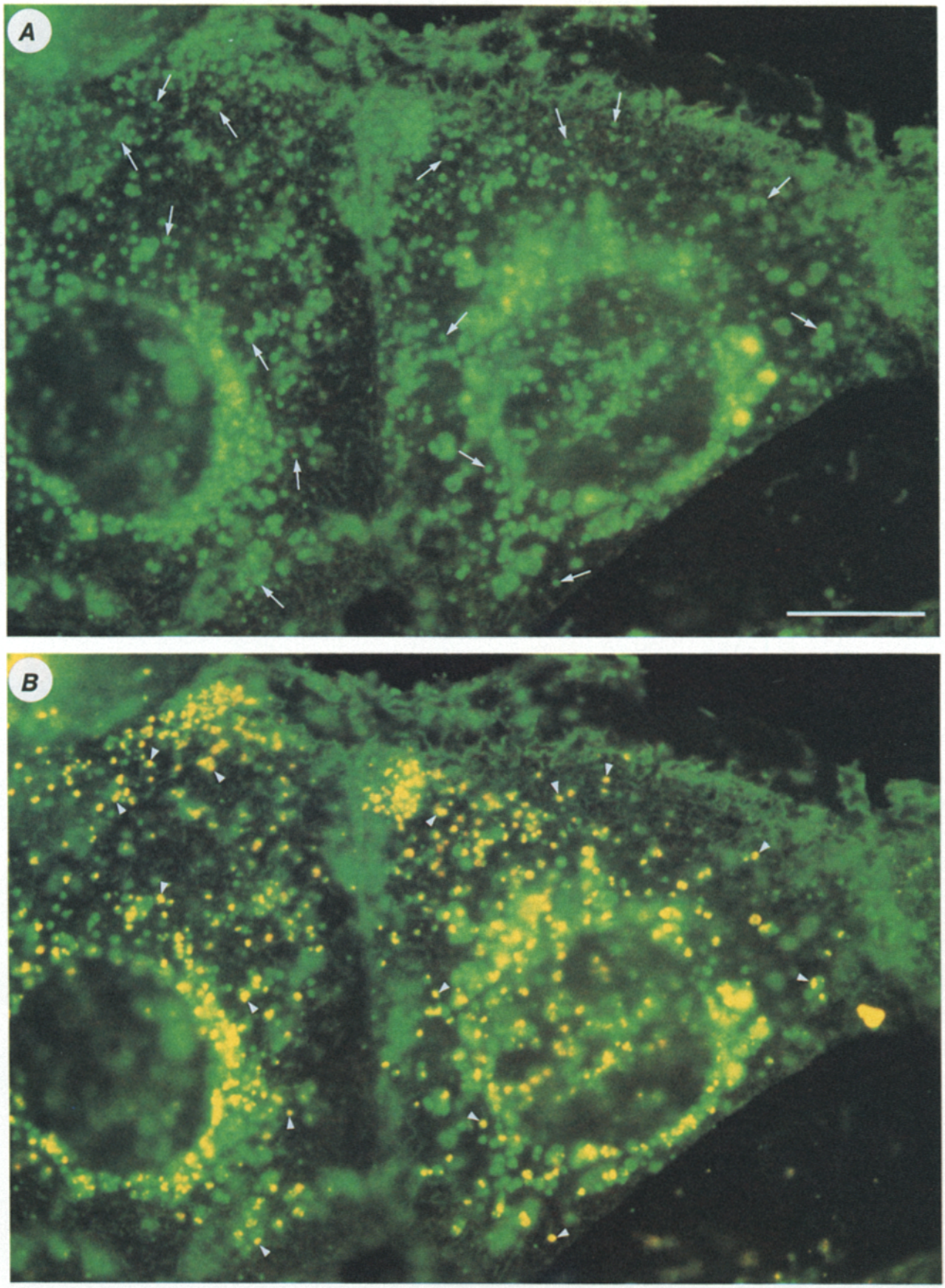

Figure 5. BR96 colocalizes with internalized TR. 184 mammary epithelial cells were pulsed and chased with TR/gold as for Fig. 4, fixed, permeabilized, and stained with BR96/FITC. Epifluorescence alone (*a*). Combination of epifluorescence and darkfield microscopy (*b*). Note that many BR96 labeled vesicles (Fig. 5 *a*, *arrows*) also contain internalized TR (Fig. 5 *b*, *arrowheads*). Bar, 20 μm.

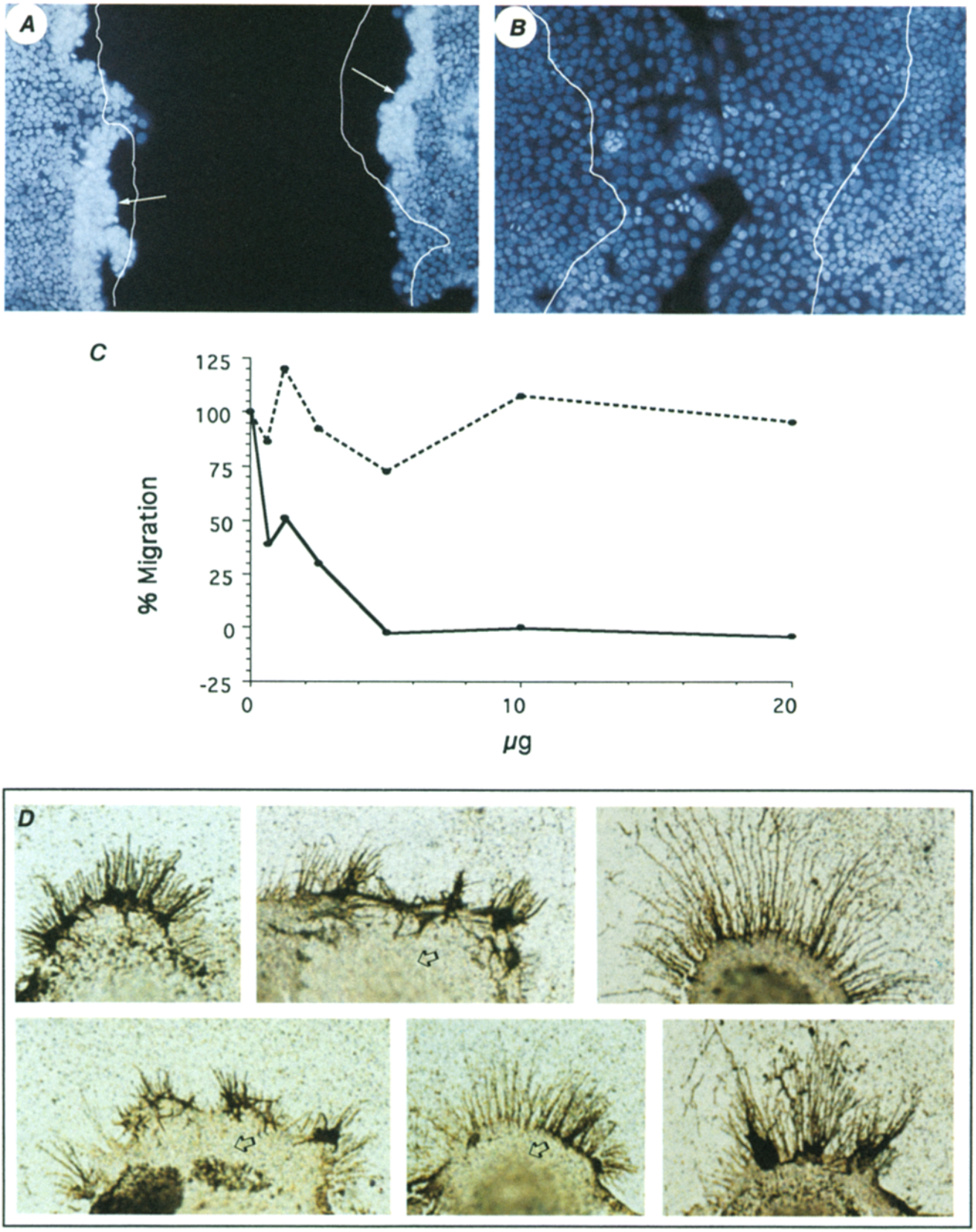

Figure 6. BR96 inhibits cell migration. Cultures of 3396 carcinoma cells were scratch wounded and treated with either BR96 (*a*) or IIG5 control (*b*) for 65 h. The cells were fixed and stained with Dapi. The white line marks the location of the wound edge just before mAb treatment. The arrows in *a* indicate areas of cell aggregation. The percent migration was calculated by measuring the change in area of the scratch which remained cell free after treatment with BR96 (*solid line*) or IIG5 (*dashed line*) as compared to media control (Fig. 6 *c*). In some experiments after BR96 treatment, the cells were cultured further (24 h) in 15% IMDM without mAb and stained with BR96/gold (*d*, *montage*). Note the prominent microspikes extending from the cell margin are heavily labeled with BR96/gold which appears dark brown; adjacent membrane regions (*d*, *open arrows*) are stained significantly less.

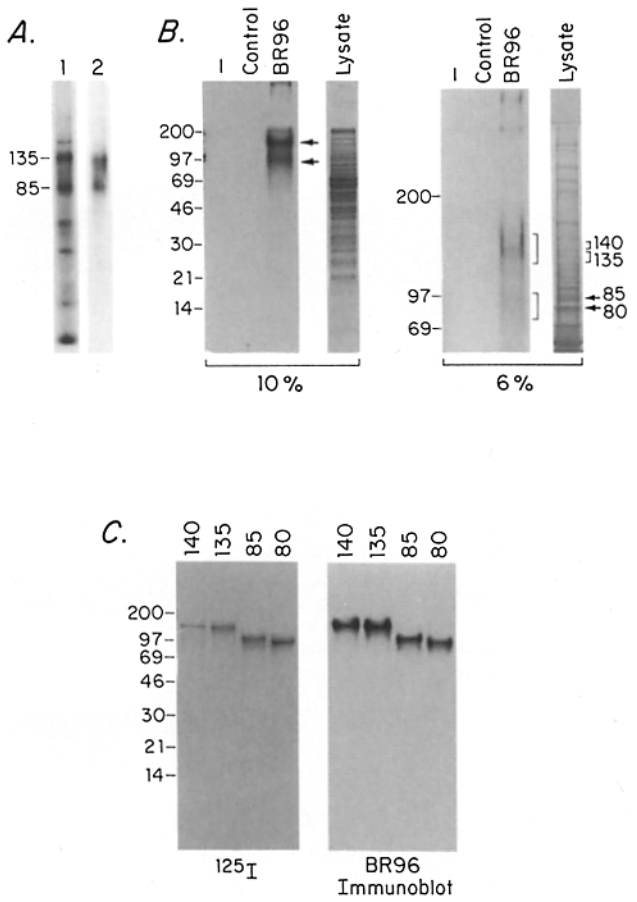

Figure 7. Characterization of the BR96 Antigen. Detergent lysates of 3396 carcinoma cells were chromatographed on a UEA I lectin column. The column was washed and eluted with 10% fucose. The eluate was iodinated and applied to a BR96 affinity column (Fig. 7 *a*). The nonbinding fraction (lane *1*) and the high pH eluate (lane *2*) were analyzed on 7.5–15% gradient SDS/PAGE (Fig. 7 *a*). Cultures of 3396 cells were metabolically labeled with [^{3}H]glucosamine, cooled, incubated with BR96 at 4°C, washed, and solubilized with detergent. Anti-Ig was added to the lysate, the immune complexes precipitated with protein A and analyzed on 10% tricine gels or 6% tris glycine gels (Fig. 7 *b*). Autoradiographs were compared to gels of 3396 lysates stained with Coomassie blue (Fig. 7 *b*). BR96 antigens were purified on 6% tris glycine preparative gels, electroeluted, and aliquots of the purified protein were either iodinated or blotted and probed with BR96 (Fig. 7 *c*).

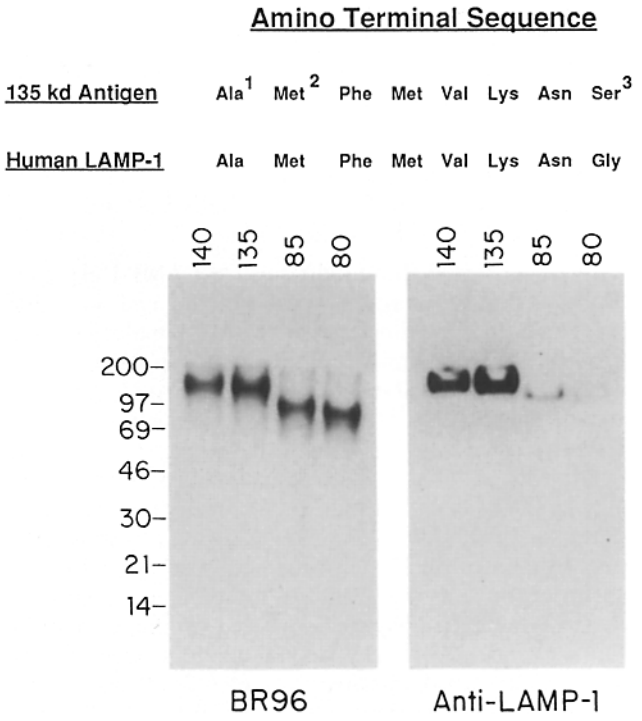

Figure 8. BR96 recognizes LAMP-1. The 135-kd BR96 antigen was purified by preparative electrophoresis or BR96 affinity chromatography as described above, electrotransferred to Problot and the amino terminal sequence determined. At amino acids designated 1,2,3 yields of Arg, Glu, and Leu were also detected. The sequence of the 135-kd antigen was similar to human LAMP-1 and a mAb to LAMP-1 reacted strongly with the 140-kd and 135-kd antigens and less with the 85-kd and 80-kd antigens.

antigen recognized by BR96 (Hellström et al., 1990). Initial antigen purification schemes, therefore, used the fucose specific lectin, UEA I in combination with mAb affinity chromatography to identify BR96 antigens from 3396 cells. Approximately ten fucosylated molecules ranging in molecular weight (mw) from <20 kd to >200 kd were isolated from 3396 cells (Fig. 7 *a*, lane *1*). Two of the molecules with approximate mw's of 135 kd and 85 kd bound specifically to the BR96 affinity column (Fig. 7 *a*, lane *2*). To determine if either of these molecules were present on the cell surface, [^{3}H]glucosamine-labeled cells were incubated with BR96 at 4°C, washed, extracted with detergent, and the immune complexes precipitated with protein A. Antigens from the intracellular compartment are, for the most part, excluded from the precipitation. As shown in Fig. 7 *b*, both the 135-kd and 85-kd antigens were precipitated; suggesting that both are cell surface receptors for BR96. A comparison of the immunoreactive BR96 antigens with total cellular proteins stained with Coomassie blue (Fig. 7 *b*) identified a number of bands which were sufficiently resolved on 6% gels for isolation purposes. Bands of 140 kd and 135 kd comigrated with the upper antigen band and proteins of 85 kd and 80 kd comigrated with the lower antigen (Fig. 7 *b*). The proteins were electroeluted from preparative gels and a portion labeled with ^{125}I to assess their purity. As shown in Fig. 7 *c*, left panel, each protein ran as a single band with the expected mw. Furthermore, each of the proteins expressed BR96 epitopes as determined by immunoblotting (Fig. 7 *c*, *right panel*).

The upper antigen purified by BR96 affinity chromatography and the 135-kd antigen (10 pmol) isolated from preparative gels were electrotransferred to PVDF and the same amino terminal sequence of eight residues was obtained for both preparations (Fig. 8 *a*). A search of the Genbank data base identified human LAMP-1 as having seven of eight matching residues. Furthermore the 140-kd and 135-kd BR96 antigens were recognized on immunoblots probed with an anti-LAMP-1 mAb (Fig. 8 *b*). The 85-kd antigen reacted significantly less with the mAb and the 80-kd showed only trace levels of immunoreactivity (Fig. 8 *b*). Since the anti-LAMP-1 mAb recognizes a core protein epitope (Mane et al., 1989), it is possible that the 85-kd and 80-kd antigens represent degradation products of the mature 135/140-kd upper antigen.

This may result in successive loss of the anti-LAMP-1 epitope on the 85-kd and 80-kd antigens, however, since LAMP-1 is heavily glycosylated, these molecules retain multiple carbohydrate epitopes recognized by BR96.

Increased BR96 Epitope Density on LAMP-1 Expressed in Carcinoma Cells Relative to Nontransformed Epithelial Cells

The data presented above indicates that LAMP-1 expressed on the surface of tumor cells may promote cell migration. This raises the question whether LAMP-1 molecules from cells with different growth potentials are the same. Experiments were performed in which a soluble LAMP-1/Ig fusion construct, consisting of the LAMP-1 extracellular domain ligated to human IgCγ1 (Fig. 9 *a*) was expressed in both tumorigenic 3396 breast carcinoma cells and in 184 mammary epithelial cells which do not form tumors in nude mice (Clark et al., 1988). In addition, we assessed why some colon carcinoma lines, like 3719, are not recognized by BR96. Soluble fusion proteins were purified by protein A Sepharose affinity chromatography and analyzed by immunoblotting (Fig. 9 *b*). The LAMP-1/Ig fusion proteins isolated from the three cell lines had identical mws (~140 kd) and reacted equivalently with anti-human IgG (Fig. 9 *b*). The LAMP-1/Ig's, therefore, appear to be glycosylated to the same extent, with carbohydrate accounting for 55% of their mass. Fusion protein blots probed with BR96 gave a different pattern of immunoreactivity as compared to results with anti-IgG. First, LAMP-1/Ig's from 184 and 3396 were recognized by BR96 while that from the BR96 negative line 3719 was not (Fig. 9 *b*). Since the fusion protein produced in 3719 has a mass equivalent to those from 3396 and 184 cells, it suggests that minor carbohydrate differences may account for the lack of BR96 immunoreactivity. Second, LAMP-1/Ig produced in 3396 cells was significantly more immunoreactive than the fusion protein produced in 184 cells. This was not due to differences in the amount of protein present since blots probed with anti-human IgG and anti-LAMP-1 gave equivalent signals with LAMP-1/Ig's expressed in 184 and 3396 cells. Third, a number of lower mw species were detected in 3396 cells, the major one of which migrated at a position (85 kd, *arrow*) identical to one of the cell surface immunoprecipitated antigens.

We next quantitated the observed difference in BR96 epitope density on LAMP-1/Ig expressed in carcinoma cells vs epithelial cells. Dilutions of the various fusion proteins were used to coat ELISA plates. The wells were incubated with either BR96, mouse monoclonal anti-human IgG, or control mouse IgG (5 μg/ml). Bound mAbs were detected with horseradish peroxidase-labeled goat anti-mouse IgG. The data reflect what is seen on the immunoblot. LAMP-1/Ig expressed in 184 cells reacted equivalently with both anti-human IgG and BR96 indicating a similar epitope density for both of these mAbs. On the other hand, it was necessary to dilute LAMP-1/Ig isolated from 3396 cells 30-fold relative to that from 184 cells to generate the same signal with BR96 (Fig. 9 *c*). Data from transient expression studies, therefore, shows conclusively that the core protein of LAMP-1 can carry carbohydrate determinants recognized by BR96 and that LAMP-1 from mammary epithelial cells has significantly fewer BR96 epitopes than LAMP-1 from breast carcinoma

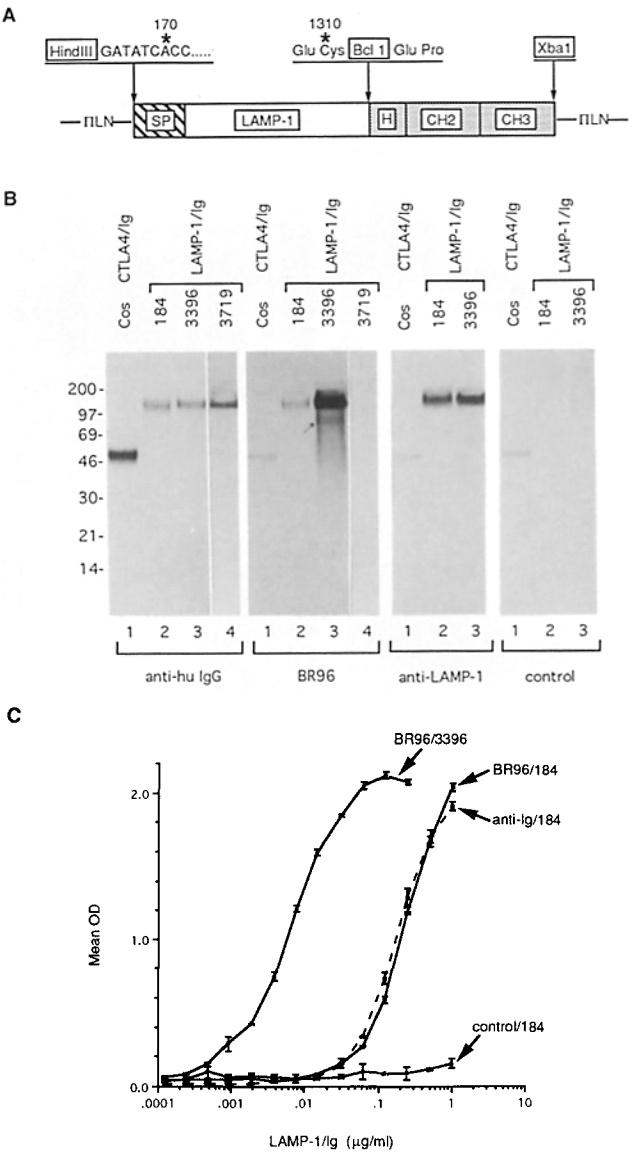

Figure 9. BR96 binding to LAMP-1/Ig fusion proteins. A cDNA coding for the LAMP-1 signal peptide (*SP*) and 98% of the LAMP-1 extracellular domain (*LAMP-1*) was fused to a cDNA coding for the hinge, CH2, and CH3 domains of human IgCγ1, cloned into the vector π LN (Fig. 9 *a*) and transiently expressed in mammary epithelial cells (184), breast carcinoma cells (3396), or colon carcinoma cells (3719). Soluble fusion proteins were purified on protein A, run on 10% tricine gels, electrotransferred, and the blots probed with anti-human Ig, BR96, anti-LAMP-1, or IIG5 control (Fig. 9 *b*). CTLA4/Ig produced in Cos cells served as a fusion protein control. Note that the quantity of fusion protein analyzed from 3396 and 184 were equal based on ELISA (not shown) and comparable reactivity with anti-Ig and anti-LAMP-1 (Fig. 9 *b*). BR96 reacted significantly more with LAMP/Ig from 3396 cells (*BR96 blot*, lane *3*) relative to LAMP-1/Ig from 184 cells (*BR96 arrow*, lane *2*). Also alternate forms of LAMP-1/Ig produced by 3396 cells were detected with BR96, the major one migrated at 85 kd (*BR96 blot*, lane *3*, *arrow*). The apparent difference in BR96 epitope density on LAMP-1/Ig expressed in 184 and 3396 cells was quantitated by ELISA (Fig. 9 *c*).

cells. Differences in glycosylation may, therefore, be one way in which LAMP-1 influences cell migration in different cell types.

Discussion

BR96 is an internalizing mAb which recognizes an antigen highly expressed on carcinoma cells and has, therefore, been used to effectively deliver drugs and toxins to tumor in mice and rats resulting in complete tumor eradication (Friedman et al., 1993; Garrigues et al., 1993; Hellström et al., 1990; Trail et al., 1993). Previously we showed that the level of BR96 antigen expression appeared to be controlled by cell density in that confluent cultures bound less BR96 than subconfluent ones (Garrigues et al., 1993). However, when confluent cultures were scratch wounded, cells at the wound edge bound BR96 suggesting that antigen expression was either inhibited by cell contact or upregulated when cells were stimulated to migrate (Garrigues et al., 1993). To investigate these possibilities further, we have now studied the distribution of BR96 at the cell surface and have looked at the effect of BR96 internalization on cell behavior.

A number of findings are consistent with the idea that BR96 recognizes an antigen which is involved in migration. First, localization studies using both light and scanning electron microscopy showed that BR96 is highly enriched on mirospikes and ruffled membranes. Cells induced to migrate by chemotactic factors or scratch wounding rapidly develop membrane ruffles and microspikes (filopodia) which continuously extend and retract as the cell advances (Guirguis et al., 1987; Trinkaus, 1985). These membrane protrusions contain parallel bundles of actin filaments which gives them tensile strength and are enriched for cytoskeletal associated proteins, e.g., villin, fimbrin, talin, a-actinin, ezrin family members (Bretscher, 1991; Small, 1988); ECM and growth factor receptors, e.g., fibronectin, laminin (Guirguis et al., 1987), transferrin (Hopkins, 1985); signal transduction molecules, e.g., G proteins (Lewis et al., 1991); and others, e.g., melanoma chondroitin sulfate proteoglycan (Garrigues et al., 1986), CD43 (Yonemura et al., 1993), p185^{neu} (Carothers Carraway et al., 1993), glucose transporter (Orci et al., 1989), and GAP-43 (Widmer and Caroni, 1993). These structures represent distinct functional microdomains of the cell surface. Microspikes, for example, can mediate centripetal particle movement and have a surveying function capable of guiding cells onto favorable substrates or away from other cells, i.e., contact inhibition (Albrecht-Buehler, 1976; Fisher et al., 1988). In addition, microspikes are the precursor structures of focal adhesions and therefore play a pivotal role in establishing links between the ECM and the cytoskeleton which, in many instances, are necessary for cell migration (DePasquale et al., 1987; Izzard and Lochner, 1980; Izzard, 1988).

We also found that BR96, when added to cells in culture, was a potent inhibitor of cell migration using conditions that favor mAb internalization. Assays to assess cell viability showed the inhibition of migration was not due to BR96 induced cell death (data not shown). When the inhibitory effect of BR96 was reversed by treating cells with fresh media, the onset of cell migration coincided with the appearance of newly formed microspikes and ruffled membranes at the cell margin. Subsequent immunogold-labeling studies showed that these protrusions contained more BR96 antigen than other regions of the cell surface. BR96 appears to affect cell migration by inhibiting the ability of cells to elaborate microspikes and lamellipodia. This is consistent with the findings of Guirguis et al. (1987) who showed that these structures are required for cell migration. BR96 also has some features in common with the motility-inhibiting antibody, MIA-15-5 which has specificity for the H carbohydrate structure [Fucα1,2 Galβ 1-R] and additionally reacts with Le^y (Miyake and Hakomori, 1991). Like BR96, MIA-15-5 blocked cell migration in vitro and it also inhibited experimental metastases in mice (Miyake and Hakomori, 1991). At the same time other anti-carbohydrate mAbs (e.g., anti-H or anti-Le^y) had no effect on cell migration indicating that mAb avidity and/or epitope position along the oligosaccharide chain may be important factors in determining whether mAbs with similar specificities can block cell migration. Miyake et al. (1992) have also looked at the binding of MIA 15-5 to human tumors and related the data to patient survival (Miyake et al., 1992). The five year survival for patients having primary lung carcinomas that expressed the MIA-15-5 epitope was significantly less (20.9% vs 58.6%) relative to patients with antigen negative tumors (Miyake et al., 1992). Perhaps the carbohydrate structures which are recognized by mAbs like MIA-15-5 and BR96 promote invasiveness and therefore adversely affect patient survival.

Interestingly, BR96 also has a number of features in common with a mAb to gp78 which recognizes autocrine motility factor (Nabi et al., 1992). For instance, they both bind to an antigen highly expressed in the endocytic compartment and on membrane protrusions, and both are internalized after antigen binding. Yet they have opposite effects on cell migration, as BR96 inhibits and anti-gp78 stimulates this process. Nabi et al. have speculated that anti-gp78 probably enhances membrane flow to the leading edge and thus increases directed locomotion (Nabi et al., 1992; Singer and Kupfer, 1986). It is not known if BR96 affects vesicle transport. Preliminary experiments however indicate that BR96 causes microfilament fragmentation which undoubtedly resulted in the cell rounding observed in this study. The formation of membrane ruffles, microspikes, and the advancement of the leading edge by migrating cells in response to ligand/receptor interactions involves the breakdown and reassembly of the cytoskeleton. Stossel (1989, 1990) has proposed that ligand induced phosphatidylinositol turnover and cytosolic calcium fluxes regulate the activation of the actin-binding proteins, gelsolin and profilin, which in turn regulate actin filament cleavage and elongation (Lester and McCarthy, 1992). Indeed, the over expression of a gelsolin cDNA in fibroblasts resulted in enhanced cell motility which was dose dependent (Cunningham et al., 1991). Other studies indicate that agonist mediated changes in the cytoskeleton can be independent of intracellular calcium levels (Greenberg et al., 1991; Hall et al., 1989; Sha'afi et al., 1986) and involve GTP-binding proteins such as rac (Ridley et al., 1992) and rho (Ridley and Hall, 1992). Additional work will address what effect BR96 has on cytoskeletal modifications caused by gelsolin, profilin, intermediates in phosphatidylinositol metabolism, or whether the inhibitory effect of BR96 is mediated by small GTP-binding proteins.

We have also presented a number of findings which indicate that LAMP-1 is a major cell surface glycoprotein bearing BR96 defined epitopes. First, BR96 recognizes a Le^y epi-

tope found on polylactosamine modified oligosaccharides which are abundant on LAMP-1. Second, BR96 is internalized and either cycle between an endocytic compartment and the cell surface or, is degraded in lysosomes with similar kinetics as mAbs to chicken LAMP-1 (Garrigues et al., 1993; Lippincott-Schwartz and Fambrough, 1987). Third, within the intracellular compartment, BR96 localizes exclusively to endosomes and lysosomes. Fourth, the NH_2-terminal sequence of the 135-kd BR96 cell surface antigen was identical to LAMP-1. And fifth, LAMP-1/Ig fusion proteins transiently expressed in Le^y positive cell lines were recognized by BR96.

Cell surface antigens immunoprecipitated with BR96 ran as diffuse bands with approximate mw's of 135 kd (upper antigen) and 85 kd (lower antigen). These broad antigen bands were further resolved into bands of 140 kd and 135 kd (upper antigen) and 85 kd (lower antigen) which most likely represent "doublet" bands due to minor glycosylation differences as LAMP-1 is heavily glycosylated. Two pieces of data suggest that the lower antigen may be derived from the upper antigen possibly as a result of degradation. First, the 140 and 135 kd antigens isolated from preparative gels reacted significantly more with anti-LAMP-1 mAb than the 85 kd form, and the 80-kd antigen was barely detected which might be due to a stepwise proteolytic loss of the anti-LAMP-1 core protein epitope (Mane et al., 1989). Second, although the major form of LAMP-1/Ig transiently expressed in 3396 cells migrated at 140 kd, BR96 immunoblots also revealed the presence of a number of lower mw forms of LAMP-1/Ig (>46 kd). Since the mass of the nonglycosylated fusion protein is ~65 kd, and modification of polylactosamines to generate the Le^y epitope is a late event in carbohydrate biosynthesis, it follows that the low mw glycosylated forms of LAMP-1/Ig recognized by BR96 may be generated via degradation of the mature (140 kd) fusion protein. Interestingly, the most abundant form of degraded LAMP-1/Ig had an identical size (85 kd) as the lower antigen immunoprecipitated with BR96 further suggesting that the latter may be a degradation product of the upper antigen. Additional work will focus on identifying the lower BR96 antigen to determine whether it is related to LAMP-1. In addition to LAMP-1, carcinomas are also known to have glycolipids which express Lewis antigens (Hakomori, 1989). A comparison of glycolipid and glycoprotein fractions from 3396 cells showed that the majority of BR96 immunoreactivity was associated with the glycoprotein fraction (Wang, W.-C., unpublished data). While our data indicate that LAMP-1 is a major cell surface antigen recognized by BR96 they do not exclude the possibility that BR96 could also bind other less abundant protein or glycolipid antigens.

The cDNA expression studies also provide information about why some carcinomas fail to react with BR96. The mass of the LAMP-1/Ig's from both BR96 antigen positive and negative cell lines was the same (140 kd) indicating that the different cell lines had glycosylated the fusion proteins equivalently. Immunoblots of LAMP-1/Ig from the BR96 negative colon carcinoma cells however failed to react with BR96, suggesting that minor carbohydrate differences, e.g., fucosylation, may control whether or not tumors react with BR96. From this data in addition to our previous observations (Hellström et al., 1990), it follows that Le^y is the only structure recognized by BR96 and that polypeptide sequences are not involved in mAb binding. BR96 recognition of tumors therefore is probably controlled at the level of polylactosamine modification rather than LAMP-1 transcription.

Although the function of LAMP-1 is unknown, some studies investigating changes in glycosylation associated with metastasis are consistent with the idea that LAMP-1 may promote cell migration (Saitoh et al., 1992). Highly metastatic cells, for example, have more polylactosamine than those from normal or nonmetastatic cells (Hubbard, 1987; Pierce and Arango, 1986; Yamashita et al., 1984; Yousefi et al., 1991). Glycosylation mutants of a metastatic cell line which were deficient in β 1,6 GlcNAc transferase V activity, a key enzyme in polylactosamine biosynthesis, showed a loss of metastatic potential but remained tumorigenic (Dennis et al., 1987). Furthermore, when polylactosamine formation was inhibited with swainsonine, metastatic tumor cell invasion of human amnion basement membrane in vitro was blocked (Yagel et al., 1989) and tumor formation in vivo was reduced (Dennis, 1986; Humphries, 1986). On the other hand, the acquisition of highly branched polylactosamine oligosaccharides resulted in increased cell surface expression of LAMP-1 and was associated with efficient tumor metastasis (Heffernan et al., 1989). In addition, studies looking at LAMP-1/ECM interactions demonstrated that the binding of LAMP-1 from the metastatic tumor MDAY-D2 to type I collagen was glycosylation dependent and enzymatic removal of sialic acid, polylactosamine, or complete N-linked sugars resulted in enhanced binding to type I collagen, laminin, and fibronectin (Laferte and Dennis, 1988). These results suggest that polylactosamines may stimulate cell migration by destabilizing cell adhesion.

It has been difficult to propose a model for how polylactosamines could influence cell migration because of a lack of information concerning their distribution relative to cell/substratum contact points or membrane domains involved in cell migration. We now show that LAMP-1, a prominent antigen recognized by BR96 and the major cell surface glycoprotein carrying polylactosamine oligosaccharides, is strategically situated on microspikes and ruffled membranes to affect the formation of focal contacts during the initial stages of cell migration. The role of LAMP-1 in cell migration might be related to its participation in membrane vesicle transport. However its localization on actin rich structures and the fact that BR96 induced LAMP-1 modulation resulted in microspike retraction and cell rounding also suggests that LAMP-1 can interact with other molecules which may regulate assembly of the cytoskeleton. If this interaction was restricted to cell surface LAMP-1, as opposed to that in endosomes and lysosomes, the migration of transformed cells might be preferentially affected since they contain more LAMP-1 at the plasma membrane than normal cells.

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References

1. Albrecht-Buehler, G. 1976. Filopodia of spreading 3T3 cells. *J. Cell Biol.* 69:275–286.
2. Bevilacqua, M. P., and R. M. Nelson. 1993. Selectins. *J. Clin. Invest.* 91:379–387.
3. Bretscher, A. 1991. Microfilament structure and function in the cortical cytoskeleton. *Annu. Rev. Cell Biol.* 7:337–374.
4. Carothers Carraway, C. A., M. E. Carvajal, Y. Li, and K. L. Carraway. 1993. Association of p185^{neu} with microfilaments via a large glycoprotein complex in mammary carcinoma microvilli. *J. Biol. Chem.* 268:5582–5587.
5. Clark, R., M. R. Stampfer, R. Milley, E. O'Rourke, K. H. Walen, M. Kriegler, J. Kapplin, and F. McCormick. 1988. Transformation of human mammary epithelial cells by oncogenic retroviruses. *Cancer Res.* 48:4689–4694.
6. Cunningham, C. C., T. P. Stossel, and D. J. Kwiatkowski. 1991. Enhanced motility in NIH 3T3 fibroblasts that overexpress gelsolin. *Science (Wash. DC).* 251:1233–1236.
7. Dennis, J. W. 1986. Effects of swainsonine and polycytidylic acid on murine tumor cell growth and metastasis. *Cancer Res.* 46:5131–5136.
8. Dennis, J. W., S. Laferté, C. Waghorne, M. L. Breitman, and R. S. Kerbel. 1987. β1-6 branching of asn-linked oligosaccharides is directly associated with metastasis. *Science (Wash. DC).* 236:582–585.
9. DePasquale, J. A., and C. S. Izzard. 1987. Evidence for an actin-containing cytoplasmic precursor of the focal contact and the timing of incorporation of vinculin at the focal contact. *J. Cell Biol.* 105:2803–2809.
10. Drebin, J. A., V. C. Link, D. F. Stern, R. A. Weinberg, and M. I. Green. 1985. Down modulation of an oncogene protein product and reversion of the transformed phenotype by monoclonal antibodies. *Cell.* 41:695–706.
11. Fell, H. P., M. A. Gayle, D. Yelton, L. Lipsich, G. L. Schieven, J. S. Marken, A. Aruffo, K. E. Hellström, I. Hellström, and J. Bajorath. 1992. Chimeric L6 anti-tumor antibody. *J. Biol. Chem.* 267:15552–15558.
12. Fisher, G. W., P. A. Conrad, R. L. DeBiasio, and D. L. Taylor. 1988. Centripetal transport of cytoplasm, actin, and the cell surface in lamellipodia of fibroblasts. *Cell Motil. Cytoskeleton.* 11:235–247.
13. Friedman, P. N., D. F. Chace, P. A. Trail, and C. B. Siegall. 1993. Antitumor activity of the single-chain immunotoxin BR96 sFv-PE40 against established breast and lung tumor xenografts. *J. Immunol.* 150:3054–3061.
14. Fukuda, M. 1991. Lysosomal membrane glycoproteins. *J. Biol. Chem.* 266:21327–21330.
15. Fukuda, M., J. Viitala, J. Matteson, and S. R. Carlsson. 1988. The cloning of cDNAs encoding human lysosomal membrane glycoproteins, h-lamp-1 and h-lamp-2. *J. Biol. Chem.* 263:18920–18928.
16. Garrigues, H. J., M. W. Lark, S. Lara, I. Hellström, K. E. Hellström, and T. N. Wight. 1986. The melanoma proteoglycan: restricted expression on microspikes, a specific microdomain of the cell surface. *J. Cell Biol.* 103:1699–1710.
17. Garrigues, J., U. Garrigues, I. Hellström, and K. E. Hellström. 1993. Le^y specific antibody with potent anti-tumor activity is internalized and degraded in lysosomes. *Am. J. Path.* 142:607–622.
18. Greenberg, S., J. El Khoury, F. Di Virgilio, E. M. Kaplan, and S. C. Silverstein. 1991. Ca^{2+}-independent F-actin assembly and disassembly during Fc receptor-mediated phagocytosis in mouse macrophages. *J. Cell Biol.* 113:757–767.
19. Guirguis, R., I. Margulies, G. Taraboletti, E. Schiffmann, and L. Liotta. 1987. Cytokine-induced pseudopodial protrusion is coupled to tumour cell migration. *Nature (Lond.).* 329:261–263.
20. Hakomori, S.-I. 1989. Aberrant glycosylation in tumors and tumor-associated carbohydrate antigens. *Adv. Cancer Res.* 52:257–331.
21. Hall, A. L., V. Warren, S. Dharmawardhane, and J. Condeelis. 1989. Identification of actin nucleation activity and polymerization inhibitor in ameboid cells: their regulation by chemotactic stimulation. *J. Cell Biol.* 109:2207–2213.
22. Heffernan, M., S. Yousefi, and J. W. Dennis. 1989. Molecular characterization of P2B/LAMP-1, a major protein target of a metastasis-associated oligosaccharide structure. *Cancer Res.* 49:6077–6084.
23. Hellström, I., H. J. Garrigues, U. Garrigues, and K. E. Hellström. 1990. Highly tumor-reactive, internalizing, mouse monoclonal antibodies to Le^y-related cell surface antigens. *Cancer Res.* 50:2183–2190.
24. Hirano, H., and T. Watanabe. 1990. Microsequencing of proteins electrotransferred onto immobilizing matrices from polyacrylamide gel electrophoresis: application to an insoluble protein. *Electrophoresis.* 11:573–580.
25. Hopkins, C. R. 1985. The appearance and internalization of transferrin receptors at the margins of spreading human tumor cells. *Cell.* 40:199–208.
26. Hubbard, S. C. 1987. Differential effects of oncogenic transformation on *N*-linked oligosaccharide processing at individual glycosylation sites of viral glycoproteins. *J. Cell Biol.* 16403–16411.
27. Humphries, M. J., K. Matsumoto, S. L. White, and K. Olden. 1986. Oligosaccharide modification by swainsonine treatment inhibits pulmonary colonization by B16-F10 murine melanoma cells. *Proc. Natl. Acad. Sci. USA.* 83:1752–1756.
28. Izzard, C. S. 1988. A precursor of the focal contact in cultured fibroblasts. *Cell Motil. Cytoskeleton.* 10:137–142.
29. Izzard, C. S., and L. R. Lochner. 1980. Formation of cell-to-substrate contacts during fibroblast motility: an interference-reflexion study. *J. Cell Sci.* 42:81–116.
30. Laferte, S., and J. W. Dennis. 1988. Glycosylation-dependent collagen-binding activities of two membrane glycoproteins in MDAY-22 tumor cells. *Cancer Res.* 48:4743–4748.
31. Larsson, L.-I. 1988. Immunocytochemistry: Theory and Practice. CRC Press, Inc. Boca Raton, FL.
32. Lester, B. R., and J. B. McCarthy. 1992. Tumor cell adhesion to the extracellular matrix and signal transduction mechanisms implicated in tumor cell motility, invasion and metastasis. *Cancer Metastasis Rev.* 11:31–44.
33. Lewis, J. M., M. J. Woolkalis, G. L. Gerton, R. M. Smith, L. Jarett, and D. R. Manning. 1991. Subcellular distribution of the a subunit(s) of G_i: visualization by immunofluorescent and immunogold labeling. *Cell. Reg.* 2:1097–1113.
34. Linsley, P. A., W. Brady, L. Grosmaire, A. Aruffo, N. K. Damle, and J. A. Ledbetter. 1991. Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and Interleukin 2 mRNA accumulation. *J. Exp. Med.* 173:721–730.
35. Lippincott-Schwartz, J., and D. M. Fambrough. 1987. Cycling of the integral membrane glycoprotein, LEP100, between plasma membrane and lysosomes: kinetic and morphological analysis. *Cell.* 49:669–677.
36. Mane, S. M., L. Marzella, D. F. Bainton, V. K. Holt, Y. Cha, J. E. K. Hildreth, and J. T. August. 1989. Purification and characterization of human lysosomal membrane glycoproteins. *Arch. Biochem. Biophys.* 268: 360–378.
37. McConahey, P. J., and F. J. Dixon. 1980. Radioiodination of proteins by the use of the chloramine-T method. *Methods Enzymol.* 70:210–213.
38. Miyake, M., and S. Hakomori. 1991. A specific glycoconjugate controlling cell motility: evidence by functional monoclonal antibodies that inhibit cell motility and tumor cell metastasis. *Biochemistry.* 30:3328–3334.
39. Miyake, M., T. Taki, S. Hitomi, and S. Hakomori. 1992. The correlation of expression of H/Le^y/Le^b antigens with survival in patients with carcinoma of the lung. *N. Engl. J. Med.* 327:14–18.
40. Nabi, I. R., H. Watanabe, and A. Raz. 1992. Autocrine motility factor and its receptor: role in cell locomotion and metastasis. *Cancer Metastasis Rev.* 11:5–20.
41. Orci, L., B. Thorens, M. Ravazzola, and H. F. Lodish. 1989. Localization of the pancreatic beta cell glucose transporter to specific plasma membrane domains. *Science (Wash. DC).* 245:295–297.
42. Pierce, M., and J. Arango. 1986. Rous sarcoma virus-transformed baby hamster kidney cells express higher levels of asparagine-linked tri- and tetraantennary glycopeptides containing [GlcNAc-β(1,6)Man-α(1,6)Man] and poly-*N*-acetyllactosamine sequences than baby hamster kidney cells. *J. Biol. Chem.* 261:10772–10777.
43. Ridley, A. J., and A. Hall. 1992. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell.* 70:389–399.
44. Ridley, A. J., H. F. Paterson, C. L. Johnston, D. Diekmann, and A. Hall. 1992. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell.* 70:401–410.
45. Saitoh, O., W.-C. Wang, R. Lotan, and M. Fukuda. 1992. Differential glycosylation and cell surface expression of lysosomal membrane glycoproteins in sublines of a human colon cancer exhibiting distinct metastatic potentials. *J. Biol. Chem.* 267:5700–5711.
46. Seed, B., and A. Aruffo. 1987. Molecular cloning of the human CD2 antigen by a rapid immunoselection procedure. *Proc. Natl. Acad. Sci. USA.* 84:3365–3369.
47. Sha'afi, R. I., J. Shefcyk, R. Yassin, T. F. P. Molski, M. Volpi, P. H. Naccache, J. R. White, M. B. Feinstein, and E. L. Becker. 1986. Is a rise in intracellular concentration of free calcium necessary or sufficient for stimulated cytoskeletal-associated actin? *J. Cell Biol.* 102:1469–1463.
48 Singer, S. J., and A. Kupfer. 1986. The directed migration of eukaryotic cells. *Annu. Rev. Cell Biol.* 2:337–365.
49. Small, J. V. 1988. The actin cytoskeleton. *Electron Microsc. Rev.* 1:155–174.
50. Stossel, T. P. 1989. From signal to pseudopod. *J. Biol. Chem.* 264:18261–18264.
51. Stossel, T. P. 1990. How cells crawl. *Am. Scientist.* 73:408–423.
52. Trail, P. A., D. Willner, S. J. Lasch, A. J. Henderson, S. Hofstead, A. M. Casazza, R. A. Firestone, I. Hellström, and K. E. Hellström. 1993. Cure of xenografted human carcinomas by BR96-Doxorubicin immunoconjugates. *Science (Wash. DC).* 261:212–215.
53. Trinkaus, J. P. 1985. Protrusive activity of the cell surface and the initiation of cell movement during morphogensis. *Exp. Biol. Med.* 10:130–173.
54. Turley, E. A. 1992. Molecular mechanisms of cell motility. *Cancer Metastasis Rev.* 11:1–3.
55. Widmer, F., and P. Caroni. 1993. Phosphorylation-site mutagenesis of the

growth-associated protein GAP-43 modulates its effects on cell spreading and morphology. *J. Cell Biol.* 120:503–512.
56. Yagel, S., R. Feinmesser, C. Waghorne, P. K. Lala, M. L. Breitman, and J. W. Dennis. 1989. Evidence that β1-6 branched asn-linked oligosaccharides on metastatic tumor cells facilitate invasion of basement membranes. *Int. J. Cancer.* 44:685–690.
57. Yamashita, K., T. Ohkura, Y. Tachibana, S. Takasaki, and A. Kobata. 1984. Comparative study of oligosaccharides released from baby hamster kidney cells and their polyoma transformant by hydrazinolysis. *J. Biol. Chem.* 259:10834–10840.
58. Yonemura, S., A. Nagafuchi, N. Sato, and S. Tsukita. 1993. Concentration of an integral membrane protein, CD43 (leukosialin, sialophorin), in the cleavage furrow through the interaction of its cytoplasmic domain with actin-based cytoskeletons. *J. Cell Biol.* 120:437–449.
59. Yousefi, S., E. Higgins, Z. Daoling, A. Pollex-Krüger, O. Hindsgaul, and J. W. Dennis. 1991. Increased UDP-GlcNAc:Galβ1-3GalNAc-R (GlcNAc to GalNAc) β-1,6-N-Acetylglucosaminyltransferase activity in metastatic murine tumor cell lines. *J. Biol. Chem.* 266:1772–1782.