Phage Display–Derived Human Monoclonal Antibodies Isolated by Binding to the Surface of Live Primary Breast Cancer Cells Recognize GRP78

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Abstract

Clinical trials using monoclonal antibodies (mAb) against cell-surface markers have yielded encouraging therapeutic results in several cancer types. Generally, however, anticancer antibodies are only efficient against a subpopulation of cancers, and there is a strong need for identification of novel targets and human antibodies against them. We have isolated single-chain human mAbs from a large naïve antibody phage display library by panning on a single-cell suspension of freshly isolated live cancer cells from a human breast cancer specimen, and these antibodies were shown to specifically recognize cancer-associated cell-surface proteins. One of the isolated human antibody fragments, Ab39, recognizes a cell-surface antigen expressed on a subpopulation of cancer cell lines of different origins. Immunohistochemical analysis of a large panel of cancerous and normal tissues showed that Ab39 bound strongly to several cancers, including 45% breast carcinomas, 35% lung cancers, and 86% melanomas, but showed no or weak binding to normal tissues. A yeast two-hybrid screen of a large human testis cDNA library identified the glucose-regulated protein of 78 kDa (GRP78) as the antigen recognized by Ab39. The interaction was confirmed by colocalization studies and antibody competition experiments that also mapped the epitope recognized by Ab39 to the COOH terminus of GRP78. The expression of GRP78 on the surface of cancer cells, but not normal cells, makes it an attractive target for cancer therapies including mAb-based immunotherapy. Our results suggest that the human antibody Ab39 may be a useful starting point for further genetic optimization that could render it a useful diagnostic and therapeutic reagent for a variety of cancers. [Cancer Res 2007;67(19):9507–17]

Introduction

Breast cancer is the most frequent cancer in women and the second leading cause of cancer deaths, exceeded only by lung cancer (1). Clinical breast cancer management has been dramatically changed with the initiation of systematic screening programs for earlier detection and the use of adjuvant hormonal therapy, antibody-based therapy, and chemotherapy (2, 3). These changes are beginning to have a major impact on patient outcome, and despite increasing incidence, breast cancer mortality is decreasing in most of the Western world. The current goal is to expand the repertoire of novel effective targeted therapies that will, in combination with predictive and prognostic tests, allow individualized and tailored treatment regimens that will minimize serious side effects.

The topography of the surface of normal cells changes during their transformation to malignant cells (4), primarily as a result of alterations in the expression levels of lineage-specific membrane proteins or membrane proteins involved in differentiation (5). These changes also result from the reappearance of fetal antigens (6) or aberrations in the tertiary structure of the exiting cell-surface antigens, such as glycosylation (7). Several of these protein changes have been correlated with tumor progression, metastasis, and patient survival rates (8). Over the past three decades, substantial efforts have focused on exploiting these cancer-associated protein alterations to develop tumor-specific therapeutic drugs. Generation of monoclonal antibodies (mAb) against surface receptors, where the expression is up-regulated on cancer cells, has been the subject of considerable scientific interest and has resulted in the development of highly effective clinical agents for immunodiagnosis and immunotherapy of several cancer types (9, 10).

Glucose-regulated protein of 78 kDa (GRP78; also referred to as BiP) is a member of the heat shock protein 70 (HSP70) family that functions as a chaperone critical for folding, maturation, and transport of polypeptides and proteins. GRP78 is also important in the unfolded protein response that ameliorates stress conditions, such as glucose starvation and hypoxia, as well as in maintaining estrogen receptor (ER) function and protecting cells against death (11, 12). Whereas GRP78 is constitutively expressed at low levels, stress induces its synthesis (11). A small pool of such synthesized GRP78 translocates to the cell surface in association with MTJ-1 (13), where it functions as a receptor for activated forms of the plasma proteinase inhibitor α_2-macroglobulin (α2M*). Further, cell-surface GRP78 may act as a coreceptor for viruses and MHC class I antigen presentation (14). The expression of GRP78 is highly elevated in many cancers, correlating with malignancy, metastasis, and drug resistance (15–18). Signaling initiated by the binding of α2M* to GRP78 on the cell surface of human prostate cancer cells has been shown to lead to pro-proliferative and antiapoptotic behaviors, and it has been proposed that increased amounts of α2M* present in some cancer patients may play a role in the aggressive behavior of some cancers. Interestingly, autoantibodies against GRP78 have been observed in sera of prostate cancer patients with aggressive disease (19), and an affinity-purified anti-GRP78 antibody preparation generated from such patients protected cancer cells from apoptosis and induced tumor cell proliferation similar to that observed following binding of α2M*

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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(20). The identification of GRP78 on the cell surface has made it an attractive target for cancer therapy (reviewed in ref. 21). Recently, synthetic peptides capable of specifically binding GRP78 have been identified and shown to target tumor cells, e.g., suppressing tumor cell growth *in vitro* when coupled to Taxol or suppressing tumor cell growth in xenograft and isogenic mouse models of prostate and breast cancer when fused to a programmed cell death–inducing sequence (22, 23).

To date, most clinically used mAbs against cancer-associated antigens have been generated by conventional mouse hybridoma technology. However, to prevent human anti-murine antibody responses, which enhances clearance of the murine antibodies from the circulation and precludes repeated administration, these murine antibodies had to be "chimerized" or "humanized." Methods wherein the entire variable region or the complementarity-determining regions (CDR) of the murine mAb are grafted onto the constant regions of human immunoglobulin Gs (IgG) are laborious and expensive and may result in antibodies that still retain some degree of immunogenicity (24, 25). Thus, although recent chimeric and humanized mAbs have provided clinical improvements, alternative methods for generating clinically applicable human mAbs should also be pursued (26).

Phage display technology is an attractive method by which completely human mAbs can be generated from both immune and nonimmune sources (27, 28). Such antibodies are generally retrieved from antibody libraries by selection against a purified relevant subcellular component. We and others have previously used this strategy to select antibodies against HIV-1 gp120, nicotinic acetylcholine receptors, and thrombospondin-1 (29, 30). However, this approach only enables isolation of antibodies against known targets. To identify mAbs against novel antigens, we previously selected antibody libraries against immobilized fixed cell lines or tissue sections (31, 32). These strategies led to successful identification of novel antibody targets, although most of these were cytoplasmic antigens. Other groups have developed selection strategies for isolation of cell-surface antigens primarily using cell lines (33, 34).

In the current study, we describe an efficient procedure for selecting human scFv antibodies to cancer-associated cell-surface antigens by carrying out selections on viable cancer cells isolated from freshly excised breast tumor tissue. To identify the antigen recognized by one of the isolated human antibodies, we screened a human testis cDNA library using the yeast two-hybrid system and identified GRP78 as the target antigen. This scFv human antibody recognized a large percentage of cancers of different origins, but did not, or only weakly, recognize normal tissues, strongly suggesting its potential clinical utility in targeted immunotherapy.

Materials and Methods

Bacterial strains and cloning. Electrocompetent amber codon suppressing *E. coli* strain TG1 (Stratagene; *supE thi-1 D(lac-proAB) D(mcrB-hsdSM)5 (rK− mK−) [F′ traD36 proAB lacIqZDM15]*) containing the F′ episome for single-strand rescue was used for transformations and preparation of phagemids and phage display libraries. The transformations of phagemid DNA were done as described (35).

ScFv phage library. The Griffin.1 library is a large naïve human scFv phagemid library (total diversity of 1.2×10^9) constructed from synthetic V-gene segments[4] and was made by recloning the heavy-chain and light-chain variable regions from the lox library vectors (36) into the phagemid vector pHEN2.[5] The Griffin.1 phagemid library resembles the Nissim library (37) but contains diversity in the light as well as heavy chains. The helper phage KM13 was prepared as described (38).

Isolation of living single cancer cells from a breast cancer patient specimen. Tumor tissue from an invasive ductal carcinoma was obtained from an 80-year-old woman immediately following therapeutic surgical resection. The characteristics of the tumor were 100% ER positive, 80% progesterone receptor positive, human epidermal growth factor receptor-2 negative, tumor size of 36 mm, and malignancy grade 1. After removal of fat and necrotic parts, the tumor tissue was kept in a Petri dish containing basal medium (DMEM, 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 1% penicillin/streptomycin, 5 mg/mL gentamicin, and 2.5 μg/mL fungizone; Invitrogen) on ice while being minced into 1- to 2-mm^3 pieces using a razor blade and small scissors. The minced tissue was rinsed with basal medium followed by disaggregation by shaking at 37°C for 16 h in basal medium containing 320 μg/mL collagenase (Sigma-Aldrich) and 500 μg/mL hyaluronidase (Sigma-Aldrich). Disaggregated cells were subsequently washed thrice in basal medium followed by centrifugation at $350 \times g$ for 10 min. After the last centrifugation, the cells were used directly for selection or resuspended in Mammary Epithelial Cell Medium (MEGM; Cambrex Bio Science) for further growth until the next panning.

Selection of phage libraries. Tumor cells were blocked with basal medium/6% bovine serum albumin (BSA) in PBS mixed 1:1 for 2 h at room temperature, after which Griffin.1 scFv phage library (10^{11}–10^{12} phages) blocked with basal medium/6% BSA in PBS mixed 1:1 for 2 h at room temperature was added and incubated with slow tilt rotation for 2 h at room temperature. Cells were washed 10 times with PBS by centrifuging for 5 min at $350 \times g$ between each wash. Phages were eluted by resuspending the cells in 500 μL of a 1 mg/mL trypsin solution in PBS followed by incubation for 10 min at room temperature with rotation. The eluted phages were added to 1 mL of TG1 ($A_{600} = 0.5$) and incubated for 30 min at 37°C without shaking followed by plating on 1× tryptone-yeast extract agar plates supplemented with 50 μg/mL carbenicillin and 1% glucose. A second round of selection on the primary tumor cells in MEGM medium was done by rescue with the helper phage KM13, as described above. The input, output, and output/input ratios were 10^{12}, 8×10^3, and 8×10^{-9}, respectively, for the first round of panning, and 1.25×10^{11}, 8.7×10^5, and 6.96×10^{-6}, respectively, for the second round of panning. In other experiments, Griffin.1 scFv phage library blocked with basal medium/6% BSA in PBS was panned two rounds against recombinant hamster GRP78 (97% identity to human GRP78) produced in bacteria (Assay Designs, Inc.). The recombinant GRP78 was coated on ELISA plates overnight at a concentration of 5 μg/mL. Phages were eluted and processed as described above. The input, output, and output/input ratios were 4×10^9, 6.8×10^5, and 1.7×10^{-4}, respectively, for the first round of panning, and 3×10^{10}, 4×10^7, and 1.33×10^{-3}, respectively, for the second round of panning.

Yeast two-hybrid screening. The DNA fragment encoding Ab39 was excised from the pHEN2 vector using *Nco*I and *Not*I restriction enzymes and cloned into the *Nco*I/*Not*I cloning site of the pSos vector (Stratagene) to generate bait plasmid pSL39. A human testis cDNA library, generated from a pool of 25 normal human whole testis tissue specimens, cloned unidirectionally into the CytoTrap pMyr XR vector, was obtained from Stratagene. Library plasmid DNA was cotransformed into temperature-sensitive cdc25h yeast (Matα, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112, cdc25-2, Gal+) with pSos bait plasmids and plated on synthetic defined agar (Clontech) supplied with 20 g/L glucose or galactose. In general, the recommendations of the manufacturer's protocols were followed unless otherwise stated. To select for the plasmids, both uracil and leucine were omitted from the media. Yeast grown for 24 to 48 h at 25°C were replica plated on galactose plates and incubated at the nonpermissive temperature of 37°C for 3 to 5 days. Individual colonies

[4] http://vbase.mrc-cpe.cam.ac.uk/?&MMN_position=1:1

[5] http://www.mrc-cpe.cam.ac.uk/index.php?module=pagemaster&PAGE_user_op=view_page&PAGE_id=12

that grew at 37°C were picked and expanded in liquid medium at 25°C. Plasmid DNA was isolated according to the manufacturer's protocol and used to transform electrocompetent XL1-blue. The transformation was plated on Luria broth-chloramphenicol (130 mg/L) agar plates; the resulting colonies were expanded in 2× tryptone-yeast extract + chloramphenicol (130 mg/L); and plasmid DNA was isolated using the miniprep kit (Promega). The purified plasmid was used to cotransform with pSos bait plasmids pSL39 into cdc25h yeast and reexamined for growth on galactose or glucose solid media plates at 25°C and 37°C. The DNA insert of the three positive library clones was amplified using the primers Myr 5′ (5′-ACTACTAGCAGCTGTAATAATAC-3′) and Myr 3′ (5′-CGTGAATGTAAGCGTGACAT-3′; Stratagene) and fingerprinted using *Bst*N1 and gel separation.

Antibody competition experiments. Tissue sections were processed and stained with Ab39, as described in the immunohistochemistry section above, except that before incubation with Ab39, sections were preincubated with affinity-purified goat anti-GRP78 antibody (C-20, Santa Cruz Biotechnology), affinity-purified rabbit anti-GRP78 antibody (H-129, Santa Cruz Biotechnology), isotype-matched controls, or diluent only. The goat anti-GRP78 antibody (C-20) recognizes the COOH terminus (amino acids 614–654) of GRP78, produces a single band in Western blotting, and also recognizes GRP78 in its native conformation. The rabbit anti-GRP78 antibody (H-129) is generated by immunization with a protein fragment (amino acids 525–655) of GRP78, but the major reactivity is thought to be directed against a region more NH_2-terminal than C20 (manufacturer's information). This antibody also produces a single band in Western blotting and recognizes the native conformation of GRP78. Bound Ab39 was detected with mouse anti-M13 antibody (GE Healthcare) and horseradish peroxidase–conjugated "Ready-to-use" EnVision+ polymer K4001 (Dako), and visualized with 3,3′-diaminobenzidine. Inhibition of Ab39 staining by the polyclonal antibody preparations was evaluated by microscopy.

Detailed Information on "Cell lines and culture conditions," "Production of phage libraries," "Production of monoclonal scFv phages," "Cell ELISA and recombinant GRP78 ELISA," "Sequence analysis," "Immunohistochemistry," "Flow cytometry," and "Cell growth inhibition assay" are provided in Supplementary data.

Results

Selection of cancer cell–binding phage-displayed scFv antibodies. To identify and clone human antibodies against breast cancer cell-surface molecules, single live breast cancer cells were obtained from a freshly excised breast tumor of a patient with ductal carcinoma and used for selection of a large naïve human scFv phage display library. Following two rounds of biopanning, a 100-fold amplification of the amount of eluted phage was observed, indicating enrichment for cell-binding clones. Single clones from the two rounds of panning were selected and screened for binding to a panel of breast cancer cell lines by direct phage ELISA, revealing a set of clones that bound at least to one of the cell lines and not with control wells containing only media. Following further analysis, antibody Ab39, obtained from the first round of panning, and antibody Ab83, obtained from the second panning round, were selected for further characterization. As shown in Table 1, analysis of the binding of phage antibodies Ab39 and Ab83 to five breast cancer cell lines of different origins and a T-cell leukemia line showed that both Ab39 and Ab83 bound to all the cancer cell lines, albeit at varying intensities. Whereas Ab39 showed efficient binding (+++/++) only to BrCa-MZ-01, MDA-MB-231, and Hs578T, Ab83 efficiently bound (+++/++) to all cell lines except MDA-MB-231. A control, AbK2, a scFv-expressing phage chosen randomly from the original library, showed no binding to the cell lines.

The DNA encoding the variable heavy-chain and light-chain genes of the two selected antibody fragments was sequenced and the amino acid sequences were deduced (Table 2). The two scFv antibodies, Ab39 and Ab83, have heavy chains from two different V_H families. In contrast, both had λ light chains from the same family, although the CDR3 regions differed.

Immunohistochemical analysis of normal and cancerous tissues. The tissue distribution of the antigens recognized by Ab39 and Ab83 was examined using a panel of formalin-fixed, paraffin-embedded cancer and normal tissues. As shown in Fig. 1 and Table 3*A*, Ab39 bound to cancers of different origins, including breast carcinoma, malignant melanoma, ovarian carcinoma, lung adenocarcinoma, lung sqamous cell carcinoma, and seminoma. Generally, however, not all cancers within a certain cancer subtype were stained with Ab39 [i.e., 45% (22 of 49) of breast carcinomas, 35% (7 of 20) of lung cancers, and 86% (6 of 7) of melanomas]. The staining intensity varied within tumors and between tumors from different patients. Ab83 also bound to cancers of different origins but recognized a higher frequency of cancers within a certain subtype compared with Ab39 [i.e. 65% (32 of 49) of breast carcinomas, 50% (10 of 20) of lung cancers, and all (7 of 7) melanomas]. Similar to Ab39, the staining intensity of Ab83 varied within tumors and between different patients. In the

Table 1. Cell ELISA showing Ab39 and Ab83 binding to different cancer cell lines

Cancer cell line	ATTC no.	Type of cancer	Original tissue	ER status	PgR status	Tumorigenicity	Invasiveness	Ab39	Ab83	AbK2
BrCa-MZ-01	—*	MBC	P	+	+	Y	n.d.	+++	+++	—
Hs578T	HTB-126	CS	P	—	—	N	Highly invasive	+++	+++	—
MCF-7	HTB-22	IDC	M (Pl)	+	+	Y	Weakly invasive	+	+++	—
MDA-MB-157	HTB-24	MBC	M (Pl)	—	—	Y	n.d.	+	++	—
MDA-MB-231	HTB-26	IDC	M (Pl)	—	—	Y	Highly invasive	++	+	—
Jurkat	TIB-152	Leukemia T-cell		nd	nd			+	+++	—
RPMI medium								—	—	—

NOTE: Characteristics of the cell lines analyzed are included. +++, strong binding; ++, intermediate binding; +, weak binding; —, no binding.
Abbreviations: PgR, progesterone receptor; MBC, medullary breast cancer; CS, breast carcinoma stellate cell type; IDC, infiltrating ductal carcinoma (breast); P, primary breast tumor; M, metastasis; Pl, pleura fluid.
*A kind gift from Prof. Möbus, Universitätklinikum, Ulm, Germany.

Table 2. Deduced amino acid sequences of the heavy-chain and light-chain CDR3 regions of Ab39, Ab83, and Ab99

Clone	Variable heavy segment	Variable heavy CDR3	Variable light segment	Variable light CDR3
Ab39	VH1-24	FELPMGCT	VL3-31	NSRDSSDHGYV
Ab83	VH2-70	MCKNYPGRFY	VL3-31	NSRDSSGDHLV
Ab99	VH3-72	RRRFKNR	VK1-O12	QQSYSSPYLT

NOTE: The closest germ-line genes and VH and VL families for each clone are also shown.

cancer specimens, Ab39 and Ab83 stained only the cancer cells, not the connective tissue, blood vessels, or muscles. The control AbK2 did not stain the cancer cells or adjacent normal tissue.

Further tissue sections of formalin-fixed, paraffin-embedded cell pellets of different cancer cell lines were examined for staining by Ab39 and Ab83. As shown in Fig. 2*A*, Ab39 and Ab83 bound intensely to the cell surface of Hs578T, MCF-7, and MDA-MB-157 cells and also stained the cytoplasm. Interestingly, not all Hs578T and MDA-MB-157 cells were stained with the same intensity, and a subpopulation of cells was not stained at all. No significant binding to Hs578T, MCF-7, and MDA-MB-157 was observed with the control AbK2.

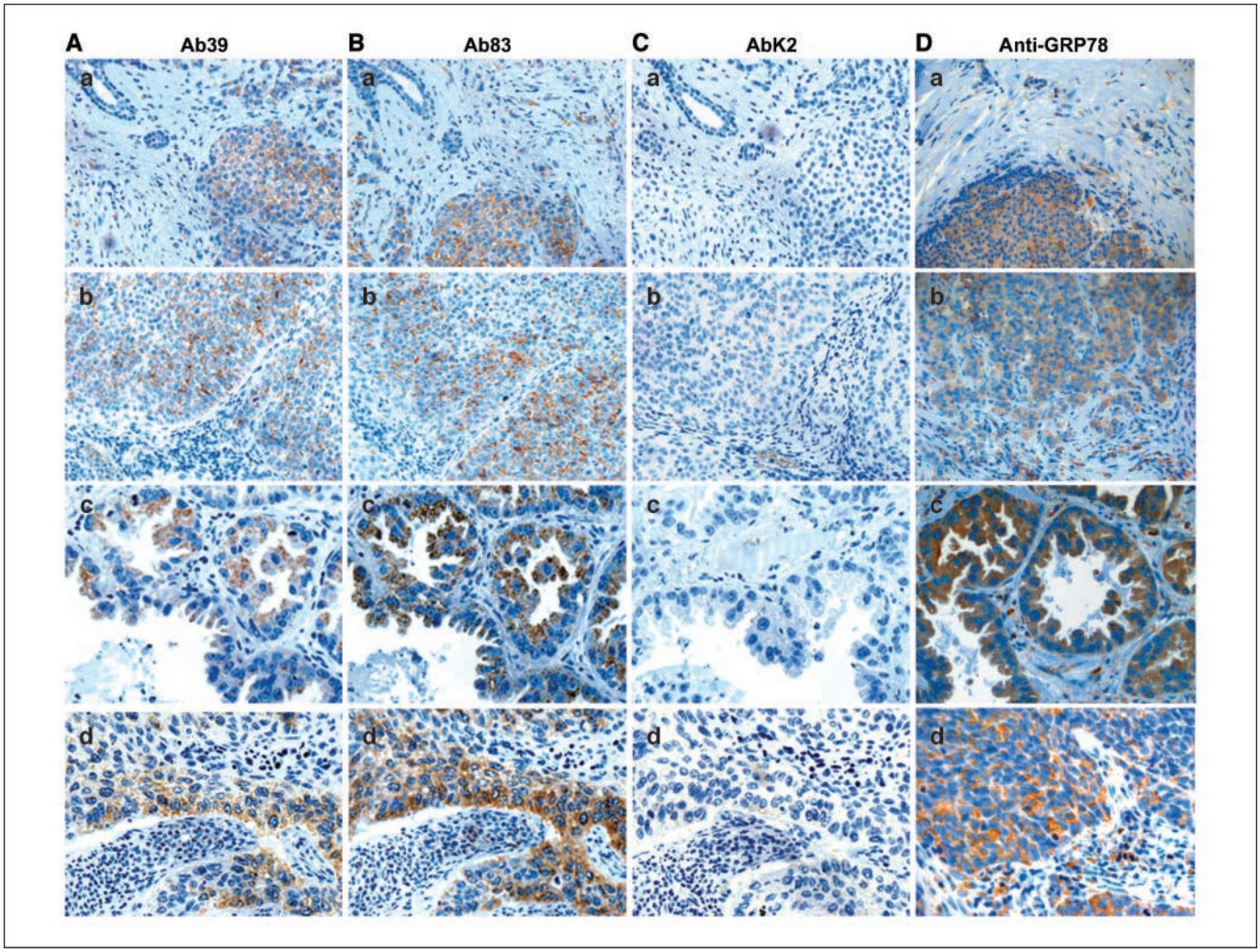


Figure 1. Staining pattern of human mAbs Ab39 and Ab83 in human cancer biopsies and its comparison with anti-GRP78 staining as determined by immunohistochemical analysis. Ab39 (*A*), Ab83 (*B*), AbK2 (negative control; *C*), and rabbit anti-GRP78 antibody (H-129; *D*) were used to stain human cancer tissues, including lobular breast carcinoma (*a*), malignant melanoma (*b*), lung adenocarcinoma (*c*), and lung squamous cell carcinoma (*d*). Ab39, Ab83, and rabbit anti-GRP78 antibody stained cancer cells in all four specimens, whereas no staining was observed in adjacent normal tissue. No staining of cancer cells or adjacent normal tissue using control AbK2 was observed (*C*).

Table 3. Immunohistochemical analysis of the reactivity of Ab39, Ab83, and anti-GRP78 antibody (H-129) with cancerous (*A*) and normal (*B*) tissues ($n_{positive}/n_{total}$)

(*A*)

Tumor type	Ab39	Ab83	AbK2 (control)	Anti-GRP78 antibody
Breast carcinoma	22/49	32/49	0/49	28/49
Lymphoma	1/2	1/2	0/2	1/2
Lung carcinomas total	7/20	10/20	0/20	8/20
Adenocarcinoma	2/4	2/4	0/4	2/4
Large-cell carcinoma	1/2	2/2	0/2	1/2
Small-cell carcinoma	1/2	1/2	0/2	1/2
Planocellular Carcinoma	3/12	5/12	0/12	5/12
Malignant fibrous Histocytoma	0/1	0/1	0/1	0/1
Malignant melanoma	6/7	7/7	0/7	6/7
Mesothelioma	0/1	0/1	0/1	0/1
Ovary carcinoma	1/1	1/1	0/1	1/1
Pheochromocytoma	0/1	0/1	0/1	0/1
Renal cell carcinoma	0/1	0/1	0/1	0/1
Seminoma	1/1	1/1	0/1	1/1
Medullary thyroid carcinoma	1/1	1/1	0/1	1/1

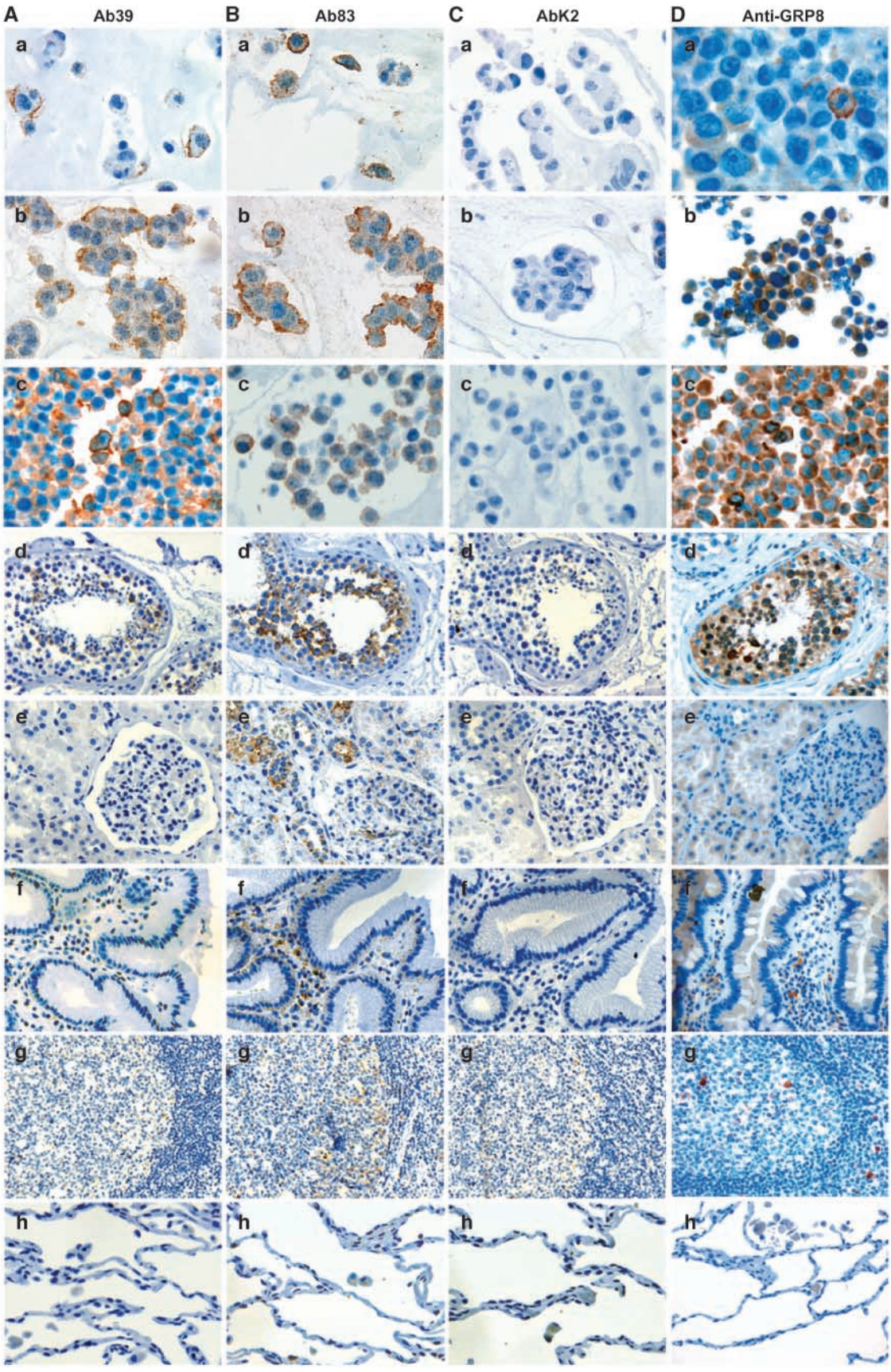
(*B*)

Tissue	Ab39	Ab83	AbK2 (control)	Anti-GRP78 antibody
Bladder	−	+	−	+
Bowel	−	−	−	−
Cerebellum	−	−	−	−
Cerebrum	−	−	−	−
Gallbladder	−	−	−	−
Kidney	−	+	−	+
Liver	−	−	−	−
Lung	−	−	−	−
Muscle	−	−	−	−
Esophagus	−	−	−	−
Ovary	−	−	−	−
Parathyroid gland	−	−	−	−
Placenta	−	+	−	+
Prostate	−	−	−	−
Rectum	−	−	−	−
Skin	−	−	−	−
Small intestine	−	+	−	+
Testis	+	+	−	+
Thymus	−	−	−	−
Thyroid gland	−	+	−	+
Tonsil	−	+	−	+
Ventricle	−	−	−	−

NOTE: AbK2 was used as negative control.

A large panel of different normal tissues was also examined for Ab39 and Ab83 staining (Fig. 2*B*; Table 3*B*). Immunohistochemical analysis showed that Ab39 did not react with any of the normal tissues except a subpopulation of cells in the seminiferous tubule of testis. These cells were determined to be predominantly spermatocytes with some spermatids, and no staining was observed in spermatogonia, spermatozoa, or Sertoli cells. In contrast, Ab83 stained cell populations in a broad variety of normal tissues, including a cell population present in the submucosa of the small instestine, the distal convoluted tubule of the kidney, and, to a lesser extent, the proximal convoluted tubule of the kidney, normal abortion tissue, placenta, spermatocytes and spermatids in normal testis, and a cell population in the lymphoid follicles of tonsil (Fig. 2*B*). No binding to these normal tissues was observed with the control AbK2.

Binding of scFv antibodies to the surface of breast cancer cell lines. Ab39 and Ab83 were tested for binding to a panel of unfixed breast cancer cell lines (Hs578T, MDA-MB-157, MCF-7, and MDA-MB-231) by flow cytometry. Ab39 bound to the cell surface of all four cell lines, whereas Ab83 bound to the Hs578T and



MDA-MB-157 cell lines, but only weakly to MCF-7 or MDA-MB-231 (Fig. 3*A*). The control antibody, AbK2, showed no significant binding to any of the cell lines.

A second set of experiments focusing on the most promising clone, Ab39, examined cell-surface binding to three other beast cancer cell lines, BrCa-Mz-01, 2C5, and 4A4, as well as to Jurkat T cells (Fig. 3*B*). Ab39 bound to all three breast cancer cell lines but showed no surface staining of Jurkat T cells. As shown in Fig. 2*A* and *B*, a complete shift in intensity of all the Ab39 or Ab83 stained cells was not always observed, suggesting that not all cells were equally stained, and some remained unstained, in agreement with our cytochemistry results.

Western blotting. To determine the approximate mass of antigens recognized by Ab39 and Ab83, lysates of the MCF-7, Hs578T, and MDA-MB-231 cell lines were separated by Western blotting and stained by the two antibodies. No specific bands were recognized by the two antibodies, suggesting that they recognized conformationally dependent epitopes.

Identification of the target antigen for Ab39 using yeast two-hybrid screening. To identify the antigen recognized by Ab39, a yeast two-hybrid screen of a large human testis cDNA library was done using the CytoTrap two-hybrid system, which allows identification of glycosylated antigens, an important aspect because the target antigen of Ab39 is expressed on the cell surface. The cDNA encoding Ab39 cloned into the pSOS to be used as bait was cotransformed with the testis library in the pMyr XR vector. The screen identified three clones that permitted growth at 37°C of yeast harboring plasmids expressing Ab39 (pSL39). Fingerprinting indicated that the cDNA inserts of these three clones were identical. Sequencing of the insert of one of the clones revealed a cDNA that encodes the 166-amino-acid COOH-terminal region of human GRP78 (amino acids 489–654; 99% identity) as the Ab39 interacting protein.

Verification of GRP78 as the target antigen and epitope mapping. To verify that GRP78 was the target antigen for Ab39, serial sections of the same tissue specimens and cell lines initially analyzed with Ab39 and Ab83 were examined using two polyclonal antibodies directed against the COOH-terminal part of GRP78. As shown in Figs. 1 and 2 and Table 3, the staining pattern of rabbit anti-GRP78 antibody (H-129) was very similar to that observed with Ab39 and Ab83. A similar staining pattern was also observed with the goat anti-GRP78 antibody (C20; data not shown). Importantly, Ab39 seemed to be better at discriminating cancer from normal tissue, perhaps due modification of the target epitope on GRP78.

To more precisely determine the epitope of Ab39 within the COOH-terminal domain of GRP78, competition experiments between Ab39 and the two polyclonal ant-GRP78 antibodies were done using serial tissue sections of tumors with high GRP78 expression. As shown in Fig. 3*C*, C20 goat anti-GRP78 antibody directed against the COOH terminus of GRP78 (amino acids 614–654) significantly inhibited Ab39 staining, whereas the H-129 rabbit anti-GRP78 antibody and isotype-matched control antibody did not, showing the epitope of Ab39 to be within the amino acid region 614–654 of GRP78.

To expand our panel of human monoclonal anti-GRP78 antibodies, we selected the large naïve human scFv phage display library against recombinant GRP78 coated on ELISA plates. One additional human anti-GRP78 scFv, Ab99, was identified from the second round of panning (Table 2). Whereas this scFv bound efficiently to recombinant GRP78, it did not bind to the control antigen, as shown by ELISA (Fig. 3*D*). Interestingly, Ab39 and Ab83 did not react significantly with the recombinant GRP78 (hamster GRP78; 99% identity to human GRP78). Comparing the sequences of human and hamster identified one amino acid change (amino acid 649, Hu:A-Ham:S) in the region where Ab39 was mapped. Further analysis of potential glycosylation sites on GRP78 identified one O-glycosylation site (amino acid T643) in the COOH terminus of GRP78 to which Ab39 was mapped. Because the recombinant hamster was produced in bacteria and thus lacks glycosylation, the ELISA results suggest that the binding of Ab39 may be influenced by glycosylation or may involve amino acid 638.

Cell growth inhibition assay. Because a human polyclonal anti-GRP78 antibody preparation purified from serum of prostate cancer patients with autoantibodies against GRP78 has been shown to increase tumor cell proliferation *in vitro* (R), we examined the effect of Ab39 and Ab83 on the growth of different breast cancer cell lines in culture with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. High concentrations of purified Ab39, Ab83, and control AbK2 scFv failed to alter the growth of MDA-MB-231, BrCa-MZ-01, or MDA-MB-157 cells (data not shown).

Discussion

The search for human mAbs against novel cell-surface molecules that may be used as targets for cancer therapies has recently led to the development of several novel technologies that efficiently generate such antibodies. Phage display is one such technology that, with the development of improved selection and screening methods, has proved very useful in isolating antibodies with specificities against undefined cellular disease markers. Antibodies against novel cell specificities have been retrieved from immune libraries developed using materials from patients with malignancies or autoimmune conditions, thus reflecting the immune repertoire in these individuals (31, 32, 39). In nonimmune libraries, the most promising application is the ability to generate human antibodies with specificities that are not normally, or only rarely, generated by the natural human antibody response or by the immune response of immunized normal or human IgG transgenic mice.

Our selection strategy uses freshly isolated live cancer cells from a clinically excised breast cancer specimen to isolate a panel of breast cancer cell-surface–reactive antibodies from a large

Figure 2. Staining pattern of human mAbs Ab39 and Ab83 in breast cancer cell lines and normal human tissues and its comparison with anti-GRP78 staining determined by immunohistochemical analysis. Ab39 (*A*), Ab83 (*B*), AbK2 (negative control, *C*), and rabbit anti-GRP78 antibody (H-129, *D*) were used to stain breast cancer cell lines Hs578T (*a*), MCF-7 (*b*), and MDA-MB-157 (*c*) and normal tissues including testis (*d*), kidney (*e*), intestine (*f*), tonsil (*g*), and lung (*h*). Ab39 and Ab83 showed cell-surface staining of the three breast cancer cell lines. In normal tissues, Ab39 did not stain a large panel of normal tissues but showed staining in testis restricted to spermatocytes and spermatids of the seminiferous tubule, and no staining in spermatogonia, spermatozoa, or Sertoli cells. Ab83 also stained spermatocytes and spermatids in normal testis (*Bd*), the distal convoluted tubule, and, to a lesser extent, the proximal convoluted tubule of the kidney (*Be*), as well as cells in the submucosa of the small instestine (*Bf*) and the lymphoid follicles of tonsil (*Bg*), but not in lung (*Bh*) or several other normal tissues. AbK2 did not stain the breast cancer cell lines or the normal tissues (*C*), whereas rabbit anti-GRP78 antibody (H-129) showed a staining pattern similar to Ab83 (*D*).

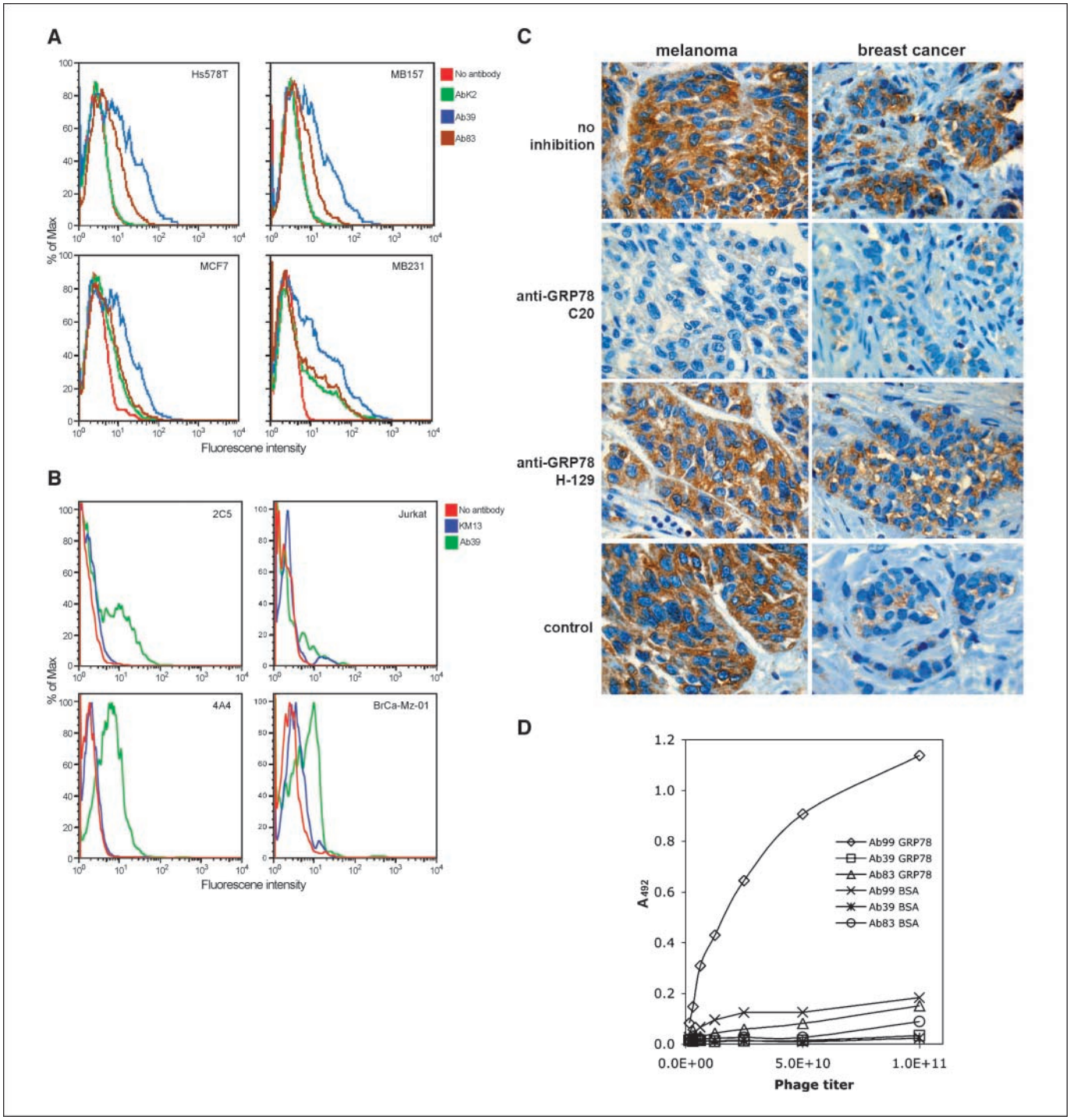


Figure 3. Biochemical characterization of human scFvs Ab39, Ab83, and Ab99. *A,* flow cytometry analysis of scFv phage antibodies Ab39 and Ab83 binding to different cancer cell lines. In one set of experiments, Ab39 and Ab83 were tested for binding to the four breast cancer cell lines MDA-MB-231, Hs578T, MDA-MB-157, and MCF-7. AbK2 was included as negative control. *B,* a second set of experiments assessed Ab39 binding to three other breast cancer cell lines, NM-2C5, M-4A4, and MDA-MB-157, and the Jurkat T-cell line. M13 phage was used as negative control. In both experiments, bound scFv phages were detected using a phycoerythrin-labeled anti-M13 antibody. *C,* inhibition of human monoclonal Ab39 staining by polyclonal anti-GRP78 antibody: Serial tissue sections of human melanoma (*first column*) and a ductal breast cancer (*second column*) were incubated with an anti-GRP78 antibody before adding Ab39. A marked reduction in Ab39 staining was observed with the C20 goat antibody directed against the COOH terminus of GRP78 (*second row*), whereas strong staining was observed when Ab39 was applied alone (*first row*), with a rabbit anti-GRP78 antibody, H-129 (*third row*), or with isotype-matched control antibody (*fourth row*). *D,* binding of scFv phage antibodies Ab39, Ab83, and Ab99 to bacterial expressed recombinant hamster GRP78 and bovine serum albumin (control) by ELISA.

nonimmune human scFv library. Two human scFv antibodies, Ab39 and Ab83, which are reactive with many tumors of different histologic origins but show no or weak binding to normal tissues, were isolated. Identification of the antigen recognized by Ab39 was less straightforward because the epitope recognized by the antibody is probably conformational dependent, and the antigen is therefore not detected in Western blots. Standard immunoprecipitation experiments often lead to insufficient material for mass

spectrometry analysis, and therefore a yeast-two hybrid system screen was done using Ab39 as bait. The CytoTrap yeast two-hybrid system was used, which monitors protein interactions at the plasma membrane through activation of a Ras-dependent signal pathway by hSos protein (40), and thus enables identification of interaction partners not identifiable by conventional yeast two-hybrid systems, including proteins that require posttranslational modification in the cytoplasm, are transcriptional activators or repressors, and are toxic to yeast in conventional two-hybrid systems. Because our immunohistochemical analysis had shown that the target antigen was expressed in normal testis, a large testis cDNA library was screened with the yeast-two hybrid system. Because GRP78 has been reported to be constitutively expressed in several normal tissues, its identification as the target antigen came as a bit of a surprise as we would have expected to see staining with Ab39 in many normal tissues. On the other hand, the result correlated well with the previous observation that increased levels of GRP78 are found on the surface of spermatocytes and some spermatids in normal testis (41) compared with other normal tissues, and that increased GRP78 levels are present in a substantial number of cancers. Comparison of the staining patterns of the two human scFv antibodies and two polyclonal anti-GRP78 antibodies also showed colocalization, with Ab83 showing identical staining with the two polyclonal anti-GRP78 antibodies in normal tissues. Although Ab39 exhibited slightly less intense staining of the cancers compared with Ab83 and the polyclonal anti-GRP78 preparations, Ab39 seemed to be better at discriminating cancer from normal tissue. This could relate to the particular epitope on GRP78 recognized by Ab39 that may be modified in cancer cells (e.g., posttranslational modification). The influence of fixation on the epitopes recognized by the two scFv should also be considered.

Flow cytometric and immunocytochemical analyses of Ab39 staining in different breast cancer cell lines revealed significant variability from cell to cell within a given cell line. Whereas some cells stained intensely, others exhibited weak or no staining, and these subpopulations could not be distinguished by size or granularity. We previously observed similar staining intensity variability in breast cancer cell lines using an antibody reactive with another antigen (cancer/testis antigen GAGE; ref. 42). In those studies, only 5% to 30% of the experimental cancer cell line stained for GAGE, showing that expression of this antigen was not associated with a specific genotype but was linked to a specific phenotype (e.g., a specific stage in the cell cycle). Because the effectiveness of new therapeutics requires that they also target the cancer stem cells, it would be important to evaluate whether our human mAbs also recognized these cells. The phenotype of breast cancer stem cells is still not fully defined, but they are generally characterized by being $CD44^+$ and $CD24^-$ (43). Two of the breast cancer cell lines, NM-2C5 and M-A4A, which were shown to be targeted by our human scFvs, indeed exhibited the $CD44^+/CD24^-$ phenotype on all cells.[6] Interestingly, this cell line also exhibited features generally observed in breast cancers of the basal-like subtype, which has been suggested to be of stem cell origin (44). These preliminary results suggest that our human anti-GRP78 mAbs will recognize cancer stem cells.

Our immunohistochemical studies were done using scFv expressed on phage, a method that has been considered problematic because the phage particles have a tendency to bind unspecifically. However, we optimized a protocol that largely overcomes this. No or very limited unspecific binding was observed using a panel of control phages that express irrelevant scFv antibodies, including AbK2, selected from the starting library and verified to express scFv antibody. The use of scFv expressed on phage instead of soluble scFv in the initial screening provides significant amplification of the immunohistochemical detection signal. A phage particle may express more than one scFv, resulting in increased avidity, which may be significant because some antibodies retrieved from naïve libraries may be of modest affinity compared with those isolated from immune libraries. Moreover, the use of an anti-M13 antibody as secondary antibody may significantly amplify the signal because the target of this antibody, protein VIII, is expressed in 2,500 to 3,000 copies on the phage surface.

Competition experiment revealed that Ab39 is directed against the COOH terminus of GRP78. The conformation and orientation of GRP78 in the cell membrane is not well characterized. GRP78, like other HSP proteins, consists of two domains: the 44-kDa NH_2-terminal domain, which is responsible for GRP78 ATPase activity, and a 20-kDa COOH-terminal polypeptide binding domain. In addition, it contains a highly helical and variable 10-kDa COOH-terminal tail of unknown function (45). Because GRP78 does not contain a transmembrane domain, it was uncertain how GRP78 became exposed on the cell surface. Recent results, however, have shown that GRP78 is likely translocated and present at the cell surface associated with the transmembrane protein MTJ-1, which is also an accessory protein for GRP78 catalytic activity (13). The J domain of MTJ-1 interacts with GRP78, stimulates its ATPase activity, and functions as its cochaperone in protein folding and translocation of newly synthesized polypeptides across ER membranes (46). Interestingly, GRP78, like other HSP70 proteins, self-associates into multiple oligomeric species (47, 48). The COOH-terminal domain of GRP78 has been shown to be responsible for the oligomeric properties of the protein (48). Binding of unfolded peptidic substrate onto the COOH-terminal domain, or binding of ATP onto the NH_2-terminal domain, promotes depolymerization and stabilization of BiP monomers (48). A recent study examining the interaction of dengue virus serotype 2 with the cell surface identified GRP78 as a interaction partner and showed that both antibodies directed against both the NH_2 and COOH termini of GRP78 altered the binding of the virus to the cell surface (49), indicating that both the NH_2 and COOH termini of GRP78 are accessible at the cell surface.

A human polyclonal antibody preparation of affinity-purified GRP78 from serum of prostate cancer patients with autoantibodies against GRP78 has been shown to increase tumor cell proliferation and inhibit apoptosis *in vitro* (20). This human polyclonal anti-GRP78 antibody was directed against the same region as that recognized by GRP78 physiologic agonist, a2M*, and mapped to amino acids 98 to 115 present in the more NH_2-terminal region of GRP78. Another study examining Kringle 5 of human plasminogen, which inhibits angiogenesis by inducing apoptosis of proliferating endothelial cells, showed that Kringle 5 also induces apoptosis of stressed HT1080 fibrosarcoma cells by binding to the NH_2 terminus of GRP78 (50). Due to these opposing observations, we tested whether Ab39 and Ab83 expressed as soluble scFvs were capable of influencing the cell growth of a panel of breast cancer cell lines, but no change in growth rate was observed. This may not come as a surprise because the scFvs bind to a different region of

[6] R. Leth-Larsen, R. Lund, and H.J. Ditzel, unpublished data.

GRP78 than the anti-GRP78 autoantibody preparation and Kringle 5 and do not diminish the potential of the human anti-GRP78 mAbs in clinical therapeutics. Recently, a study using a cyclic GRP78 binding peptide showed that its linkage to Taxol resulted in specific and efficient tumor growth suppression *in vitro* (23). In another study, using a different GRP78 binding peptide fused to a programmed cell death–inducing sequence resulted in efficient tumor growth suppression in xenograft and isogenic mouse models of prostate and breast cancer (22). Similarly, the human anti-GRP78 mAbs may be used for cancer-specific delivery of cytotoxic agents and radioisotopes. In addition, whole anti-GRP78 mAbs may potentially mediate antibody-dependent cellular cytotoxicity dependent on the surface expression levels of GRP78 and the affinity of mAb.

In conclusion, our efficient selection strategy led to the isolation of the human scFv mAb Ab39 that may have clinical applications as both a therapeutic drug and an imaging agent. Because the antibodies are isolated from human antibody repertoires, they should not be immunogenic when given to patients. Following affinity maturation by genetic engineering, it may be beneficial to transform Ab39 into a whole human IgG antibody to increase the recruitment of effector cells to the targeted cancer cells, whereas the use of single-chain molecules may have advantage as an imaging agent.

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