The Breast Cancer $\beta_4$ Integrin and Endothelial Human CLCA2 Mediate Lung Metastasis*

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Adhesion of blood-borne cancer cells to the endothelium is a critical determinant of organ-specific metastasis. Here we show that colonization of the lungs by human breast cancer cells is correlated with cell surface expression of the $\alpha_6\beta_4$ integrin and adhesion to human CLCA2 (hCLCA2), a $\text{Ca}^{2+}$-sensitive chloride channel protein that is expressed on the endothelial cell luminal surface of pulmonary arteries, arterioles, and venules. Tumor cell adhesion to endothelial hCLCA2 is mediated by the $\beta_4$ integrin, establishing for the first time a cell-cell adhesion property for this integrin that involves an entirely new adhesion partner. This adhesion is augmented by an increased surface expression of the $\alpha_6\beta_4$ integrin in breast cancer cells selected in vivo for enhanced lung colonization but abolished by the specific cleavage of the $\beta_4$ integrin with matrilysin. $\beta_4$ integrin/hCLCA2 adhesion-blocking antibodies directed against either of the two interacting adhesion molecules inhibit lung colonization, while overexpression of the $\beta_4$ integrin in a model murine tumor cell line of modest lung colonization potential significantly increases the lung metastatic potential. Our data clearly show that the $\beta_4$/hCLCA2 adhesion is critical for lung metastasis, yet expression of the $\beta_4$ integrin in many benign breast tumors shows that this integrin is insufficient to bestow metastatic competence on cells that lack invasiveness and other established properties of metastatic cells.

Colonization of secondary organs by blood-borne cancer cells marks the final, usually fatal stage in a long, multistep cascade of tumor progression that is propelled by an array of acquired, cumulative, genetic abnormalities and promoting tissue microenvironmental cues (1–5). Increasing evidence suggests that tumor cell targeting of preferred, secondary organs for metastasis is mediated by distinct endothelial cell adhesion molecules (6–8). These molecules are expressed constitutively (or-gan-specifically) on the endothelial cell luminal surface of select vascular compartments (e.g. capillaries, arterioles, and/or venules) (7). By binding blood-borne cancer cells at high affinity, these molecules mediate vascular arrest of tumor cells under hydrodynamic conditions (6–9) and, as shown recently, promote intravascular growth to form tumor colonies at these secondary target sites (10, 11). Using a unique large vessel endothelial cell system, in which an organ-specific vascular phenotype can be induced by growing “neutral” bovine aortic endothelial cells on matrix extracts of that organ (12), a lung-specific endothelial cell adhesion molecule, termed Lu-ECAM-1 (lung-endothelial cell adhesion molecule-1) was isolated, purified, and cloned by our laboratory (13–15). Lu-ECAM-1 is the prototype of a newly discovered mammalian family of proteins (termed LCAs, for $\text{Cl}^-$ channel proteins, $\text{Ca}^{2+}$-activated) (13), which, similar to the cystic fibrosis transmembrane conductance regulator (16, 17), serve the dual function of mediating chloride conductance and cell-cell adhesion (16–18). Lu-ECAM-1 protein, like all other members of the CLCA family, is synthesized as an $\sim$125-kDa precursor protein that, upon membrane incorporation, is rapidly processed into N-terminal 90-kDa and C-terminal 35-kDa components (15, 18). The 90-kDa polypeptide is responsible for the adhesion qualities of LCAs, promoting the $\text{Ca}^{2+}$-dependent adhesion of a variety of lung metastatic cancer cells but not cancer cells that metastasize to other organ sites (12–15).

In this report, hCLCA2 cloned from a lung cDNA library (19) is identified as the human counterpart of Lu-ECAM-1. We show that hCLCA2 is expressed by endothelia from different lung vascular compartments and that lung colonization of established human breast cancer cell lines is dependent upon the tumor cells’ ability to interact with hCLCA2. Breast cancer cell adhesion to hCLCA2 is mediated by the $\beta_4$ integrin, which is prominently expressed in breast cancer cells that are able to colonize the lungs upon tail vein injection of nude mice (20). Cell-to-cell adhesion assays, adhesion-blocking assays with antibodies generated against either of the two interacting molecules, and overexpression of the $\beta_4$ in a murine model tumor cell line are used to confirm involvement of the $\beta_4$ integrin/hCLCA2 adhesion mechanism in lung metastasis. Together, our data confirm that the $\alpha_6\beta_4$ integrin is a lung metastasis-associated gene (21) and establish for the first time a cell-to-cell adhesion property for the $\beta_4$ integrin that involves an entirely new integrin adhesion partner.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Anti-Lu-ECAM-1 mAb 6D3 was produced in BALB/c mice (22) and selected for adhesion blocking of B16-F10 melanoma cells to Lu-ECAM-1-expressing bovine aortic endothelial cells (13, 14). Rabbit polyclonal antibodies (pAbs) 4 and 18 were gener-

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1 The abbreviations used are: hCLCA2, human CLCA2; mCLCA1, mouse CLCA1; mAb, monoclonal antibody; pAb, polyclonal antibody; HMVEC-L, human lung microvascular endothelial cells; HAE, human aortic endothelial cells; HUVEC, human umbilical vein endothelial cells; HEK, human embryonic kidney; RT, reverse transcriptase; FCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; HRP, horseradish peroxidase; FACS, fluorescence-activated cell sorting; DMEM, Dulbecco’s modified Eagle’s medium.

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and HUVEC (endothelial cells) is shown by RT-PCR amplification of an 800-base pair HMVEC-L stained with rabbit anti-hCLCA2 pAb 4 (reading frame from HMVEC-L RNA) and of the full-length 2.9-kilobase pair hCLCA2 open reading frame corresponding to the 5' (ttcttacaatgaccagggagc and catg-gaagcttgtggaag as 5' and 3' primers, respectively, and Taq polymerase (Life Technologies). The full-length 2.9-kilobase pair open reading frame of hCLCA2 was amplified from HMVEC-L RNA, using primers corresponding to the 5' and 3' (gacagctgtatattttattataggct) ends of the hCLCA2 open reading frame. Both primer sets were tested on cloned plasmid DNA to ascertain that they would not recognize other CLCA homologs. RNA extracted from HEK293 cells and processed in parallel served as negative control.

Expression, Myc Tagging, Immunoprecipitation, and Purification of hCLCA2—Lung expression of hCLCA2 was analyzed by staining sections of paraffin-embedded, formaldehyde-fixed lung tissue blocks or rabbit anti-hCLCA2 pAb 4 at a dilution of 1:100 (A, C, and E) or rabbit preimmune IgG (B, D, and F). Bound antibody was detected by HRP-conjugated goat anti-rabbit IgG antibodies and diaminobenzidine as substrate. A positive staining reaction is observed in small arteries (A), arterioles (C), and venules of interlobular septa (E). Comparable vessels stained with preimmune serum are negative (B, D, and F). Bar, 100 μm. G and H, expression of hCLCA2 in cultured endothelial cells is shown by RT-PCR amplification of an 800-base pair hCLCA2 product from total RNA of HMVEC-L (lane 1), HAEC (lane 2), and HUVEC (lane 3) but not HEK293 (lane 4) (see “Experimental Procedures”) and of the full-length 2.9-kilobase pair hCLCA2 open reading frame from HMVEC-L RNA (G) and by FACs analysis of HMVEC-L stained with rabbit anti-hCLCA2 pAb 4 (open histogram) or rabbit preimmune IgG (closed histogram) and fluorescence isothiocyanate-conjugated goat anti-rabbit IgG (H).

FIG. 1. Endothelial cell expression of hCLCA2. A–F, sections (2 μm thick) from paraffin-embedded, formaldehyde-fixed lung tissue blocks were stained with rabbit anti-hCLCA2 pAb 4 at a dilution of 1:100 (A, C, and E) or rabbit preimmune IgG (B, D, and F). Bound antibody was detected by HRP-conjugated goat anti-rabbit IgG antibodies and diaminobenzidine as substrate. A positive staining reaction is observed in small arteries (A), arterioles (C), and venules of interlobular septa (E). Comparable vessels stained with preimmune serum are negative (B, D, and F). Bar, 100 μm. G and H, expression of hCLCA2 in cultured endothelial cells is shown by RT-PCR amplification of an 800-base pair hCLCA2 product from total RNA of HMVEC-L (lane 1), HAEC (lane 2), and HUVEC (lane 3) but not HEK293 (lane 4) (see “Experimental Procedures”) and of the full-length 2.9-kilobase pair hCLCA2 open reading frame from HMVEC-L RNA (G) and by FACs analysis of HMVEC-L stained with rabbit anti-hCLCA2 pAb 4 (open histogram) or rabbit preimmune IgG (closed histogram) and fluorescence isothiocyanate-conjugated goat anti-rabbit IgG (H).
Transfection of K-Balb/3T3 Cells with β4 cDNA—Wild-type β4 cDNA cloned into the expression vector pRC-CMV was from Dr. F. G. Giancotti. K-Balb/3T3 cells at 70% confluence were stably transfected with β4 cDNA by electroporation and selected for G418 resistance. Controls were K-Balb/3T3 cells transfected with vector alone. Cells were used for (a) FACS to quantify β4 surface expression (25), (b) adhesion to immunopurified mCLCA1 (14), and (c) lung colony assays (12–14).

FACS Analyses, Adhesion, and Lung Colony Assays—FACS analyses, adhesion assays, and lung colony assays were performed as previously described in detail by our laboratory (12–14, 25).

RESULTS
hCLCA2 Expression by Endothelia of the Lung Vasculature—Human CLCA2 was cloned from a human lung cDNA library, and its amino acid sequence, protein processing, transmembrane topology, and channel properties are described elsewhere (19). Northern blot hybridization and/or RT-PCR revealed epithelial expression of hCLCA2 in the mammary gland and trachea (19), while RT-PCR and immunohistochemistry demonstrated endothelial cell expression in the lungs (Fig. 1). In the latter, hCLCA2 protein was expressed selectively in endothelia of small pulmonary arteries, arterioles, and subpleural and interlobular venules (Fig. 1, A–F), while endothelia in other tissues including brain, liver, pancreas, kidney, alimentary tract, testis, ovary, adrenal gland, thyroid, and skeletal muscle were negative (data not shown). Strong hCLCA2 expression was also observed in cultured HMVEC-L lung microvascular endothelial cells, while a weak hCLCA2 expression was recorded for endothelial cells derived from human aorta (HAEC) and umbilical vein (HUVEC) (Fig. 1G). Expression of hCLCA2 protein in HMVEC-L was confirmed by FACS, using the same polyclonal antibody that had been employed in the immunohistochemical studies (Fig. 1H).

hCLCA2 Mediates Adhesion of Human Breast Cancer Cells via the β4 Integrin—The selective expression of hCLCA2 on endothelia of lung blood vessels, which recently were implicated with location of tumor cell arrest and early intravascular micrometastasis formation by in situ epifluorescence microscopy (11), suggested that hCLCA2 could serve as the human counterpart of Lu-ECAM-1 and might play a major role in lung metastasis of blood-borne human cancer cells. To test this hypothesis, we selected three human breast cancer cells with different biological behaviors for adhesion to recombinant Myc-tagged hCLCA2 immunopurified from transfected HEK293 cells. The first cell line was MDA-MB-231, which efficiently colonizes the lungs of nude mice following tumor formation from cancer cells injected into mammary fat pads (orthotopic xenografts) or intravenous injection; the second cell line was MDA-MB-435, which only forms lung metastases from orthotopic xenografts but not after intravenous injection; and the third cell line was MCF7, which is nonmetastatic by either of the two modalities (20). Consistent with the proposed role of hCLCA2 in lung metastasis, only MDA-MB-231 cells adhered in strong numbers to recombinant hCLCA2 (Fig. 2A). Adhesion correlated with the amount of Myc-tagged hCLCA2 protein present in elution fractions from anti-Myc mAb 9E10 immunoaffinity column and was dependent upon serum activation of tumor cells (Fig. 2B). To identify the tumor cell molecule that served as the ligand for hCLCA2, surface-biotinylated MDA-MB-231 cancer cells were allowed to bind to confluent monolayers of Myc-tagged hCLCA2- or vector-transfected HEK293 cells, yielding tumor cell adhesion values of ~75% for Myc-hCLCA2-HEK293 monolayers and 25% for vector-transfected HEK293 monolayers. Myc-hCLCA2-HEK293 monolayers were extracted together with bound tumor cells, and extracts were subjected to immunoprecipitation with anti-

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Fig. 2. The β4 integrin mediates adhesion of breast cancer cells to endothelial hCLCA2. A, adhesion of MDA-MB-231, MDA-MB-435, and MCF7 human breast cancer cells to hCLCA2 was tested in microtiter plates coated with ~3 μg/ml recombinant hCLCA2 (overnight; 4 °C). B, adhesion of MDA-MB-231 cells to Myc-tagged hCLCA2 from successive elution fractions of an anti-Myc mAb 9E10-immunoaffinity column. Protein G beads conjugated with anti-Myc mAb 9E10 were incubated with extracts from HEK293 cells transfected with Myc-tagged hCLCA2 (overnight; 4 °C), and bound protein was eluted with 200 μl glycine (pH 2.8) in 150 mM NaCl and 0.5% octyl-β-glucoside. Elutes were collected in 1-ml fractions in 0.1 volume of 1 M Tris (pH 11) to yield a final pH of 8.2. Each fraction was evaluated for MDA-MB-231 adhesion and protein content by Western blotting with anti-Myc mAb 9E10. Adhesion values correlate well with the amount of hCLCA2 protein in the column fraction. C, surface-biotinylated or untreated MDA-MB-231 cells bound to HEK293 cell monolayers transfected with Myc-hCLCA2 (lanes 1) or HEK293 cell monolayers transfected with vector alone (lanes 2) (see "Experimental Procedures") were extracted in lysis buffer containing 5 mM EGTA. Extracts were subjected to immunoprecipitation with anti-Myc mAb 9E10, and SDS-PAGE-resolved and blotted precipitates were probed with streptavidin-HRP (left panel), anti-β4 mAb 3E1 (middle panel), or anti-Myc mAb 9E10 (right panel). D, anti-β4 pAb H101 immunoprecipitate from extracts of MDA-MB-231 bound to Myc-tagged hCLCA2-transfected (lanes 1) or vector-transfected (lane 2) HEK293 monolayers were probed by Western blot with anti-Myc mAb 9E10 (left panel) or anti-β4 mAb 3E1 (right panel).
Myc mAb 9E10. Precipitated proteins resolved by SDS-PAGE and blotted to nitrocellulose were then probed with streptavidin-HRP. A single band of molecular size 205 kDa was identified that by Western analysis with anti-\(\beta_4\) mAb 3E1 was shown to be \(\beta_4\) integrin (Fig. 2C). Subsequent immunoprecipitation of Myc-hCLCA2-HEK293/MDA-MB-231 cell extracts with anti-\(\beta_4\) pAb H101 and Western probing of the precipitate with anti-Myc mAb 9E10 identified hCLCA2, further confirming the \(\beta_4\) integrin/hCLCA2 adhesion (Fig. 2D). Controls conducted with MDA-MB-231 cancer cells bound unspecifically to vector-transfected HEK293 monolayers did not yield any precipitate. Expansion of our initial hCLCA2/tumor cell adhesion studies to MDA-MB-435L2, MDA-MB-468, and MDA-MB-453 breast cancer cell lines supported the close correlation between surface expression of the \(\beta_4\) integrin, hCLCA2 adhesion, and lung colonization (Fig. 3, A–D). For example, the consistently high lung colonization potential of the MDA-MB-231 cell line correlated with high levels of \(\beta_4\) integrin expression and hCLCA2 adhesion, while modest lung colonization of the MDA-MB-435L2 cell line was associated with modest \(\beta_4\) expression and hCLCA2 adhesion (Fig. 3, A–D). All other breast cancer cell lines were unable to form lung colonies and, with the exception of the MDA-MB-468 cell line, expressed low or nondetectable levels of the \(\beta_4\) integrin and adhered poorly to hCLCA2 (Fig. 3, A–D). In the metastatically incompetent MDA-MB-468 cell line, an intermediate level of \(\beta_4\) expression correlated with a well differentiated, near normal cellular phenotype in vitro and slow adenomatous growth in vivo, implying that the MDA-MB-468 cell line represents an early stage in tumor progression.

Since isolation of the \(\beta_4\) integrin/hCLCA2 complex from MDA-MB-231/Myc-hCLCA2-HEK293 extracts and hCLCA2 affinity purification of the \(\beta_4\) integrin from MDA-MB-231 extracts (data not shown) were unable to rule out participation of an unknown, intermediary molecule in the binding of hCLCA2 to \(\beta_4\) integrin, the \(\beta_4\)/hCLCA2 partnership was further examined by Far Western analysis. To accomplish this, adhesion receptor and ligand were first immunopurified from hCLCA2-transfected HEK293 cells and MDA-MB-231 cells, respectively, and their purity was assessed by SDS-PAGE and silver staining. After subjected the SDS-PAGE resolved, blotted \(\beta_4\) integrin to cycles of denaturing and renaturing, blots were probed with Myc-tagged hCLCA2, and hCLCA2-binding to \(\beta_4\) was visualized by anti-Myc antibodies. hCLCA2 strongly and specifically bound to \(\beta_4\) integrin, but not to the control \(\beta_4\) integrin subunits \(\beta_1\) and \(\beta_3\) (Fig. 4A, lanes 1, 3, and 5). Control blots incubated with anti-Myc antibody alone were negative (Fig. 4A, lanes 2, 4, and 6). Western blotting (Fig. 4A, lanes marked W) confirmed positions and equal loading amounts of the three \(\beta_4\) integrins. To further scrutinize the specificity of the \(\beta_4\)/hCLCA2 adhesion, Far Western analyses were also conducted with blot-immobilized hCLCA2 (Fig. 4B, lane W) that was probed with soluble \(\beta_4\) integrin immunopurified from MDA-MB-231 cell extracts. Binding of the \(\beta_4\) integrin to hCLCA2 was confirmed by staining with anti-\(\beta_4\) pAb H101 (Fig. 4B, lane 1), while blots that were stained with antibody alone in the absence of \(\beta_4\) integrin were negative (Fig. 4B, lane 2).
a control substrate, while both anti-β4 antibodies 3E1 and 81435 inhibited laminin adhesion of MDA-MB-231 cells (Fig. 5, A and B). To exclude a possible participation of the α6β1 integrin, expressed in all human breast cancer cell lines used in this study, in the hCLCA2 adhesion, functional anti-β1 antibodies (mAb 2253) were tested and found to be ineffective in blocking the adhesion between MDA-MB-231 cells and hCLCA2 but effective in blocking the adhesion to placenta laminin (Fig. 5, C and D). Western blot (lane 1), Western blot treated with 1% KOH for 2 h at 55 °C. Tyrosine-phosphorylated proteins are visualized by autoradiography. *, Student's t test; p < 0.01 (mean ± S.D. from four experiments).

In a second series of experiments, we show that selective cleavage of the β4 integrin ectodomain with matrixin (30) totally abolishes MDA-MB-231 adhesion to hCLCA2 yet had a negligible effect on the adhesion to placental laminin (Fig. 5D). These adhesion data were supported by Western analyses showing specific cleavage of the 205-kDa β4 protein but not the β1 integrin in matrixin-treated tumor cells. Finally, we examined whether the MDA-MB-231 β4 integrin was activated selectively when tumor cells were plated onto surfaces coated with hCLCA2 (Fig. 5E). Data showed prominent tyrosine phosphorylation of β4 integrin in tumor cells bound to hCLCA2 and to placental laminin (31, 32). In contrast, fibronectin generated only a weak tyrosine phosphorylation reaction, and BSA and poly-L-lysine had no effect (Fig. 5F).

**Lung Metastasis Is Inhibited by β4/hCLCA2 Adhesion-blocking Antibodies**—To test whether the adhesion-inhibitory effects of anti-hCLCA2 and anti-β4 integrin antibodies extended to an inhibition of lung metastasis, we performed lung colony assays in nude mice with the lung metastatic breast cancer cell line MDA-MB-231 in the presence of these antibodies. Prior to conducting these assays, we established that human MDA-MB-231 cells were able to adhere to mCLCA1 (Table I), the mouse counterpart of hCLCA2, and that this adhesion was inhibited with anti-Lu-ECAM-1 mAb 6D3 (22) (cross-reacts with mCLCA1) and anti-β4 integrin mAb 3E1. Anti-β3, mAb 3E1 was preincubated for 30 min and injected together with tumor cells, while mAb 6D3 was injected with tumor cells without preincubation. Control experiments were conducted in the presence of nonimmune mouse IgG. Mice sacrificed 15 weeks later revealed that both antibodies effectively blocked the colonization of the lungs by MDA-MB-231 cells, causing an 84% inhibition of lung metastasis with mAb 6D3 and a 100% inhibition with mAb 3E1 relative to mlgG-treated controls (Table I).

**Effect of in Vivo Selection for Lung Metastatic Efficiency Versus β4 Integrin Transfection**—To test whether in vivo selection for increased lung metastatic performance was associated with increased β4 expression, we compared the α6, β3, and β4 expression patterns of the selected cell line MDA-MB-435L2 (20) with those of the parental MDA-MB-435 cell line and the β4-transfected MDA-MB-435β4 cell line (24). The parental MDA-MB-435 cell line exhibited strong expression of the α6 and β1 integrin subunits but only background levels of the β4 integrin. Accordingly, these tumor cells adhered strongly to both placental and EHS laminins but poorly to hCLCA2 (5 ± 3%; Fig. 6A). The selected MDA-MB-435L2 expressed comparable levels of the α6 and β1 integrin subunits and a modest increase in surface expression of the β4 integrin. In accordance with this expression pattern, MDA-MB-435L2 cells adhered strongly to the two laminins and exhibited an increased adhesion to hCLCA2 (25 ± 3%; Fig. 6A). These data were contrasted with those from the parental MDA-MB-435 cell line that had been transfected with human β4 integrin and then selected for antibiotic resistance and by FACS for efficient stable expression of β4 (24). Transfectant cells expressed significantly higher levels of β4 integrin than MDA-MB-435L2 cells and adhered in higher numbers to hCLCA2 but in similar numbers to the two laminins, since the expression levels for both α6 and β1 remained unchanged. Transfection of MDA-MB-435 cells with tailless β4 integrin (β4Δcyt) underscored requirement of the “complete” β4 integrin subunit in hCLCA2 binding, since adhesion to hCLCA2 did not improve relative to that of parental cells (7 ± 3%; Fig. 6A), albeit the expression level of the truncated β4 was equal to that of wild-type β4 expression in MDA-MB-231 cells. Consistent with published data, adhesion of β4Δcyt-transfected MDA-MB-435 cells to murine EHS laminin was also abolished (24), but not to human placental laminin (33).

As shown in Fig. 3, β4 cell surface expression and hCLCA2 adhesion correlated well with lung colonization of the established cell lines MDA-MB-231, MDA-MB-435L2, and MDA-MB-435. Median and range of the number of lung colonies were 30 (5–100), 5 (0–17), and 0 (0–3), respectively. To our surprise, however, the β4-transfected cell line MDA-MB-435β4 was unable to produce lung colonies following a 15-week incubation period in nude mice, although the parental cell line MDA-MB-435 is known to produce lung metastases after orthotopic tu-
mor growth in mammary fat pads of nude mice and in vivo selection of these cells yielded a cell line with transiently enhanced lung colonization potential (MDA-MB-435L2) (20), which was lost gradually with increasing passage number. To explore whether differences in the quality of the β4 integrin expression on the surface of MDA-MB-231 and MDA-MB-435β4 cancer cells may have accounted for the discrepancy in the metastatic behavior, we examined the association between the β4 integrin and its presumed α6 partner in the two cell lines. Surface-biotinylated cancer cells were first subjected to immunoprecipitation with anti-β4 pAb H101. As expected, the amounts of β4 detected in streptavidin-HRP-probed blots were comparable with that identified by FACS (Fig. 6, A and B). Next, the same tumor cell extracts were subjected to immunoprecipitation with anti-α6 mAb GoH3, and precipitates were analyzed for β4-co-immunoprecipitation. Surprisingly, only the β4 of MDA-MB-231 cells was effectively co-precipitated with α6, while negligible amounts of β4 were co-precipitated from MDA-MB-435β4 cells and none from MDA-MB-435 (Fig. 6B, lanes 1–3). Since β4Δcyt is also effectively co-immunoprecipitated with α6 from MDA-MB-435β4Δcyt extracts (Fig. 6B, lane 4), our data imply that wild-type β4 transfected into MDA-MB-435 cells may interact with an intrinsic protein that affects co-immunoprecipitation with α6 and metastasis but not in vitro adhesion to hCLCA2.

**Transfection of Kirsten-Ras-transformed Balb/3T3 Cells with β4 Promotes Adhesion to hCLCA2 and Lung Colonization**—To determine whether overexpression of the β4 integrin in a cell line that expresses low levels of β4 integrin and, accordingly, has modest, yet consistent, lung metastatic capabilities, we chose a murine over a human tumor model. The former had the significant advantage that the metastatic performance could be tested in a syngeneic rather than a heterogeneic animal. Moreover, the induction time of generating macroscopically detectable lung colonies was only 3–4 weeks in syngeneic animals (13–15) versus a minimum of 15 weeks in a human/mouse model (20). The cell line we selected was the Kirsten-Ras-transformed Balb/3T3 cell line, which expressed low levels of the β4 integrin and consistently produced a moderate number of lung colonies upon tail vein injection. These tumor cells were transfected with β4 integrin cDNA or vector alone, and stable transfectants were selected based on antibiotic resistance. Expression of the β4 integrin was confirmed by FACS and surface biotinylation, both methods indicating a significantly increased surface expression of β4 integrin, which was co-immunoprecipitable with α6 in β4-transfected relative to vector-transfected cells (Fig. 7A). Prior to conducting a lung colony assay, adhesion assays were performed with mCLCA1, the mouse counterpart of hCLCA2. Adhesion of β4-transfected K-Balb cells to mCLCA1 was 66 ± 5% relative to 9 ± 4% for mock-transfected K-Balb cells (Fig. 7B). These adhesion data paralleled the metastatic performance of the two cell lines. The β4-transfected K-Balb cells injected at 2 × 10^5 cells/mouse generated a median number of >100 colonies (69–>100), while the mock-transfected cell line only generated 24 (15–29) lung colonies (Fig. 7B). This difference was also reflected in the average lung weights of the two experimental groups, measuring 0.71 ± 0.22 g in β4 transfectants and 0.45 ± 0.02 g in mock transfectants.

**DISCUSSION**

In this report, we describe a novel adhesion receptor/ligand pair that mediate colonization of the lungs by human breast cancer cells and possibly other cancer cell types. The pair consists of lung endothelial cell hCLCA2 and breast cancer cell β4 integrin. Human CLCA2 is expressed by endothelia lining arterial and venous branches, all presumably derived from the bronchial artery, while prominent expression of the β4 integrin has been associated with the invasive and metastatic phenotypes of breast cancer cells (24, 34–37). The location of MDA-MB-231 breast cancer metastases in mouse lungs is consistent with the vascular expression pattern of the mouse counterpart of hCLCA2 (mCLCA1) (10) as well as the recently established pattern of lung metastases by in situ epifluorescence microscopy (11). The binding interaction between the two lung metastasis-promoting adhesion molecules is documented by co-immunoprecipitation of the adhesion receptor/ligand pair from extracts of hCLCA2-transfected HEK293 monolayers to which MDA-MB-231 cells were bound, by hCLCA2 affinity chromatography, and by adhesion and metastasis inhibition experiments using functional antibodies. Participation of an “intermediary binding molecule” in the β4 integrin/hCLCA2 adhesion was excluded by Far Western analyses, using blotted immunopurified β4 or Myc-hCLCA2 and the corresponding purified adhesion partner as probe. Strong and specific binding between blotted β4 integrin and Myc-hCLCA2 indicated that the β4 integrin was able to recognize its endothelial cell receptor even after undergoing a vigorous denaturing/renaturing treatment, suggesting that the usually required interaction between α and β integrin subunits (38, 39) and/or other interacting cell surface and intracellular molecules (40, 41) is not a mandate for the β4 integrin/hCLCA2 adhesion function in vitro. This behavior is similar to that recently reported for the β4 integrin/Shc adhesion, using blotted β4 integrin under denaturing/renaturing Far Western conditions (42).

Molecular cloning of hCLCA2 from a lung cDNA library and biochemical and functional characterization of hCLCA2 protein have shown that the molecule shares many of the characteristics of the other CLCA family members (18). The adhesion function of CLCA channel proteins is perplexing but not without precedent. Studies involving the cystic fibrosis transmembrane conductance regulator have shown that this chloride

| Cell line | Treatment | Adhesion to mCLCA1 | Mice with lung metastases | Lung metastasis median (range) |
|-----------|-----------|--------------------|--------------------------|-------------------------------|
| MDA-MB-231 | mIgG ^a | 65 ± 5 | 9/9 | >100 (32–100) |
| MDA-MB-231 | mAb 6D3 ^d | 9 ± 2 | 2/5 | 16 (0–38) |
| MDA-MB-231 | mAb 3E1 ^c | 8 ± 3 | 1/5 | 0 (0–1) |

* mCLCA1 was immunopurified from mCLCA1-transfected HEK293 cells using anti-Lu-ECAM-1 mAb 6D3.
* Nude mice were injected via the lateral tail vein with 5 × 10^6 tumor cells/mouse/0.3 ml of DMEM in the presence of mouse nonimmune IgG, anti-Lu-ECAM-1 mAb 6D3, or anti-β4 Mab 3E1, all at 200 μg/mouse. Animals were sacrificed 15 weeks after tumor cell injection, and the number of lung colonies was counted under a dissecting microscope. Two mice in the mouse IgG-treated group died due to metastatic disease before termination of the experiment, the first at 8 weeks, the second at 12 weeks after injection of tumor cells.
* Mouse nonimmune IgG.
* Mouse anti-Lu-ECAM-1 mAb 6D3 cross-reacts with mCLCA1.
* Mouse anti-human β4 mAb 3E1.

**TABLE I**

Inhibition of lung colonization by the human breast cancer cell line MDA-MB-231 with anti-mCLCA1 and anti-β4 integrin antibodies

Average lung weights of the two experimental groups, measured at the termination of the experiment, the first at 8 weeks, the second at 12 weeks after injection of tumor cells.
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channel protein is also a cellular adhesion receptor for *Pseudomonas aeruginosa* (16) and *Salmonella typhimurium* (17). In cancer metastasis, a novel concept is the possible involvement of a CLCA-mediated Cl⁻ conductance in cancer cell extravasation by induction of apoptosis in the endothelium of the target organ. Support for such involvement came from recent observations that breast cancer cells seeded atop a monolayer of hCLCA2-expressing HUVEC apparently induce apoptosis in apposed endothelial cells (43). Endothelial apoptosis appears to involve expression and activation of chloride channels (44, 45), leading to intracellular acidification and, in turn, activation of endonucleases and chromatin digestion (44). The advantage of a selective induction of apoptosis in those endothelial cells to which tumor cells are bound is obvious, since reduction of the endothelial cell by apoptotic vesiculation may create an avenue for invasion of perivascular tissues by tumor cells. The notion that these events are initiated by β₄/hCLCA2 adhesion is supported by our preliminary observation that endothelial cells incubated with immunopurified β₄ integrin rapidly undergo apoptosis. The apoptotic index of β₄-treated endothelial cells was 26%, relative to 4% in untreated endothelial cells.

The β₄ integrin has previously been linked to metastatic disease (13, 46–49) and is confirmed here as a lung metastasis-associated gene in breast cancer. Consistent with the involvement of multiple genes in metastasis (1–8), the β₄ integrin, like other metastasis-associated genes including MMP-2, CD44, α₃β₁ integrin, and α₆ integrin (50), is by itself incapable of conferring mastery of the complex, multistep cascade of metastasis. This is exemplified by the MDA-MB-468 breast cancer cell line, which expresses the α₃β₁ integrin at relatively high levels and, accordingly, is able to adhere to hCLCA2 in vitro but fails to produce metastases upon tail vein inoculation (Fig. 3). When this cell line is compared with a metastatically competent, β₄-expressing cell line such as MDA-MB-231, the former expresses a phenotype that is comparable with the spontaneously immortalized, nontumorigenic β₄ integrin-expressing breast epithelial cell line MFC-10A (51), while the latter expresses an aggressive, invasive, and metastatic phenotype (20). This difference is manifested by the formation of a contact-inhibited, cobblestone-like monolayer in vitro and adenoma-tous growth in vivo by MDA-MB-468 cells but anaplastic, criss-crossed, and multilayered growth in vitro and the formation of invasive and metastatic tumors in vivo by MDA-MB-231 cells (20). Genotype analyses indicate that the latter cell line expresses an array of gene abnormalities that have been associated with metastasis such as overexpression of c-erbB-2 (52), MT1-MMP (53, 54), MT1-MMP (55), vimentin (56), α₆ integrin (57), and VEGF (58) and down-regulation or loss of E-cadherin (59), nm23-H1 (60), and MUC1 (61), while most of these genes are expressed at normal or near normal levels in MDA-MB-468 cells (50, 53, 55, 56, 62). These data imply that β₄ integrin expression leads to lung metastasis only in those cancer cells possessing a genotype that is otherwise compatible with metastasis. A similar scenario as described for the β₄ integrin has also been reported for other metastasis-associated proteins including MMP-2, CD44, α₃β₁ integrin, α₆ integrin, Rho

![Diagram A](imageA.png)

**Fig. 6.** Effect of in vivo selection versus β₄-transfection on hCLCA2 adhesion of human breast cancer cells. A, MDA-MB-231, MDA-MB-435, MDA-MB-435L2, MDA-MB-435β₄, and MDA-MB-435β₄Δcyt were analyzed for α₆, β₁, and β₄ expression by FACS, and the expression patterns were contrasted with adhesion to hCLCA2 and EHS (bars 1) and placental (bars 2) laminins (coated at 3 μg/ml, 20 μg/ml, and 7.5 μg/ml, respectively). In vivo selection of MDA-MB-435 for enhanced lung colonization (MDA-MB-435L2) (20) and stable β₄ transfection of MDA-MB-435 (MDA-MB-435β₄) (24) increases β₄ expression and adhesion to hCLCA2, while adhesion to the two laminins remains unchanged. Stable transfection of MDA-MB-435 with tailless β₄ (MDA-MB-435β₄Δcyt) (24) increases β₄ expression as detected with anti-β₄ mAb 3E1 (directed against the extracellular domain of the β₄ integrin), but β₄Δcyt-transfectants adhere in similarly poor numbers to hCLCA2 as parental cells. Adhesion of MDA-MB-435β₄Δcyt to EHS laminin (bar 1) is abolished, but not adhesion to placental laminin (bar 2).

![Diagram B](imageB.png)

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2. B. extracts from surface-biotinylated MDA-MB-231, MDA-MB-435, MDA-MB-435Δcyt, and MDA-MB-435 cells were subjected to immunoprecipitation with antihuman β₄ mAb H101 (upper panel) or anti-α₆ mAb GoH3 (lower panel). Immunoprecipitates were resolved by SDS-PAGE (6%) and blotted to nitrocellulose and then probed with Streptavidin-HRP. Lane 1, MDA-MB-231; lane 2, MDA-MB-435β₄; lane 3, MDA-MB-435; lane 4, MDA-MB-435β₄Δcyt.
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While an early stage of tumor progression (benign, well differentiated tumor) may have accounted for lack of metastasis in β2-expressing MDA-MB-468 cells, an altered modulation of the β3 integrin by lateral associations with other membrane (and/or cytoplasmic) proteins (reviewed in Refs. 40 and 41) in β2-transfected MDA-MB-435 cells versus MDA-MB-231 cells may have been responsible for the observed discrepancy in the metastatic behavior of the two cell lines. Although we have as yet no evidence of such a differential lateral association of the β2 integrin in the two cell lines, we show here that anti-α6 antibodies fail to co-immunoprecipitate the β2 integrin from β2-transfected MDA-MB-435 cell extracts but effectively do so from MDA-MB-231 cell extracts (as well as from extracts of immortalized normal breast epithelial cells MCF-10A and benign breast tumor cells MDA-MB-468; data not shown). This differential partitioning of the β2 integrin in MDA-MB-231 and MDA-MB-435β2 cells, which incidentally express virtually identical amounts of α6 and β2 integrins, and only a slightly reduced level of β3 in the MDA-MB-435β2 cell line is difficult to explain. However, it is possible that under our extraction conditions (1% Triton X-100), lateral association of the β3 integrin with an as-yet undetermined membrane or cytoplasmic protein in β4-transfected MDA-MB-435 cells may have weakened the binding interaction between the α6 and β2 integrin subunits, resulting not only in failure of the two integrin subunits to co-immunoprecipitate but also in inability to metastasize. Control co-immunoprecipitation of α6 and β2Δεyt suggests that such association is mediated by the cytoplasmic tail of the β4 integrin. Alternatively, our data may have been affected by the unlikely event that the β3 integrin subunit may associate with an as yet unidentified a chain to permit cell surface expression in β4-transfected MDA-MB-435 tumor cells.

Several considerations have important experimental consequences for metastasis research, since transfection of a gene suspected to play a primary role in metastasis into a tumor cell line that is nonmetastatic may not yield the expected result, since the introduced gene, even if it appropriately associates with other membrane proteins to achieve proper function, may not be sufficient in endowing tumors with mastery over the complete metastatic cascade. Therefore, we have relied in our transfection studies on a cell line that has a low lung metastatic potential and, thus, expresses a gene array that is conducive to lung metastasis including a low level of β4 integrin expression. When the β4 integrin is overexpressed in these cells, the number of lung colonies generated from intravenously injected tumor cells increases proportional to the level of α6 co-immunoprecipitable β4 integrin. Consistent with involvement of the β4 integrin gene in metastasis, blockade of the β4/hCLCA2 adhesion abrogates metastasis. Similar effects are achieved by blocking other metastasis-associated genes each facilitating a different step in the metastatic cascade, e.g., RhoC, metalloproteinases, heparinase, angiogenic factors, dipeptidyl peptidase IV (68–75).

In conclusion, we have provided molecular evidence in support of the observed link between β4 expression and malignant progression (reviewed in Ref. 21), ascribing a key role to the adhesion mechanism between β4 integrin and vascular endothelial cell hCLCA2 in lung metastasis. Although only a few examples of integrin involvement in cell-to-cell adhesion are known (e.g. leukocyte integrins α1β2 and α2β1 bind to endothelial cell ICAM-1, integrins α4β1 and α4β3 to VCAM-1, and αEβ7 to E-cadherin (reviewed in Ref. 39)), we have identified and cloned for the first time a β4 integrin-binding protein that is an integral membrane protein and that is entirely new as an integrin-binding partner (15, 18). Our discovery that hCLCA2 has an important function in heterotypic cell-to-cell adhesion in addition to that in Ca2+-sensitive chloride conductance extends the basic knowledge about this protein and indicates that ion channels can have multiple, seemingly unrelated functions (16–18, 44, 76).

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