The Interacting Binding Domains of the $\beta_4$ Integrin and Calcium-activated Chloride Channels (CLCAs) in Metastasis*

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CLCA (chloride channel, calcium-activated) proteins are novel pulmonary vascular addressins for blood-borne, lung-metastatic cancer cells. They facilitate vascular arrest of cancer cells via adhesion to $\beta_4$ integrin and promote early, intravascular, metastatic growth. Here we identify the interacting binding domains of endothelial CLCA proteins (e.g. hCLCA2, mCLCA5, mCLCA1, and bCLCA2) and $\beta_4$ integrin. Endothelial CLCAs share a common $\beta_4$-binding motif ($\beta_4$M) in their 90- and 35-kDa subunits of the sequence F(S/N)R(I/L/V)(S/T)S, which is located in the second extracellular domain of the 90-kDa CLCA and near the N terminus of the 35-kDa CLCA, respectively. Using enzyme-linked immunosorbent, pull-down, and adhesion assays, we confirmed the interaction with the 35-kDa CLCA, respectively. Using enzyme-linked immunosorbent, pull-down, and adhesion assays, we showed that glutathione S-transferase fusion proteins of $\beta_4$BM from the 90- and 35-kDa CLCA subunits bind to the $\beta_4$ integrin in a metal ion-dependent manner. Fusion proteins from fibronectin and the integrins $\alpha_1$ and $\alpha_2$ served as negative controls. $\beta_4$BM fusion proteins competitively blocked the $\beta_4$/CLCA adhesion and prevented lung colonization of MDA-MB-231 breast cancer cells. A disrupted $\beta_4$BM in hCLCA1, which is not expressed in endothelia, failed to interact with $\beta_4$ integrin. The corresponding CLCA-binding domain of the $\beta_4$ integrin is localized to the specific determining loop (SDL). Again enzyme-linked immunosorbent, pull-down, and adhesion assays were used to confirm the interaction with CLCA proteins using a glutathione S-transferase fusion protein representing the C-terminal two-thirds of $\beta_4$SDL (amino acids 184-205). A chimeric $\beta_4$ integrin in which the indicated SDL sequence had been replaced with the corresponding sequence from the $\beta_4$ integrin failed to bind hCLCA2. The dominance of the CLCA ligand in $\beta_4$ activation and outside-in signaling is discussed in reference to our previous report that $\beta_4$/CLCA ligation elicits selective signaling via focal adhesion kinase to promote metastatic growth.

Distinct vascular addressins, so-called addressins, have been implicated in playing a major role in the colonization of select organs by blood-borne cancer cells (for reviews, see Refs. 1–3). A recently discovered pulmonary vascular addressin is the multifunctional hCLCA2 molecule (4), a member of a putative Ca$^{2+}$-activated chloride channel family (for reviews, see Refs. 5 and 6). This molecule has been identified by a monoclonal antibody selected for its ability to block the adhesion between integrin metastatic cancer cell and lung matrix-modulated bovine aortic endothelial cells expressing the functional counterpart of hCLCA2, i.e. bCLCA2 (previously named Lu-ECAM-1) (7–9). Human CLCA2 is expressed on the luminal surface of endothelial cells lining distinct pulmonary vascular branches (e.g. arterioles and postcapillary venules) (4, 7, 9) where it mediates vascular arrest of lung metastatically competent cancer cells and promotes early intravascular tumor colony growth (4, 10). The surface molecule by which lung-metastatic cancer cells recognize hCLCA2 (and its murine equivalents mCLCA1 and mCLCA5) is the $\beta_4$ integrin, establishing for the first time a cell-cell adhesion function for this integrin that involves an entirely new adhesion partner (4). Accordingly, all lung-metastatic human and mouse cancer cell lines available to us (e.g. MDA-MB-231, 4T1, CSML-100, B16-F10, and LLC) prominently expressed this integrin on their surfaces, while tumor cells that were unable to colonize the lungs upon tail vein injection of syngeneic or xenogeneic immunocompromised mice (e.g. MCF7, T47D, 67NR, and CSML-0) did not express $\beta_4$ integrin and were unable to bind to endothelial CLCAs.3 Adhesion to endothelial CLCAs is augmented by an increased surface expression of the $\alpha_4/\beta_4$ integrin in cancer cells selected in vivo for enhanced lung colonization (4) but abolished by the specific cleavage of the $\beta_4$ integrin with matrixin (4). $\beta_4$/hCLCA2 adhesion-blocking antibodies directed against either of the two interacting adhesion molecules inhibit lung colonization (4, 7, 8), while overexpression of the $\beta_4$ integrin in Kirsten murine sarcoma virus-transformed Balb/c/3T3 tumor cells significantly increases the lung metastatic performance (4). Association of the $\beta_4$ integrin with a metastatic cancer phenotype was further underscored by cDNA microarray analyses in a murine pulmonary metastasis model (11). The $\beta_4$ integrin showed a 5.6-fold overexpression in a tumor cell line that was selected for increased lung metastatic performance and that had a spontaneous lung metastatic rate of 93.3%.

3 The abbreviations used are: CLC, chloride channel, calcium-activated (prefix: h, human; m, mouse; b, bovine); BSA, bovine serum albumin; $\beta_4$BM, $\beta_4$-binding motif; $\beta_4$BMcl/CLCA50, $\beta_4$BM of the 90-kDa CLCA; $\beta_4$BMcl/CLCA55, $\beta_4$BM of the 35-kDa CLCA; $\beta_4$BMhCLCA2(90), $\beta_4$BM of the 90-kDa hCLCA2; $\beta_4$BMhCLCA2(35), $\beta_4$BM of the 35-kDa hCLCA2; FACS, fluorescence-activated cell sorter; FN, fibronectin.

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versus the parental cell line that exhibited only a 33.3% spontaneous lung metastatic rate (11).

Despite intense scrutiny (4, 10), the role of β4/CLCA in metastasis of human cancers has been met with skepticism. This skepticism is fueled by the "unusual" binding interaction of the β4 integrin involving a putative chloride channel protein to achieve pulmonary vascular arrest of blood-borne cancer cells and to promote early metastatic growth and by the notion that β4 is perceived as a "non-obligatory" molecule in metastasis. However, integrins are rapidly emerging as important regulators of ion channels (for a review, see Ref. 12). In such cases, such regulation involves the direct interaction between integrin and channel protein as exemplified by the binding of the β1 integrin to the RGD domain of inwardly rectifying K+ channels (GIRK1 and GIRK2) (13). Moreover increasing numbers of reports identify plural roles for ion channels that reach beyond their channel function (for reviews, see Refs. 5, 6, and 12). An example of this functional diversity is the Na+/-H+ exchanger. In addition to regulating intracellular pH homeostasis and cell volume, the Na+/-H+ exchanger NHE1 acting independently of its transporter function is critical for the dynamic reorganization of the cortical cytoskeleton in response to extracellular signals, regulating the assembly of focal adhesions, the formation of actin stress fibers, and the cell shape and serving as an anchor of the actin-based cytoskeleton to the plasma membrane (for a review, see Ref. 14). Such a multifunctional role has also been reported for the cystic fibrosis transmembrane conductance regulator (15, 16) and is rapidly emerging for CLCA family members (17, 18). In addition to their putative Ca2+--activated chloride secretion (19), CLCA molecules also serve as tumor suppressor genes (20–22) and as potent adhesion and signaling molecules in metastasis (3–10).

A similar controversy surrounds the status of the β4 integrin as an obligatory molecule in cancer metastasis, albeit the β4 integrin has been associated with malignant progression and metastasis in several human cancers (for a review, see Ref. 23). In breast cancer, the association between β4 integrin and metastasis has been studied most thoroughly, and the reported data are consistent with a mechanistic role of the β4 integrin in the complex process of metastasis. These data include the following. (i) The β4 integrin is associated consistently with breast cancers originating from basal cells, which are well known for their aggressive behavior including metastasis, but rarely with the more "benign" tumors of luminal origin (24). (ii) Individuals that express both β4 and laminin-5 in their primary tumors have the poorest prognosis among breast cancer patients (25). (iii) More than 50% of dormant cancer cells isolated from bone marrow express the α6 and/or β4 integrin subunits (26). (iv) Lymph node metastases originating from β4-negative primary breast cancers often stain positive for the β4 integrin (27). (v) The MDA-MB-231 human breast cancer cell line known for its invasive and metastatic behavior prominently expresses β4 integrin (4). Other cancers also provide support for a link between β4 and metastasis including the expression of β4 at the invasive front of gastric cancers (28), the de novo expression and association with lymph node metastasis of β4 integrin in papillary thyroid cancers (29), and the co-expression of β4 and its ligand laminin-5 in colon cancers (30).

Here we identify the interacting binding domains of the β4 integrin and CLCA-type vascular addressins. We show that β4 recognizes a common binding motif that is present in both the 90- and 35-kDa subunits of CLCA addressins including hCLCA2, mCLCA1, mCLCA5, and bCLCA2 (Lu-ECAM-1) (for cloning and partial structural and functional characterization of these molecules, see Refs. 5, 6, and 17–19). The β4 recognition site is located within the specific determining loop (SDL) of the I-domain of the β4 integrin subunit (31–33). A dramatic inhibition of metastasis of MDA-MB-231 breast cancer cells by a fusion protein containing the β4-binding motif of hCLCA2 further advances the role of the β4/CLCA2 adhesion in pulmonary metastasis.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents

Antibodies against the β4 integrin ectodomain were mouse α-human monoclonal antibody (mAb) 3E1 (from Dr. E. Engvall, The Burnham Institute, La Jolla, CA), rabbit α-human polyclonal antibody (pAb) H-101 (Santa Cruz Biotechnology, Santa Cruz, CA), and rat α-mouse mAb346–11A (BD Pharmingen), and against the β4 cytoplasmic domain rabbit α-human pAb19292 (Chemicon, Temecula, CA). Mouse α-human β4 integrin mAb (clone 18) was from BD Pharmingen, and α-human β4 mAb65E11 was from Chemicon. Mouse mAb93E10 was against the Myc protein tag (Calbiochem), mouse mAbF-7 was against the HA tag (Santa Cruz Biotechnology), and rabbit pAb(B-14) and mouse mAb65Z-5 were against glutathione S-transferase (GST) (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated goat α-mouse, α-rat, and α-rabbit IgG antibodies were from Jackson Immunoresearch Laboratories (West Grove, PA). Anti-bCLCA2 (Lu-ECAM-1) pAb6303 was produced in BALB/c mice (7, 35) and selected for blocking the adhesion of lung-metastatic cancer cells (e.g., R3230AC-MET; and B16-F10) to bCLCA2-expressing bovine aortic endothelial cells (7, 8). The antibody cross-reacts with mCLCA1 (7–9). Rat plasma fibronectin was from Invitrogen. Purified β1 (α6β1) and β3 (α6β3) integrins were purchased from Chemicon. Human placental and EHS laminins as well as all other reagents were from Sigma. Genemed Synthesis, Inc. (South San Francisco, CA) prepared synthetic peptides of β4(184–203) and β3(207–213).

Plasmid Constructs

GST-bCLCA2 Fragments—To generate GST fusion proteins from bCLCA2 fragments that together span the length of the 90-kDa bCLCA2 proteins, bCLCA2 cDNA was cut with unique restriction enzymes (36): (i) GST-HX, HindIII and Xhol; (ii) GST-HV, HindIII and PvuII; (iii) GST-HP, HindIII and PstI; (iv) GST-NE, Ndel and EcoRV; (v) GST-VX, PvuII and Xhol; (vi) GST-PX, PstI and Xhol; and (vii) GST-BX, BstXI and Xhol. Blunt-ended restriction fragments were electrophoretically purified and ligated at either SmaI or blunt EcoRI sites to linearized pGEX-2T vector (Amersham Biosciences).

GST-β4BMCLCA290 (β4-binding Motif of hCLCA2)—PROTOMAT was used to search for conserved motifs in the 90- and 35-kDa subunits of hCLCA2 as described previously (37, 38). Identified sequences AFSR-RISSTGTG, located at amino acids 479–488 of the 90-kDa hCLCA2 subunit (β4BMHCLCA290), and GFSRVSNGGS, located at amino acids 730–739 of the 35-kDa hCLCA2 subunit (β4BMCLCA235) (39), both tagged at their C termini with HA, were generated by primer extension with Taq polymerase and inserted into the EcoRI and HindIII sites of pGEX-KG. β4(184–203) and β3(197–219) GST Fusion Constructs—The SDL sequences of the β4 integrin subunit (amino acids 184–203) and the β3 integrin subunit (amino acids 197–219) (31), tagged at the C terminus with HA, were generated by PCR and inserted into the EcoRI and HindIII sites of pGEX-KG. β4(184–203) and β3(197–219) GST Fusion Constructs—The SDL sequences of the β4 integrin subunit (amino acids 184–203) and the β3 integrin subunit (amino acids 197–219) (31), tagged at the C terminus with HA, were generated by PCR and inserted into the EcoRI and HindIII sites of pGEX-KG.

β4(184–203) Chimeric Integrin—Amino acids 184–203 of the SDL of the β4 integrin domain were substituted for the corresponding sequence of the β3 integrin (amino acids 197–219) (31) by PCR using the unique restriction sites Ndel in the RecCMV vector backbone and BspMI in the β4 cDNA with the high fidelity DNA polymerase Herculease (Stratagene, La Jolla, CA). The sequence of the chimeric integrin was verified.

Cell Lines and Transfections

The MDA-MB-231L breast cancer cell line was from Dr. J. A. Price (The University of Texas M. D. Anderson Cancer Center, Houston, TX), 4T1 was from Dr. F. R. Miller (Karmanos Cancer Institute, Detroit, MI), and human embryo kidney (HEK) 293 cells were from ATCC (Manassas, VA). All cell lines were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum. HEK293 cells were transiently transfected with Myc-tagged bCLCA2, α6, β4, or vector alone using LipofectAMINE Plus as described by the manufacturer (Invitrogen). Transfection rates assessed by green fluorescent protein co-transfection were 40–50%. Cells were used in the various assays 48 h after transfection unless otherwise stated.
β4/CLCA-binding Domains and Metastasis

Purification of GST Fusion Proteins

GST-hCLCA2 fragments, GST-β4(184–203)-HA, GST-β4(197–219)-HA, GST-β4(BM_hCLCA290)-HA, and GST-β4(BM_hCLCA290)-HA fusion proteins were purified according to the manufacturer’s instructions (New England Biolabs, Beverly, MA) (37). Briefly, 2 liters of Escherichia coli culture were centrifuged after a 2-h isopropyl-β-D-thiogalactoside induction (0.3 mM) and passed through a 10-cm column. Columns were washed with 10 volumes of CB and eluted with 10 mM glutathione in CB. The purity of the eluates was evaluated by Coomassie Blue staining of SDS-polyacrylamide gels and Western blotting with α-GST and/or α-HA mAbs. Protein concentrations were measured by the Bradford method (Bio-Rad). Alternatively, HA-tagged GST fusion proteins were purified with α-HA mAb-conjugated protein G-agarose beads. Control GST fusion proteins were P14 and PEDA derived from fibronectin (FN) III (14) (amino acids 1774–1791), respectively, as described previously (37).

Purification of hCLCA2 and β4 Integrin

Myc-tagged hCLCA2 was immunopurified from transfected HEK293 cells 48 h after transfection, and the β4 integrin was immunopurified from MDA-MB-231L cells as described previously (4, 10). Cells were lysed in Tris-buffered saline (TBS) lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.01% aprotinin, 1 mM benzamidine, and 1% octyl-glucoside (OG)) for 1 h at 4 °C, and lysates were centrifuged at 15,000 rpm (4 °C) for 20 min at 4 °C. Supernatants were diluted 1:3 in CB and passed through a 10-cm column. Columns were washed with 10 volumes of CB and eluted with 10 mM glutathione in CB. The purity of the eluates was evaluated by Coomassie Blue staining of SDS-polyacrylamide gels and Western blotting with α-GST and/or α-HA mAbs. Protein concentrations were measured by the Bradford method (Bio-Rad). Alternatively, HA-tagged GST fusion proteins were purified with α-HA mAb-conjugated protein G-agarose beads. Control GST fusion proteins were P14 and PEDA derived from fibronectin (FN) III (14) (amino acids 1774–1791), respectively, as described previously (37).

Enzyme-linked Immunosorbent Assay (ELISA)

An ELISA was used to measure the binding of GST-β4(BM_hCLCA290)-HA to the β4 integrins β1, β2, and β3. Wells of microtitration plates were coated with integrins, the control substrate fibronectin, or the blocking substrate Integrin BSA (all at 10 μg/ml) overnight at 4 °C. After blocking with 3% skim milk (for 2 h at room temperature) and several washes with phosphate-buffered saline, GST-β4(BM_hCLCA290)-HA was added at various concentrations and incubated for 1 h at room temperature. Bound GST-β4(BM_hCLCA290)-HA was determined colorimetrically using α-HA mAbP7, horseradish peroxidase-conjugated goat α-mouse IgG antibody, and the horseradish peroxidase substrate o-phenylenediamine. For ELISA binding studies between GST-β4(BM_hCLCA290)-HA and GST-β4-HA, GST-β4-HA was biotinylated, and GST-β4(BM_hCLCA290)-HA-bound GST-β4-HA was detected by streptavidin-horseradish peroxidase chromogen as described previously (37).

Pull-down Assay

Pull-down assays were performed essentially as described by Puzon-McLaughlin and Takada (40). In brief, immunopurified full-length β4 integrin, GST-β4(184–203)-HA, and GST-β4(197–219) were immobilized on protein G-Sepharose beads conjugated with α-β4 mAb1922 or glutathione-conjugated agarose beads, respectively (4). Beads with bound β4 or GST fusion proteins were washed extensively with lysis buffer containing 1 mM MnCl2 at a final detergent concentration of 0.5% OG (4). Conversely, beads coated with α-Myc mAb1921 and bound GST-β4(BM_hCLCA290)-HA or GST-β4(BM_hCLCA290)-HA were used to pull down the β4 integrin from lysates of HEK293 cells co-transfected with the α-Myc and β4 integrin subunits or surface-biotinylated MDA-MB-231 cells (both cell lysates were prepared in the same 0.5% OG-containing buffer as above). For detection of bound protein, beads were washed extensively with washing buffer and boiled in SDS sample buffer, and bound material was detected by SDS-PAGE and Western blotting.

FACS Analyses and Adhesion and Lung Colony Assays

FACS analyses, adhesion assays, and lung colony assays were performed as described previously in detail by our laboratory (4, 7–9, 37). Tumor cell binding of GST-CLCA(90)-β4 BM was determined by incubating MDA-MB-231 cells (or 4T1) in end-over-end culture with GST-β4(BM_hCLCA290)-HA for 20 min at room temperature in Dulbecco’s modified Eagle’s medium containing 1% BSA. After washing, tumor cells were stained with α-HA mAb (or mlgG as control) and subjected to FACS analysis. β4/CLCA adhesion inhibition experiments were conducted with both hCLCA2- and β4-derived polypeptides. Human CLCA2-derived polypeptides (GST-β4(BM_hCLCA290)-HA and GST-β4(BM_hCLCA290)-HA) or control polypeptides (GST-P14-HA and GST-PEDA-HA) were preincubated with tumor cells for 20 min, and tumor cell adhesion to hCLCA2-coated dishes was performed in the presence or absence of polypeptide. β4/CLCA-derived polypeptides (β4(BM_hCLCA290)-HA, control) (37)). Tumor cells (1 × 105 cells/mouse) were incubated with hCLCA2 polypeptide (for 20 min at 37 °C) prior to intravenous injection together with polypeptide (0.5 mg/mouse). Female SCID/beige (MDA-MB-231) and BALB/c (4T1) 4-week-old mice were used (eight mice/experimental condition).

RESULTS

CLCA Proteins Contain Binding Domains for β4-expressing Tumor Cells—To identify the CLCA sequence that is responsible for the β4/CLCA-mediated adhesion of lung-metastatic human (MDA-MB-231) and mouse (4T1) breast cancer cells (4), we first examined the binding behavior of the β4/CLCA adhesion-blocking mAb6D3 (7–9) using a series of polypeptides encompassing the length of the 90-kDa subunit of the CLCA prototype bCLCA2 (Lu-ECAM-1) (36) (Fig. 1A). Polypeptides were prepared as GST fusion proteins in E. coli, and the fusion proteins were biotinylated on a glutathione column (data not shown) was not restricted to the 90-kDa protein but was also mediated by the 35-kDa subunit of bCLCA2 (Fig. 1C). The shortest bCLCA2 fragment recognized by mAb6D3 was GST-BX, localizing the antibody-binding domain to the second extracellular domain of bCLCA2 (36). Next we examined whether the same bCLCA2 fragments that supported binding of the β4/CLCA adhesion-blocking antibody also harbor the binding domain for β4-expressing MDA-MB-231 cells. To do so, we coated wells of microtitration plates with GST-BX, GST-PX, GST-BX, and GST-BX but not GST-HV, GST-HP, and GST-NE (Fig. 1C). The shortest bCLCA2 fragment recognized by mAb6D3 was GST-BX, localizing the antibody-binding domain to the second extracellular domain of bCLCA2 (36). Next we examined whether the same bCLCA2 fragments that supported binding of the β4/CLCA adhesion-blocking antibody also harbor the binding domain for β4-expressing MDA-MB-231 cells. To do so, we coated wells of microtitration plates with GST-BX, GST-PX, GST-NE, and GST-BX and seeded coated wells with MDA-MB-231 cells. Analogous to the binding characteristics of the adhesion-blocking antibody, MDA-MB-231 bound to GST-PX and GST-BX but not to GST-HV and GST-NE (Fig. 1D). Binding of MDA-MB-231 to GST-PX and GST-BX was equally as strong as the adhesion to the 90-kDa natural processing product of bCLCA2. Unexpectedly, adhesion of MDA-MB-231 as well as mAb6D3 (data not shown) was not restricted to the 90-kDa protein but was also mediated by the 35-kDa subunit of bCLCA2 (Fig. 1D). Curious whether this adhesion behavior was specific for bCLCA2 or extended to other CLCA proteins, we tested the adhesion of the 90- and 35-kDa subunits of hCLCA2 for adhesion to MDA-MB-231 cells. Both products bound the cancer cells (Fig. 1D) but not mAb6D3, which is consistent with its specificity for bCLCA2 and mCLCA1 (7–9). Identical binding data were also obtained for other lung-metastatic cancer cell lines including 4T1, B16-F10, and CSM/L-100 using the 90- and 35-kDa subunits of either bCLCA2 (Lu-ECAM-1) or hCLCA2 in...
static adhesion assays (data not shown).

The β4 Integrin-binding Motif of CLCAs—The above adhesion data suggest that the 90- and 35-kDa subunits of hCLCA2 (and hCLCA2) harbor a common binding motif for the β4 integrin. To test this hypothesis, we used the PROTOTMAT motif search program (37). The sequences AFSLRSSGTG in the 90-kDa and GFSRVSSGGS in the 35-kDa subunits of hCLCA2 were identified as the single, common motif (Fig. 2A). One fragment was located at amino acid residues 479–488 of hCLCA2 (39), placing it within the GST-BX fragment of hCLCA2 (AFS-RISSRSG) recognized above as the shortest bCLCA2 fragment to mediate binding of lung-metastatic cancer cells (Fig. 1D). The second fragment is located at amino acids 740–749 of hCLCA2 located near the N terminus of the 35-kDa hCLCA2 (39). To prove that this motif is binding MDA-MB-231 cells via the β4 integrin, we generated a HA-tagged GST fusion protein of the 90-kDa β4-binding motif of hCLCA2 (termed β4BMhCLCA2(90)) and tested its binding ability for the β4 integrin by ELISA. β4BMhCLCA2(90) bound to immobilized β4 integrin but not to β1 integrin, β3 integrin, fibronectin, or BSA. The same result was achieved in pull-down assays (Fig. 2B). Immobilized on glutathione-conjugated agarose beads, β4BMhCLCA2(90) pulled down β4 but not β1 and β3 from solutes (Fig. 2C), and, in reverse, β4 integrin but not β1 integrin, immobilized by anti-integrin antibodies on protein G-conjugated agarose beads, pulled down soluble β4BMhCLCA2(90) (Fig. 2D). Identical results were obtained with the 35-kDa β4-binding motif of hCLCA2 (termed β4BMhCLCA2(35)) (data not shown). To test whether the addition of β4BMhCLCA2(90) to β4 integrin was dose-dependent, we coated wells of microtiter plates with a standard concentration of immunopurified β4 integrin (10 μg/ml) and determined the adhesion of increasing concentrations of β4BMhCLCA2(90) by ELISA. Our data showed a linear increase in adhesion of β4BMhCLCA2(90) from 1 to 1,000 ng (Fig. 3A). This adhesion was dependent upon the presence of Mn2+, but not Mg2+ or Ca2+, in the assay medium (Fig. 3B).

β4BMhCLCA2(90) Binds to Lung-metastatic Cancer Cells and Inhibits Adhesion to hCLCA2—To establish hCLCA2-β4BM as a β4/hCLCA2 adhesion-blocking polypeptide, we first examined the ability of the polypeptide to bind to the surface of lung-metastatic MDA-MB-231 cancer cells. β4BMhCLCA2(90) was incubated with tumor cells for 20 min at room temperature, and bound polypeptide was detected by α-GST antibody and quantified by FACS analysis. Data showed strong binding of β4BMhCLCA2(90) to tumor cell surfaces, while the control polypeptide P14 (37) did not adhere (Fig. 4A, a). The FACS histogram generated by bound β4BMhCLCA2(90) was similar to that generated by α-β4 antibody staining of MDA-MB-231 cells (Fig. 4A, b), concurring with the interaction between β4BMhCLCA2(90) and the β4 integrin. In accordance, β4BMhCLCA2(90) as well as β4BMhCLCA2(35) immobilized on the well bottom of microtiter plates supported adhesion of β4BMhCLCA2(90)-treated mouse had two small lung colonies 4BMhCLCA2(90)-treated animal had two small lung colonies. The FACS histogram of β4BMhCLCA2(90)-treated mouse had two small lung colonies. The FACS histogram generated by bound β4BMhCLCA2(90) was similar to that generated by α-β4 antibody staining of MDA-MB-231 cells (Fig. 4A, b), concurring with the interaction between β4BMhCLCA2(90) and the β4 integrin. In accordance, β4BMhCLCA2(90) as well as β4BMhCLCA2(35) immobilized on the well bottom of microtiter plates supported adhesion of MDA-MB-231 cancer cells to the same extent as full-length, immunopurified hCLCA2, while BSA and GST did not support tumor cell adhesion (Fig. 4B). Finally β4BMhCLCA2(90) and β4BMhCLCA2(35) were tested for their abilities to block the adhesion of lung-metastatic MDA-MB-231 cells to hCLCA2 in vitro. Both β4BMhCLCA2(90) and β4BMhCLCA2(35) were preincubated with hCLCA2-coated wells for 20 min at room temperature, completely blocked the adhesion of MDA-MB-231 cells to hCLCA2 (Fig. 4C). The control polypeptides P14 and PEDA were unable to block tumor cell adhesion to hCLCA2. Identical results were obtained for lung-metastatic 4T1 murine breast cancer cells (data not shown).

β4BMhCLCA2(90) Blocks Lung Colonization of MDA-MB-231 Cells—To examine the effect of the β4BMhCLCA2(90)-GST fusion protein on lung colonization of MDA-MB-231 cells, we incubated MDA-MB-231 cells for 20 min with β4BMhCLCA2(90) and then injected tumor cells together with the fusion protein into the lateral tail vein of 4-week-old, female Scid/beige mice (2). MDA-MB-231 cells were treated mice. The experiment was terminated. Autopsy and lung colony counting revealed a median number of >100 (from 77 to >100) tumor colonies in the control group and zero (from 0 to 2) colonies in the β4BMhCLCA2(90)-treated animal group (Fig. 5A). Gross examination of the lungs showed numerous tumor nodules throughout the lungs as well as in mediastinal and bronchial lymph nodes in GST-treated mice and normal, tumor-free lung in seven of eight β4BMhCLCA2(90)-treated mice. Histological examination of the lungs confirmed the gross findings. There was massive tumor involvement in the lungs of GST-treated mice but no evidence of metastatic disease in seven of eight β4BMhCLCA2(90)-treated mice. The remaining β4BMhCLCA2(90)-treated mouse had two small lung colonies.
colonies. This outcome was not the result of diminished growth and survival rates of tumor cells exposed to hCLCA2, but not GST, adheres to β4 integrin-coated dishes (15 μg/ml) in a dose-dependent manner (assay medium: phosphate-buffered saline + 1 mM MnCl2). B, Mn2+, but not Mg2+ and Ca2+, promotes adhesion of β4BMhCLCA2(90) (50 ng/ml) to β1 integrin (15 μg/ml). *, p < 0.01 relative to GST control.

**Fig. 3.** Adhesion of β4BMhCLCA2(90) to β4 integrin. A, β4BMhCLCA2(90) but not GST adheres to β4 integrin-coated dishes (15 μg/ml) in a dose-dependent manner (assay medium: phosphate-buffered saline + 1 mM MnCl2). B, Mn2+, but not Mg2+ and Ca2+, promotes adhesion of β4BMhCLCA2(90) (50 ng/ml) to β1 integrin (15 μg/ml). *, p < 0.01 relative to GST control.

**Fig. 2.** Identification of the β4 integrin binding-domain of hCLCA2. A, a PROTMAT-identified conserved sequence motif in the 90- and 35-kDa processing products of hCLCA2 is compared with the corresponding sequences in mCLCA5, mCLCA1, and bCLCA1, all expressed by pulmonary endothelia (boxed motifs, principal test sequences; underlined motifs, not tested). B, GST-AFSRISSGTG-HA (100 ng/ml) representing the β4-binding motif of the 90-kDa hCLCA2 (termed β4BMhCLCA2(90)) was generated and tested for adhesion to dishes coated with β4 integrin, β1 integrin, β3 integrin, fibronectin, and BSA (10 μg/ml) using anti-GST antibody ELISA to detect bound β4BMhCLCA2(90). Notice that β4BMhCLCA2(90) bound only to β4-coated dishes, p < 0.01 relative to BSA. C, pull-down (PD) of soluble β4 but not β1 and β3 (remain in flow-through (FT) with β4BMhCLCA2(90)) immobilized on glutathione beads. α-β4 mAbH101, α-β4 mAb (clone 18), α-β3 mAb2E11 were used for Western blot detection. D, pull-down of β4BMhCLCA2(90) by immunobead-immobilized β4 integrin but not by β1 integrin. α-GST pAbB-14 was used for Western blot detection.
not GST-β4 was able to pull down hCLCA2 (Fig. 6C). To examine whether GST-β4 and β4BM(hCLCA2)(90) were the interacting binding domains of the β4 integrin subunit and hCLCA2, microtitration plates were coated with skim milk (blocking agent), β4BM(hCLCA2)(90), or the control polypeptide PEDA. Biotinylated GST-β4 selectively bound to β4BM(hCLCA2)(90) but not to PEDA (Fig. 6D). GST-β1 did not bind to any of the three substrates (data not shown). Similarly the chimeric β4 protein β4.1.4, in which the C-terminal two-thirds of the β4 SDL domain were replaced with the corresponding region of the β1 integrin subunit failed to bind to hCLCA2 (Fig. 6E). Finally synthetic peptides of β4(184–203) and β1(207–213) were evaluated for their ability to block the adhesion of MDA-MB-231 and 4T1 breast cancer cells to hCLCA2 and mCLCA1, respectively. The β4 polypeptide, but not the β1 polypeptide, blocked adhesion of both MDA-MB-231 and 4T1 cells to the respective human and mouse CLCA proteins (Fig. 7, A and D). Polypeptides were equally efficient in their inhibitory activities when they were preincubated with CLCA-coated wells prior to seeding of tumor cells or when they were present throughout the adhesion assay. Polypeptides had no effect on the binding of tumor cells to placental (Fig. 7B) and EHS (Fig. 7, C and E) laminins.

The 90-kDa Protein of hCLCA1 Harbors a Disrupted β4BM and Fails to Bind β4 Integrin—In contrast to the highly conserved β4BM subunits of the 90-kDa subunits of hCLCA2, mCLCA5, mCLCA1, and hCLCA2, the hCLCA1 90-kDa protein exhibited a disrupted β4BM of the sequence AFGALSSGNG in which the amino acids RS are substituted by GA (Fig. 8A). However, hCLCA1 contains a relatively well conserved β4BM motif in its 35-kDa processing product (CFSRTSSGGS) (Fig. 8A). Thus, β4 integrin should not be able to bind to the 90-kDa hCLCA1 protein but might bind to the unprocessed 125-kDa and the processed 35-kDa proteins of hCLCA1. To examine this premise, we transfected HEK293 cells with Myc-tagged hCLCA1 and purified the protein by α-Myc immunofluorescence chromatography. Four fractions were collected from the affinity column. Fractions 1 and 4 contained only the 90-kDa processing product, while fractions 2 and 3 contained the 90-kDa processing product as well as the 125-kDa full-length, unprocessed hCLCA1 (the untagged 35-kDa hCLCA1 was lost in the column flow-through) (Fig. 8B). To test these fractions for adhesion of MDA-MB-231 cells, wells of microtitration plates were coated with the four fractions, then seeded, and incubated for 20 min with MDA-MB-231 cells. Tumor cells strongly bound to fractions 1 and 3 but failed to bind to fractions 2 and 4, indicating that they did not recognize the disrupted “β4BM” sequence but recognized the sequence of the conserved β4BM in the 35-kDa fragment of full-length hCLCA1 protein (Fig. 8C). A pull-down assay using GST-β4 immobilized on glutathione-agarose beads confirmed these data showing the inability of β4 to pull down the 90-kDa hCLCA1 but an excellent pull-down of the 90-kDa hCLCA2 (Fig. 8D).

DISCUSSION

The CLCA family comprises a multifunctional group of proteins (for reviews, see Refs. 17–19). One of their functions is the cell-cell adhesion of select CLCA molecules (for reviews, see Refs. 5 and 6). Expressed on the surface of pulmonary endo-
therial cells, CLCA molecules (e.g. hCLCA2, mCLCA1, mCLCA5, and bCLCA2) have been shown to serve as vascular addresses for hematogenously disseminating, \(/<\text{H}9252\)4-expressing cancer cells (4). By engaging in high affinity bonds with tumor cell \(/<\text{H}9252\)4 integrin, CLCA molecules mediate vascular arrest and promote early, intravascular, metastatic growth (4, 10). Here we have disclosed the interacting binding domains of the two molecules, using two independent, yet complementary approaches. In the first approach, we took advantage of the availability of an anti-bCLCA2 monoclonal antibody that blocked the adhesion of lung-metastatic, \(/<\text{H}9252\)4-expressing cancer cell lines (e.g. B16-F10, 4T1, R3230AC-MET, and MDA-MB-231) to bCLCA2-expressing lung matrix-modulated bovine aortic endothelial cells (7, 8). This antibody identified the second extracellular domain of bCLCA2 to harbor its binding site (amino acids 448–511). Not surprisingly, this region also supported binding of lung-metastatic MDA-MB-231 cancer cells. In the second approach, we examined the 90- and 35-kDa subunits of hCLCA2, which both supported adhesion of MDA-MB-231 cancer cells, for a common binding motif using the PROTOMAT algorithm (38). A single common motif was identified in the two proteins. Complementing the findings of our scanning of bCLCA2 fragments for MDA-MB-231 adhesion, the motif of the 90-kDa protein was located in the second extracellular domain of hCLCA2 (amino acids 479–488). Detailed biochemical and functional analyses conducted with GST fusion proteins that harbored the motif of the 90- (AFSRISSGTG) or 35-kDa (GFSSRVSSGGS) subunits of hCLCA2 showed that these sequences indeed serve as binding domains for the \(/<\text{H}9252\)4 integrin. Both motif polypeptides of the hCLCA2 subunits blocked adhesion between tumor cells and hCLCA2 and prevented lung and lymph node colonization of MDA-MB-231 cells using a standard lung colony assay. The importance of the identified motif in \(/<\text{H}9252\)4 adhesion is underscored by the binding behavior of hCLCA1. This molecule has a disrupted \(/<\text{H}9252\)4-binding motif of the sequence AFGALSSGNG. Substitution of two successive amino acids with uncharged polar (Ser) and basic (Arg) side chains with amino acids with nonpolar side chains results in failure to bind \(/<\text{H}9252\)4 integrin. Thus, the \(/<\text{H}9252\)4-binding motif has the following predicted sequence: F(S/N)R(I/L/V)(S/T)S.

Interestingly the \(/<\text{H}9252\)4-binding domain of CLCA proteins (hCLCA2, mCLCA5, mCLCA1, and bCLCA1) is located within a von Willebrand’s factor A (vWFA)-like domain (41–44). This domain was discovered by searching the Conserved Domain Database with the structure-sensitive CD Search BLAST tool of the National Center for Biotechnology Information (NCBI) Web site (www.ncbi.nlm.nih.gov:80/Structure/cdd/wrpsb.cgi) using the complete amino acid sequence of hCLCA2 as the query. The remaining CLCA family members produced the same domain match despite considerable overall sequence di-
vergence. Although the vWFA-like domain may be a signatory feature of the CLCA family, its presence in an area where CLCAs presumably loop through the plasma membrane into the cytoplasm and out again (U-shape loop) (34, 36, 39, 45) questions whether its functional and structural characteristic are similar to those of genuine vWFA domains in other proteins (e.g. integrins) (42–44).

Nonetheless like other vWFA domains it encompasses a puta-

**FIG. 6.** The CLCA-binding domain is located in the SDL of β4 integrin. A, scheme of the β4 integrin: I–27, signal sequence; 111–343, putative I domain-like structure; 184–203, predicted loop region of β4 (see Ref. 27). Sequences of the loop regions of β4(184–203) and β1(197–219) are displayed (boxed). B, hCLCA2 binding assay. Myc-tagged hCLCA2 (β4 μg/ml) is bound to uncoated wells (black column), GST- (gray column), GST-β1(197–219)- (dashed column), or GST-β4(184–203)-coated wells. Bound hCLCA2 is detected by anti-Myc antibody. hCLCA2 binds to GST-β4(184–203)-coated wells as well as uncoated wells (positive control) but not to wells coated with GST-β1(197–219) or GST. C, pull-down assay. GST-β1 and GST-β4 fusion polypeptides are immobilized on glutathione beads and then tested for pull-down of hCLCA2 from lysates of transfected HEK-293 cells. Notice that only GST-β4 is able to pull down hCLCA2. D, GST-β4 binds to GST-β4BMhCLCA2(90). The wells of microtitration plates were coated with β4BMhCLCA2(90) or control polypeptide PEDA (10 μg/ml each) and tested for binding of biotinylated GST-β4(30 μg/ml) by ELISA. E, chimeric β4-1-4 fails to bind hCLCA2. β4-1-4 was generated as described under “Experimental Procedures” and transfected together with αc into HEK293 cells (positive control: β4 + αc, negative control: αc). Lysates from transfected HEK293 cells were incubated with anti-β4 pAbH101-conjugated protein G beads (overnight at 4 °C). Beads were then washed and incubated with immunopurified Myc-tagged hCLCA2. Bound material was detected by Western blotting using anti-Myc mAb 9E10. WB, Western blot.
The sequence CTSEQNC of the SDL region of the hCLCA2 sequence 184–203 in the β4 integrin (184–203) and β4 (197–219) were added to substrate-coated wells and incubated for 30 min at room temperature. Polypeptides were either removed by washing prior to the addition of tumor cells (adhesion blocking) or were present throughout the tumor cell adhesion assay (adhesion competition). Notice a complete inhibition of adhesion (by blocking or competition) was observed for both MDA-MB-231 and 4T1 cells with the β4 polypeptide. No effect was recorded for the binding to placental and EHS laminins. *, p < 0.01 relative to adhesion to substrate alone.

**Fig. 7. Inhibition of the β4/CLCA adhesion with a β4 SDL polypeptide.** Adhesion assays were performed as described in detail previously (4, 33). In brief, wells of microtitration plates were coated with substrate (hCLCA2 (A), mCLCA1 (D), EHS laminin (C and E), or placental laminin (B)) overnight at 4 °C at the indicated concentration, then seeded with MDA-MB-231 (A, B, and C) or 4T1 (D and E) breast cancer cells, and incubated for 20 min at 37 °C. The number of bound tumor cells was determined by a colorimetric method (4, 33). Polypeptide β4 (184–203) and β4 (197–219) were added to substrate-coated wells and incubated for 30 min at room temperature. Polypeptides were either removed by washing prior to the addition of tumor cells (adhesion blocking) or were present throughout the tumor cell adhesion assay (adhesion competition). Notice a complete inhibition of adhesion (by blocking or competition) was observed for both MDA-MB-231 and 4T1 cells with the β4 polypeptide but not the β4 polypeptide. No effect was recorded for the binding to placental and EHS laminins. *, p < 0.01 relative to adhesion to substrate alone.
molecules (AFSRISS). In contrast, laminin-5 ligation to β4 SDL induces association of the β4 cytoplasmic tail with the adaptor protein Shc and an as yet unidentified 105-kDa phosphoprotein in outside-in signaling (33). Since laminin-5 binding to β4 was sensitive to point mutations K177A and Q182L (33), located at the N terminus of the SDL domain and proximal to the loop sequence analyzed in this study (amino acids 184–203), and since neither the αβ, β3, nor γ2 chains of laminin-5 contained a β4BM similar to that in CLCAs, it is likely that CLCAs and laminin-5 interact with different SDL motifs. However, this needs to be confirmed since changes in loop configuration resulted in outside-in signaling (33). Since laminin-5 binding to β4 and 4BM similar to that in CLCAs, it is likely that CLCAs and laminin-5 interact with different SDL motifs. However, this needs to be confirmed since changes in loop configuration resulted in outside-in signaling (33).

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The Interacting Binding Domains of the $\beta_4$ Integrin and Calcium-activated Chloride Channels (CLCAs) in Metastasis
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