Focal Adhesion Kinase Activated by $\beta_4$ Integrin Ligation to mCLCA1 Mediates Early Metastatic Growth*

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Early metastatic growth occurs at sites of vascular arrest of blood-borne cancer cells and is entirely intravascular. Here we show that lung colonization by B16-F10 cells is licensed by $\beta_4$ integrin adhesion to the mouse lung endothelial Ca$^{2+}$-activated chloride channel protein mCLCA1. In a manner independent of Met, $\beta_4$ integrin-mCLCA1 ligation leads to complexing with and activation of focal adhesion kinase (FAK) and downstream signaling to extracellular signal-regulated kinase (ERK). FAK/ERK signaling is Src-dependent and is interrupted by adhesion blocking antibodies and by dominant-negative (dn)-FAK mutants. Levels of ERK activation in B16-F10 cells transfected with wild-type or mutant FAK are closely associated with rates of proliferation and bromodeoxyuridine (BrdUrd) incorporation of tumor cells grown in mCLCA1-coated dishes, the ability to form tumor cell colonies on CLCA-expressing endothelial cell monolayers, and the extent of pulmonary metastatic growth. Parallel with the transfection rates, B16-F10 cells transfected with dn-FAK mutants and injected intravenously into syngeneic mice generate approximately half the number and size of lung colonies that vector-transfected B16-F10 cells produce. For the first time, $\beta_4$ integrin ligation to its novel CLCA-adhesion partner is shown to be associated with FAK complexing, activation, and signaling to promote early, intravascular, metastatic growth.

Hematogenous metastases originate from tumor cells arrested in the vasculature of select target organs. This arrest is tumor- and tissue-specific and is mediated at least in part by distinct tumor cell/endothelial cell adhesion ligand/receptor pairs (reviewed in Refs. 1 and 2). Studies in our laboratory have shown that lung metastatic human breast cancer cells colonized the lungs following adhesion to hCLCA2, a Ca$^{2+}$-sensitive chloride channel protein that is expressed on the endothelial cell luminal surface of human pulmonary arteries, arterioles, and interlobular venules (3). Similar to the unique adhesion functions of other channel proteins (reviewed in Ref. 4), CLCA proteins mediate adhesion via the $\beta_4$ integrin tumor cell ligand, which for the first time has been associated with a cell-cell adhesion function (3, 4). This novel adhesion interaction between members of CLCA family of proteins (e.g. bCLCA2 (Lu-ECAM-1), hCLCA2) and the $\beta_4$ integrin has been scrutinized by a variety of stringently controlled biochemical and functional assay procedures (3, 5, 6). These assays included (i) the co-immunoprecipitation of the $\beta_4$-hCLCA2 complex from extracts of lung metastatic MDA-MB-231 breast cancer cells bound to monolayers of hCLCA2-transfected human embryonic kidney (HEK) cells (3); (ii) the selective binding of immunopurified, recombinant hCLCA2 to membrane-immobilized, reconstituted $\beta_4$ integrin in Far Westerns (3); (iii) the increased expression of the $\beta_4$ integrin in breast cancer cell lines selected in vivo for enhanced lung colonization and the concomitant increased adhesion of the selected cells to hCLCA2 (3); (iv) the loss of hCLCA2 adhesion of breast cancer subjected to selective cleavage of the $\beta_4$ integrin with the metalloproteinase matrilysin (3, 7) or tumor cells transfected with mutant $\beta_4$ integrin (tail-less) (3); (v) the inhibition of pulmonary metastasis by $\beta_4$-hCLCA2 adhesion-blocking antibodies directed against either of the interacting adhesion molecules (3, 5, 6); and (vi) the increased lung metastatic performance of tumor cells overexpressing the $\beta_4$ integrin (3). These data are in agreement with previous reports showing that overexpression of the $\beta_4$ integrin is associated with an aggressive/metastatic cancer phenotype in several malignancies (reviewed in Ref. 8) and, more recently, with the finding that selection for enhanced lung colonization concurs with prominent overexpression of the $\beta_4$ integrin gene in a murine metastasis model analyzed by cDNA microarray (9).

Here, B16-F10 melanoma cells, characterized by strong surface expression of the $\beta_4$ integrin (10, 11) and consistently high lung colonization potential (12), were employed to explore whether the $\beta_4$ integrin interacts with a murine CLCA family member to promote metastasis of mouse lungs. Although the existence of a murine counterpart of hCLCA2 has been suspected by the positive immunohistochemical staining of endothelia of mouse pulmonary blood vessels with anti-bCLCA2 mAb6D3 and by the anti-metastatic effect of mAb6D3 (5, 6, 13, 14), cloning of this molecule was not achieved due to unavail-

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1 The abbreviations used are: HEK, human embryonic kidney; RT, reverse transcription; FAK, focal adhesion kinase; Pyk2, proline-rich tyrosine kinase-2; P13K, phosphatidylinositol 3-kinase; ERK, extracellular signal-regulated kinase; BAKC, bovine aortic endothelial cells; dn, dominant-negative; mAb, monoclonal antibody; pAb, polyclonal antibody; HA, hemagglutinin; pY, phosphotyrosine; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; wt, wild-type; GFP, green fluorescent protein; PLL, poly-L-lysine; BSA, bovine serum albumin; OG, o-glyceroldehy; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; MBP, myelin basic protein; IP, immunoprecipitation; WB, Western blotting; FRNK, FRAK-related non-kinase; EHS, Engbrith-Hom-Swarm; pMBP, phosphi-MBP.
ability of a pure pulmonary endothelial cell source. We report now the isolation and cloning of this molecule, which establish identity with the previously cloned mCLCA1 (15), illustrate the mCLCA1 expression pattern in the lung vasculature both by RT-PCR and by immunohistochemistry, and characterize the specific binding interaction between mCLCA1 and murine β₄ integrin. Intrigued by studies in a novel pulmonary metastasis model that allowed the in situ tracking of blood-borne cancer cells in perfused rodent lungs and the finding that lung metastases arose exclusively from endothelial cell-bound tumor cells by intravascular growth (16), we then explored whether the β₄-mCLCA1 adhesion by activating distinct, growth-promoting signaling pathways could account for the observed intravascular tumor cell proliferation. Our effort was focused on three signaling targets that may operate immediately downstream of the establishment of focal adhesions and promote cell growth. These targets were focal adhesion kinase (FAK), proline-rich tyrosine kinase-2 (Pyk2), and phosphatidylinositol 3-kinase (PI3K) (reviewed in Refs. 17–19). We found that β₄ integrin ligation to mCLCA1 selectively caused complexing with and activation of FAK that did not require participation of the Met oncogene (20). Downstream of FAK the extracellular signal-regulated kinase (ERK) was activated to promote tumor cell proliferation on surfaces coated with recombinant mCLCA1 and on bovine aortic endothelial cells (BAEC) that constitutively express bCLCA2 protein (5). FAK/ERK signaling was abrogated by β₄-mCLCA1 adhesion-blocking antibodies and by transfection of B16-F10 with dominant-negative (dn)-FAK mutants. These dn-FAK mutants also suppressed the metastatic growth of B16-F10 cells by down-regulating intravascular tumor cell proliferation, showing for the first time that FAK signaling initiated by tumor cell β₄ integrin ligation to its novel endothelial cell mCLCA1 adhesion partner is critical during the initial steps of metastasis formation.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents—**Antibodies against the β₄ integrin ectodomain were rat mAb54-11A (BD Pharmingen, San Diego, CA) and mouse mAb E1 (Dr. B. Nilius, CU Leuven, Leuven, Belgium) by extractions with TRIzol (Invitrogen). B16-F10 and HEK293 cells served as negative controls. 1 μg of RNA was reverse-transcribed (Superscript, Life Technologies) using random hexamers. cDNA was subjected to PCR (93 °C, 30 min; 55 °C, 30 min; 72 °C, 30 min; 35 cycles) with degenerate primers based on bCLCA2 (Lu-ECAM-1) amino acids 36–45 (LGATTTGAATACCCATCCGCACCAAGG) and 161–175 (5'-GCTATCCTCCTCAGAACCTCCCCA-3') (26). The 414 bp PCR products were inserted into pGEM-T (Promega) and sequenced.

**Pull-down Assay—**Pull-down assays were performed essentially as described by Puzon-McLaughlin and Takada (27). In brief, β₄ integrin immunoprecipitated from B16-F10 or HEK293 cells co-transfected with the α₅ and β₃ integrin subunits was immobilized on Protein G-Sepharose beads conjugated with anti-β₃ mAb1929 (3). Beads with bound β₄ integrin were washed extensively with lysis buffer containing 1 mM CaCl₂, 2 mM MgCl₂, and 0.5%, instead of 1% OG (washing buffer), then incubated overnight at 4 °C. Immune complexes were washed extensively with cold TBS lysis buffer (0.5% OG) and analyzed by SDS-PAGE and Western blotting, using streptavidin-HRP and anti-β₃, mAb6D3 was produced in BALB/c mice (22) and selected for blocking activity of a pure pulmonary endothelial cell source. We report now the isolation and cloning of this molecule, which establish identity with the previously cloned mCLCA1 (15), illustrate the mCLCA1 expression pattern in the lung vasculature both by RT-PCR and by immunohistochemistry, and characterize the specific binding interaction between mCLCA1 and murine β₄ integrin. Intrigued by studies in a novel pulmonary metastasis model that allowed the in situ tracking of blood-borne cancer cells in perfused rodent lungs and the finding that lung metastases arose exclusively from endothelial cell-bound tumor cells by intravascular growth (16), we then explored whether the β₄-mCLCA1 adhesion by activating distinct, growth-promoting signaling pathways could account for the observed intravascular tumor cell proliferation. Our effort was focused on three signaling targets that may operate immediately downstream of the establishment of focal adhesions and promote cell growth. These targets were focal adhesion kinase (FAK), proline-rich tyrosine kinase-2 (Pyk2), and phosphatidylinositol 3-kinase (PI3K) (reviewed in Refs. 17–19). We found that β₄ integrin ligation to mCLCA1 selectively caused complexing with and activation of FAK that did not require participation of the Met oncogene (20). Downstream of FAK the extracellular signal-regulated kinase (ERK) was activated to promote tumor cell proliferation on surfaces coated with recombinant mCLCA1 and on bovine aortic endothelial cells (BAEC) that constitutively express bCLCA2 protein (5). FAK/ERK signaling was abrogated by β₄-mCLCA1 adhesion-blocking antibodies and by transfection of B16-F10 with dominant-negative (dn)-FAK mutants. These dn-FAK mutants also suppressed the metastatic growth of B16-F10 cells by down-regulating intravascular tumor cell proliferation, showing for the first time that FAK signaling initiated by tumor cell β₄ integrin ligation to its novel endothelial cell mCLCA1 adhesion partner is critical during the initial steps of metastasis formation.
for each transfectant, the number of GFP° bated on the BAEC monolayers in DMEM were scored for anti-BrdUrd staining. The percent BrdUrd (incubated with anti-BrdUrd mAb (1:100) in PBS/1% BSA for 30 min at PBS/1% BSA/goat anti-mouse IgG (1:100) for 30 min at 37 °C were thoroughly washed with PBS, blocked with a 10-min treatment with 2 x HCl, completely abolishing the GFP fluorescence. Cells were washed with PBS, blocked with PBS/1% BSA/goat anti-mouse IgG (1:100) for 30 min at 37 °C, and the secondary antibody was fluorescence isothiocyanate-conjugated goat anti-mouse IgG (1:500 in PBS/1% BSA, 30 min, 37 °C) (ICN). The fields recorded for GFP° cells were then rephotographed, and cells were scored for anti-BrdUrd staining. The percent BrdUrd° cells per GFP° cells was determined (a minimum of 200–300 green cells were evaluated per transfectant).

Tumor Cell Proliferation on mCLCA1-coated Dishes and BAEC Monolayers—Serum-starved B16-F10 cells co-transfected with GFP and wtFAK, FRNK, FAKY397F, or vector alone were seeded at a concentration of 200 green fluorescent cells/well into 96-well microtiter plates (Corning) coated with mCLCA1 (~3 µg/ml, overnight). The mean number of green fluorescent cells was counted 48 h after seeding (96 h after transfection) for each transfectant and expressed as the percentage of GFP° cells in mock transfected B16-F10 cells. For tumor cell growth on endothelial cell monolayers, lung matrix-modulated BAEC (5 x 10^3/cm²) (5, 24) were seeded into 48-well plates and grown to confluence, then seeded with 3 x 10^4 B16-F10 cells co-transfected with GFP/wtFAK or GFP/FRNK. Tumor cells were incubated on the BAEC monolayers in DMEM + 1% FBS for 24, 48, 72, 96, and 120 h at 37 °C. Ten random areas (100 x) were photographed daily for each transfectant, the number of GFP° cells per field was counted, and the counts were averaged.

Adhesion and Lung Colony Assays—Adhesion and lung colony assays were performed as described previously (3, 5, 6). To determine the effects of wt and mutant FAK on the lung colony efficiency, B16-F10 were injected into the lateral tail vein of 6-week-old male C57BL/6 mice (eignt mice/experiment) 48 h after transfection with wtFAK, FRNK, FAKY397F, or vector alone. Mice were sacrificed 21 days after tumor cell injection. Median and range of the number of lung colonies and mean ± S.D. of the lung weights were determined for each transfectant.

RESULTS

Lung Colonization by B16-F10 Melanoma Cells Is Mediated by Endothelial mCLCA1—Previous studies in our laboratory showed that intravenous co-injection of the anti-bCLCA2 (Lu-ECAM-1) mAb6D3 and [125I]iododeoxyuridine-labeled B16-F10 cells into syngeneic mice caused an almost complete clearance of tumor cells from mouse lungs within 3–5 days and a concomitant dramatic reduction in the number of lung metastases (6) (Fig. 1A). In contrast, co-injection with mIgG resulted in incomplete tumor cell clearance and large numbers of metastases (Fig. 1A). These observations strongly suggested that mouse pulmonary endothelia express a CLCA family member that facilitates lung colonization by B16-F10 cells. To explore this possibility, we performed RT-PCR on RNA isolated from mouse lungs and cultured mouse pulmonary endothelial cells using degenerate primers based on the bCLCA2 amino acid sequence. A 414-bp PCR product was amplified from these sources in addition to RNA from mouse aortic endothelial cells, but not B16-F10 and HEK293 cells (Fig. 1B). Sequencing of individual PCR products revealed 100% identity with the previously cloned mCLCA1 (15). The mCLCA1 protein products were subsequently isolated from extracts of mouse lungs, whose endothelia had been surface-biotinylated by in situ right heart perfusion, using mAb6D3 immunoprecipitation and Western analysis by streptavidin-HRP; lanes 2 and 3, same as in lane 1 but with Western blotting with rat pAb(rat4) (lane 2) and preimmune rat IgG (lane 3); and lane 4, typical protein processing of recombinant mCLCA1 immunoprecipitated with mAb6D3 from extracts of transfected HEK293 cells: 125-kDa precursor protein and 90- and 35-kDa proteolytic processing products (15). D, B16-F10 adhesion to wild-type and recombinant mCLCA1 protein and inhibition by mAb 6D3. *p < 0.01, Student’s t test.

FIG. 1. Lung colonization of B16-F10 is mediated by mCLCA1. A, lung colony formation of intravenously injected B16-F10 melanoma cells in the presence of mIgG or mAb6D3 (anti-bCLCA2/Lu-ECAM-1); 238 ± 43 colonies (mIgG control); 25 ± 28 colonies in the presence of mAb6D3. B, RT-PCR was performed with RNA isolated from lungs (L), mouse pulmonary endothelia (LEC), mouse aortic endothelia (AEC), B16-F10 (B16), and HEK293 (HEK) and degenerate primers based on the bCLCA2 amino acid sequence (see “Experimental Procedures”); 414-bp bands were observed in L, LEC, and AEC. C, mCLCA1 protein isolation and characterization: lane 1, 125- and 90-kDa mCLCA1 protein products were isolated from lungs, whose endothelia had been surface-biotinylated by in situ right heart perfusion, using mAb6D3 immunoprecipitation and Western analysis by streptavidin-HRP: lanes 2 and 3, same as in lane 1 but with Western blotting with rat pAb(rat4) (lane 2) and preimmune rat IgG (lane 3); and lane 4, typical protein processing of recombinant mCLCA1 immunoprecipitated with mAb6D3 from extracts of transfected HEK293 cells: 125-kDa precursor protein and 90- and 35-kDa proteolytic processing products (15). D, B16-F10 adhesion to wild-type and recombinant mCLCA1 protein and inhibition by mAb 6D3. *p < 0.01, Student’s t test.

β4 Integrin Is the B16-F10 Ligand for mCLCA1—To determine whether endothelial mCLCA1 serves as the adhesion receptor for the β4 integrin expressed at high levels on lung metastatic B16-F10 cells (10, 11) (Fig. 2A), we seeded surface-biotinylated B16-F10 cells onto monolayers of HEK293 cells transfected with Myc-tagged mCLCA1 or lung matrix-modulated, bCLCA2-expressing BAEC and incubated cells for 30 min at 37 °C. After removing unbound tumor cells by washing, we successfully co-immunoprecipitated the β4-mCLCA1 complex from cell extracts, using antibodies directed against
**FIG. 2. β₄ integrin is the mCLCA1 ligand.** A, expression of α₅β₄ in B16-F10: Surface-biotinylated B16-F10 cells were extracted and immunoprecipitated with anti-α₅ mAb4G8 (lane 1) and anti-β₄ mAbG34-11A (ectodomain) (lane 2), and anti-β₄ pAb1922 (cytoplasmic domain) (lane 3), and immunoprecipitates were also probed by Western blotting (WB) with pAb1922. B, co-precipitation of β₄-mCLCA1: Monolayers of HEK293 cells transfected with mCLCA1-Myc (lane 1), surface-biotinylated B16-F10 cells (lane 2), and surface-biotinylated B16-F10 cells seeded onto monolayers of HEK293 cells transfected with mCLCA1-Myc and incubated for 30 min at 37°C (lane 3), were extracted and subjected to immunoprecipitation with anti-Myc mAb9E10. Immunoprecipitates were Western probed with anti-Myc mAb9E10 (top panel), streptavidin-HRP (middle panel) and anti-β₄ mAbG34-11A (bottom panel). C, mCLCA1 pull-down with bead-immobilized β₄ integrin: β₄ integrin from B16-F10 extracts was immobilized on anti-β₄ pAb1922-conjugated protein G-Sepharose beads (top panel). β₄ beads were incubated overnight at 4°C with immunopurified mCLCA1 isolated from HEK293 cells transfected with mCLCA1-Myc (upper middle panel) or lysates from surface-biotinylated BAEC monolayers constitutively expressing bCLCA2 (lower middle panel), both solubilized in TBS buffer containing 1 mM CaCl₂, 2 mM MgCl₂, and 0.5% OG. The β₄-bound protein (lane 1) and protein bound to anti-β₄ antibody-conjugated beads alone (lane 2) were detected by Western blot using anti-Myc mAb9E10 or streptavidin-HRP. GluH3 expressed and isolated from stably transfected HEK293 cells served as negative control (CL = cell lysate Western probed with anti-GluH3 antibody). Notice that both mCLCA1 and bCLCA2 were readily precipitated by immobilized β₄ integrin, whereas GluH3 was not. D, β₄ pull-down with bead-immobilized mCLCA1-Myc: mCLCA1-Myc was immobilized on protein G-Sepharose beads via the Myc tag of mCLCA1 and mAb6D3, respectively. The specificity of the β₄-mCLCA1 interaction was underscored by the co-precipitation of a single, biotinylated membrane protein of 205-kDa molecular mass with mCLCA1 that by Western blotting was identified as β₄ integrin (Fig. 2B). Because co-precipitations of the β₄-mCLCA1 complex under the detergent conditions employed in our experiments (1% Triton X-100 or OG in TBS lysis buffer) has been perceived as a non-standard approach in studies of integrin-ligand interactions, we examined the binding interaction between the β₄ integrin and mCLCA1 in a pull-down assay using conditions established for the pull-down of the β₁ integrin from cell lysates by Sepharose beads immobilized fibronectin (TBS buffer containing 1 mM CaCl₂, 2 mM MgCl₂, and 0.5% OG) (27). Accordingly, after binding the B16-F10 β₄ integrin to anti-β₄ antibody-conjugated protein G-Sepharose beads, β₄ beads were used to successfully pull-down recombinant mCLCA1 immunopurified from HEK293 cells transfected with Myc-tagged mCLCA1 or bCLCA2 from lysates of surface-biotinylated BAEC that constitutively expressed bCLCA2, whereas beads conjugated with anti-β₄ antibody alone were unable to do so (Fig. 2C). The control, multilamennembrane-spanning channel protein GluR3, which has similar hydrophobicity characteristics to mCLCA1, had no binding affinity for the β₄ integrin. Similar results were obtained, when Myc-tagged mCLCA1 was immobilized on beads, and the β₄ integrin was pulled down from extracts of surface-biotinylated B16-F10 cells or HEK293 cells transfected with αβ₄, both extracts again adjusted to 0.5% OG (Fig. 2D).

The β₄-mCLCA1 Adhesion Promotes Growth via FAK Signaling—The well known formation of predominantly subpleural tumor colonies by B16-F10 melanoma cells injected subcutaneously or intravenously into C57BL/6 mice is closely associated with a most prominent expression of mCLCA1 in subpleural venules (13). The early colony growth appeared to emerge from endothelial cell-bound tumor cells and was entirely intravascular (Fig. 3C). As time progressed, tumor cells occupied the entire vessel lumen (Fig. 3, D and E), then obliterated the vascular wall and penetrated along the entire vascular circumference into surrounding subendothelial tissues (Fig. 3, F and G). These findings suggested that early intravascular tumor cell growth might be initiated by the adhesion between tumor cell β₄ integrin and endothelial mCLCA1. To test this hypothesis, we plated serum-starved B16-F10 cells onto mCLCA1-coated dishes and examined three signaling targets known to operate immediately downstream of the formation of focal adhesions and to support growth. These targets were FAK, Pyk2, and PI3K. Only FAK was strongly activated upon adhesion of the serum-starved B16-F10 cells to mCLCA1-coated dishes (Fig. 4A), whereas Pyk2 and PI3K were not activated by the β₄-mCLCA1 adhesion (data not shown). FAK activation plateaued after 30 min of adhesion of B16-F10 cells to mCLCA1 and was sustained for the length of the 2h test period (Fig. 4A). No activation of FAK was observed when cells were plated onto PLL-coated dishes. The dependence of FAK activation upon β₄ ligation to mCLCA1 was substantiated by the selective co-immunoprecipitation of FAK with mCLCA1-ligated β₄, but not non-ligated β₄ (Fig. 4B). In complex with the β₄ integrin, activation of FAK presumably occurred by autophosphorylation (31) and was independent of the recently reported β₄ integrin/Met cooperation in outside-in signaling (20). Although Met was expressed in B16-F10 cells, it failed to co-precipitate with either mCLCA1-ligated β₄ integrin or FAK (Fig. 4C).

In addition to FAK, Src and ERK were also active in β₄-mCLCA1-ligated tumor cells (Fig. 4, D–F). Although the activation of ERK followed a similar time pattern as that of FAK with only a minor delay in its maximal state of activation (Fig. 4, A, E, and F), Src was active independent of the coating surface, except for a slightly increased activity after 2 h of B16-F10 adhesion to mCLCA1, implying that Src is constitutively active in our tumor model (Fig. 4D). ERK activation was paralleled by increased BrdU incorporation into tumor cells bound to mCLCA1-coated dishes, which was comparable to that of tumor cells adherent to fibronectin-coated dishes (Fig. 4G). In contrast, cells bound to PLL-coated dishes expressed background levels of activated FAK and ERK and incorporated little or no BrdU (Fig. 4, A, E, F, and G).

**FAK/ERK Signaling Is Inhibited by β₄-mCLCA1 Adhesion-blocking Antibodies and by the dn-FAK Mutant FRNK**—The
importance of a β₄-mCLCA1-triggered FAK/ERK signaling was scrutinized by examining the effects of adhesion-blocking antibodies as well as β₄ adhesion substrates other than mCLCA1. β₄-mCLCA1 adhesion-blocking antibodies directed against either mCLCA1 (mAb6D3) or β₄ integrin (mAb3E1) totally blocked activation of FAK and dramatically impaired ERK activation, although tumor cells were kept in intimate contact with the mCLCA1-coated dish surface by centrifugation (Fig. 5A). Consistent with the lack of FAK activation, tumor cells remained rounded throughout the experiment and failed to spread on the mCLCA1-coated dishes in the presence of the adhesion-blocking antibodies. Adhesion of B16-F10 cells to the control substrate laminin (e.g. EHS laminin, placental laminin) failed to trigger FAK activation (Fig. 5B). Accordingly, there was no downstream signaling to ERK, and BrdUrd incorporation was similar to that measured for B16-F10 cells incubated in PLL-coated dishes (Fig. 4G).

To further prove that FAK was the principal signaling molecule that was activated by the β₄-mCLCA1 adhesion, we transiently transfected B16-F10 with the dn-FAK-related non-kinase FRNK, which competes with endogenous FAK in focal contacts and lacks any downstream signaling capability (25, 31) and compared FAK signaling in these cells with that in wtFAK- and mock (vector)-transfected tumor cells. Following stimulation of the transfected cells by mCLCA1 ligation, FAK activation was significantly decreased in FRNK-transfected tumor cells, whereas transfection with wtFAK caused increased FAK expression and activation (Fig. 5C). This activation was mirrored by the downstream activation of ERK, showing an ~60% inhibition of ERK activation in FRNK- relative to vector-transfected tumor cells and an ~40% increase in ERK activation in wtFAK transfectants (Fig. 5C).

The FAK-mediated Signaling to ERK Is Src-dependent—To test whether the FAK-mediated activation of ERK was Src-dependent, we compared FAK signaling in β₄-mCLCA1 adhesion-stimulated B16-F10 cells transfected with HA-tagged wtFAK or dn-FAKY397F. By competing with endogenous FAK in focal contacts, the FAKY397F mutant significantly reduces complexing of Src family protein-tyrosine kinases with endogenous FAK and thus impedes Src-dependent downstream signaling to ERK (25, 32). Before testing Src involvement in FAK/ERK signaling, we verified that both wtFAK-HA and FAKY397F-HA were expressed equally in B16-F10 transfectants (Fig. 6, IP: α-HA) and showed by anti-β₄ immunoprecipitation that both FAK constructs were associated with mCLCA1-ligated tumor β₄ integrin (Fig. 6, IP: α-β₄). Src involvement in FAK/ERK signaling was then confirmed by Western analyses of anti-Src immunoprecipitates prepared from extracts of serum-starved B16-F10 cells transfected with wtFAK-HA or FAKY397F-HA and stimulated by a 30-min ligation to mCLCA1 (Fig. 6, IP: α-Src). Western blotting with anti-Src and anti-pY antibodies revealed equal expression of Src and confirmed the constitutively active state of this kinase in the two transfectants. However, a dramatically decreased co-precipitation of FAKY397F-HA with Src was observed relative to wtFAK-HA, which was abundantly co-precipitated with Src. To further confirm the involvement of Src in FAK/ERK signaling, we show that antibodies against the adaptor protein Grb2 co-immunoprecipitated wtFAK-HA but not FAKY397F-HA (Fig. 6, IP: α-Grb2), implying that wtFAK was phosphorylated on residue Tyr-925 by Src bound to FAK phosphorytrosine 397 to allow Grb2 binding, whereas the FAK substitution mutant F397Y was unable to promote such an interaction (25, 31–33). Together, these data strongly support that a significantly decreased activation of ERK in FAKY397F relative to wtFAK transfectants is Src-dependent. Consistent with the β₄-mCLCA1-mediated FAK/Src/Grb2/ERK signaling, we found a strong phosphorylation of wtFAK in anti-β₄, anti-HA, anti-Src, and anti-Grb2 immunoprecipitates prepared from extracts of wtFAK-HA-transfected B16-F10 and a lack thereof in FAKY397F-HA-transfected tumor cells (Fig. 6). Similar FAK signaling results were also obtained for other lung metastatic tumor cells, including MDA-MB-231 human breast cancer cells that strongly express β₄ integrin and colonize the lungs of nude mice at high efficiency (3).

2 M. Abdel-Ghany, H.-C. Cheng, R. C. Elble, and B. U. Pauli, unpublished data.
To examine whether the β1-mCLCA1-mediated FAK/ERK signaling promotes metastatic growth, the lung colonization potential of FRNK- and FAKY397F-transfected B16-F10 cells was compared with that of vector-transfected B16-F10 melanoma cells in a standard lung colony assay. Corresponding with a 40–50% transient transfection rate of the B16-F10 cells, metastatic growth was significantly retarded in mice injected with FRNK- and FAKY397F-transfected B16-F10 cells relative to vector-transfected cells (Fig. 7A). Significantly decreased lung weights in mice injected with FRNK- and FAKY397F-transfected B16-F10 transfectants reflected a dramatic decrease in the tumor burden, which was the result of fewer numbers and smaller sizes of lung colonies relative to those generated by vector-transfected tumor cells (Fig. 7A). These data were not the result of a differential adhesion of mock-, wtFAK-, FRNK-, and FAKY397F-transfected B16-F10 cells to recombinant mCLCA1 (Fig. 7B) but correlated well with decreased rates of proliferation (Fig. 7C) and BrdUrd incorporation (Fig. 7D) in FRNK- and FAKY397F-transfected relative to mock- and wtFAK-transfected B16-F10 cells incubated on mCLCA1-coated surfaces. Proliferation of tumor cells transfected with the two dn-FAK mutants was ~60% of that of mock-transfected and 50% of that of wtFAK-transfected B16-F10 cells (Fig. 7C). Similar decreases were recorded for BrdUrd incorporation. Comparison between B16-F10 cells co-transfected with GFP/wtFAK, GFP/FRNK, GFP/FAKY397F, and GFP/vector showed that BrdUrd staining was observed less often in green cells (transfected cells) of GFP/FRNK transfectants (55% of GFP/mock) and GFP/
blebbing became noticeable in a few tumor cells (Fig. 8, 120 h of incubation, colonies began to loosen up and apoptotic
 Colonies consisted of 4–8 cells and were evenly distributed over the entire BAEC monolayer (Fig. 8, upper panel). After 120 h of incubation, colonies began to loosen up and apoptotic blebbing became noticeable in a few tumor cells (Fig. 8B, upper panel). To further prove that the growth promotion of lung metastatic B16-F10 occurred at the level of the endothelium, we seeded B16-F10 cells co-transfected with GFP/wtFAK or GFP/FRK into BAEC monolayers (Fig. 8A, 0 h). B16-F10 co-transfected with GFP/wtFAK readily proliferated and produced multicellular colonies on the BAEC monolayers that were most prominent 48–72 h after tumor cell seeding (Fig. 8, A and B, upper panel). Colonies consisted of 4–16 cells and were evenly distributed over the entire BAEC monolayer (Fig. 8B, upper panel). After 120 h of incubation, colonies began to loosen up and apoptotic blebbing became noticeable in a few tumor cells (Fig. 8B, upper panel). In contrast, B16-F10 co-transfected with GFP/FRK did not proliferate and remained as mostly single cells on the BAEC monolayers throughout the experiment (Fig. 8, A and B, lower panel). By 120 h, many tumor cells were depicted as brightly fluorescent, condensed spheres. Blebbing and cell fragmentation was prominent among these tumor cells (Fig. 8B, lower panel, 120 h, 10×). However, these decreased
DISCUSSION

Using the B16 pulmonary metastasis model (12), we have previously shown that \( ^{125}\text{I} \)iododeoxyuridine-labeled B16-F10 melanoma cells injected intravenously into syngeneic mice initially arrested in large numbers in the lungs (6, 34). However, most of the arrested cancer cells were cleared from the lungs within 3–5 days to leave only those cells behind that eventually developed into lung tumor colonies (6). Clearance was most dramatic among tumor cells that lodged unspecifically in the capillary bed, where tumor cells underwent apoptosis within 24–48 h following intravenous injection (35, 36). In contrast, tumor cells arrested in subpleural, peribronchial, and parenchymatous venules, which all were lined with endothelium that stained positively for mCLCA1, appeared to survive, and during the first few days following injection underwent a rapid growth proliferation that was almost exclusively intravascular (5, 13). In the present study, attempts were made to elucidate the molecular mechanisms that governed the early metastatic growth within pulmonary blood vessels lined with mCLCA1 \(^{-}\) endothelium. Our hypothesis was that the adhesion between tumor cell \( \beta_4 \) integrin and endothelial cell mCLCA1 that originally mediated the vascular arrest (3, 5, 6) might be responsible for initiating tumor cell growth at the sites of vascular arrest. We found that B16-F10 cells, upon adhesion to mCLCA1, responded with an almost immediate activation of FAK. For the first time, FAK activation was shown to be induced by FAK complexing with mCLCA1-ligated \( \beta_4 \) integrin, which was nullified by \( \beta_4 \)-mCLCA1 adhesion-blocking antibodies. Co-precipitation and activation of FAK was unique for mCLCA1-ligated \( \beta_4 \) integrin and was not achieved by tumor cell binding to laminin. Downstream of FAK, ERK was activated in a Src-dependent manner. By binding to phosphoryrosine 397 of \( \beta_4 \)-mCLCA1-activated wtFAK, Src phosphorylated wtFAK at Tyr-925, creating an SH2-binding site for the adaptor protein Grb2 and promoting downstream signaling to ERK (25, 31–33). This process was barred in tumor cells transfected with \( \text{dn-FAK}^{\text{Y397F}} \), a mutant that disallows FAK/Src complexing to prevent phosphorylation of the FAK residue Tyr-925 by Src and Grb2 binding. Thus, similar to the adhesion-dependent activation of FAK by other integrins, the first step in FAK activation involved complexing with mCLCA1-ligated \( \beta_4 \) integrin followed by FAK autophosphorylation at residue Tyr-397. In a second step, Src was then recruited to complex with FAK by binding to FAK phosphoryrosine 397, thereby promoting phosphorylation of FAK at other tyrosine residues such as Tyr-925 and full activation of FAK (25, 31–33).

The adaptor protein Shc, recently shown to participate in FAK-independent signaling from antibody-ligated \( \beta_4 \) integrin to ERK (37) and from Met receptor tyrosine kinase-activated \( \beta_4 \) integrin to Ras- and PI3K-dependent pathways (20, 38), seemed not to be involved in the \( \beta_4 \)-mCLCA1-mediated signaling in our tumor model. Anti-\( \beta_4 \), anti-HA, and anti-Grb2 immunoprecipitates prepared from FAK-HA-transfected B16-F10 cells bound to mCLCA1 failed to co-precipitate and activate Shc. 

FIG. 8. B16-F10 transfected with wt-FAK form multicellular colonies on BAEC monolayers, whereas B16-F10 cells transfected with FRNK do not. B16-F10 co-transfected with GFP/wtFAK and GFP/FRNK were seeded onto monolayers of BAEC that constitutively express bCLCA2 shown to mediate strong B16-F10 adhesion (5, 6). Both transfectants adhered in equal numbers to BAEC monolayers (A, 0 h). However, B16-F10-wtFAK rapidly proliferated (A) and formed multicellular colonies on BAEC that were most prominent 48–72 h after seeding (B), whereas B16-F10-FRNK remained as single cells that did not proliferate (A and B). Apoptosis became obvious in both transfectants at 120 h after seeding but was significantly more prominent in B16-F10-FRNK (B, 120 h, 10×). *, \( p < 0.01, \) Student’s \( t \) test.
(data not shown). Using serum-starved B16-F10 melanoma cells in our signaling work, the present data show that in the absence of exogenous growth factors mCLCA1 ligation to the \( \beta_4 \) integrin was sufficient in promoting \( \beta_4 \)-FAK complexing and activation of FAK by autophosphorylation, suggesting that \( \beta_4 \) is more than a mere adaptor protein for Met or other receptor tyrosine kinases (20, 38–40), but can signal following ligand interaction. In the case of Met, it is interesting to notice that selection of parental B16-F1 cells for increased lung colonization (B16-F10) did not result in an increased expression of this oncogene (41). In contrast, selection of B16-F1 for increased liver colonization (B16-L59) resulted in a significant up-regulation of Met, suggesting a causal role in liver but not lung metastasis (41, 42).

Consistent with the outside-in signaling of other integrins (reviewed in Refs. 17, 18, 31, 43), the \( \beta_4 \)-mCLCA1-triggered, FAK-mediated downstream activation of ERK is closely associated with increased rates of proliferation and BrdUrd incorporation of B16-F10 cells bound to mCLCA1-coated dishes as well as the clonal growth of tumor cells bound to BAEC monolayers that constitutively express bCLCA2 (5). Again, these effects are lost when B16-F10 cells are transfected with the dn-FAK mutants FAKY397F and/or FRNK and were most impressive for tumor cells grown on BAEC. During the first 72 h following seeding onto BAEC monolayers, B16-F10 cells transfected with wtFAK form numerous tumor colonies that emerge all over the endothelial cell monolayer, whereas FRNK-transfected tumor cells remain as single cells on the endothelial monolayers. The same result is achieved when the lung metastatic growth of B16-F10 cells is compared with that of B16-F10 transfected with FRNK or FAKY397F. Mock-transfected B16-F10 melanoma cells exhibit rapid growth within pulmonary blood vessels, whose endothelium stained positively for mCLCA1 (13). However, interruption of the growth-promoting signaling pathway from \( \beta_4 \)-mCLCA1-activated FAK to ERK by the dn-FAK mutants FRNK and FAKY397F led to a significantly retarded lung colony growth. Decreased tumor burdens were the result of fewer macroscopically detectable tumor colonies resulting in lung weights that in animals injected with the two dn-FAK mutants were about half of the lung weights of animals inoculated with mock-transfected tumor cells. A similar anti-metastatic effect was achieved, when FAK tyrosine phosphorylation of B16 cells was suppressed by (−)-epigallocatechin-3-gallate (44). Together these data show that activation of FAK is essential for pulmonary colony growth in the present and possibly other (e.g. MDA-MB-231 breast cancer cells) pulmonary metastasis models. In tumor cells transfected with the dn-FAK mutants FRNK and FAKY397F, blood plasma, surrounding endothelial cell-bound cancer cells with a rich source of growth factors and other stimuli, was apparently unable to rescue blockage of the \( \beta_4 \)-mCLCA1-mediated FAK/ERK signaling and concomitant tumor cell growth suppression. This notion is consistent with the observations that serum was ineffective in activating ERK2 in integrin-adherent cells harboring dn-FRNK or dn-FAKY397F (45) and that hepatocyte growth factor enhancing of the FAK-dependent migration of MDCK cells was unable to rescue the migration-suppressive effect of dn-FRNK (46). Based on histopathological analyses revealing exclusive, intravascular tumor cell growth and no extravasation during early metastases formation (Fig. 3, C–E), the \( \beta_4 \)-mCLCA1-mediated activation of FAK seemed not to induce a migratory/invasive tumor cell phenotype as observed in other FAK functional studies (reviewed in Refs. 17, 18, 31, 43). However, these findings do not preclude that a \( \beta_4 \)-mediated activation of alternative signaling pathways (e.g. via PI3K, reviewed in Ref. 19) could be involved in promoting tumor cell invasion during later stages of colony growth, when invasive cell behavior dominated metastatic the metastatic stage (see Fig. 3, F and G).

The finding that the \( \beta_4 \) integrin-mCLCA1 adhesion is responsible not only for docking of blood-borne cancer cells in the vasculature of the organ to be metastasized (3, 5, 6, 13) but also for initiating signaling cascades leading to intravascular tumor growth may change some of our current thinking of the early steps of hematogenous metastasis. Widespread consensus still prevails that, following adhesion to endothelium, tumor cells exit the vascular compartment in a manner similar to inflammatory cells (reviewed in Ref. 2). However, different from inflammatory cells, which are unable to divide and are “pulled” from the vasculature by powerful chemotactic agents released from tissue inflammatory foci (review in Ref. 47), cancer cells operate in the absence of such “pulling” forces but respond keenly to growth-promoting signals leading to rapid intravascular tumor cell growth (13, 16). This concept of early metastatic growth is not novel but is backed by a number of studies seemingly lost in the vast metastasis literature (48, 49). For example, a large study involving 95 mammary adenocarcinomas shows that pulmonary metastases arise exclusively from intravascular growth (48). These observations were recently substantiated and detailed in an elegant pulmonary metastasis model that allowed the \( \text{in situ} \) tracking of blood-borne cancer cells by epifluorescent microscopy of isolated, perfused rodent lungs (16). In this latter study, lung metastases arose exclusively from endothelial cell-bound tumor cells by intravascular growth. Extravasation of tumor cells was rare, and it seemed that the few transmigrated tumor cells were rapidly removed extensively from endothelial cell-bound tumor cells by \( \text{in situ} \) metastasis growth involves signaling from distinct tumor cell/endothelial cell adhesion molecules, may provide a new basis in the development of therapeutic tools for the control of metastatic growth.

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