A Novel Consensus Motif in Fibronectin Mediates Dipeptidyl Peptidase IV Adhesion and Metastasis*

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Lung endothelial dipeptidyl peptidase IV (DPPIV/CD26) is a vascular address for cancer cells decorated with cell-surface polymeric fibronectin (poly-FN). Here, we identified the DPPIV-binding sites in FN and examined the effect of binding site peptides on DPPIV/poly-FN adhesion and metastasis. Using proteolytic fragments and maltose-binding protein fusion proteins that together span full-length FN, we found DPPIV-binding sites in type III repeats 13, 14, and 15 (FNIII13, -14, and -15, respectively). DPPIV binding was mediated by the consensus motif T(L/I)TGL and was confirmed by swapping this motif in FNIII13, -14, and -15 with the corresponding region in FNIII12, which did not bind DPPIV. DPPIV binding was lost in swapped FNIII13, -14, and -15 and gained in swapped FNIII12 (FNIII12(14)). Peptides containing the DPPIV-binding domain of FNIII14 blocked DPPIV/poly-FN adhesion and impeded pulmonary metastasis. This study adds to the classes of cell-surface adhesion receptors for FN and will help in the further characterization of the functional implications of the DPPIV/poly-FN adhesion in metastasis and possibly in cell-mediated immunity involving DPPIV-expressing lymphocytes.

Dipeptidyl peptidase IV (DPPIV/CD26) is a 110-kDa type II transmembrane sialoglycoprotein. It is anchored to the plasma membrane via a single transmembrane domain that, together with a six-amino acid cytoplasmic tail, is part of the putative uncleaved signal sequence (1–4). DPPIV is expressed in various epithelial tissues (e.g. liver bile canaliculi, kidney proximal tubules, prostate gland, and intestine), endothelia (e.g. lung capillaries, kidney vasa recta, and spleen sinusoids), and T-cells (4–7). The DPPIV functions are diverse and include three major activities. The best known and most widely researched function is that of its serine exopeptidase activity. DPPIV cleaves dipptides from the N terminus of polypeptides that have either proline or alanine in their penultimate positions (3, 4). The DPPIV exopeptidase activity accounts for a variety of regulatory processes, including chemokine regulation (8–10) and glucose homeostasis (11, 12). The catalytic sequence is GWSYG and is located at amino acids 627–631 of rat DPPIV.

The second major function of DPPIV is its ability to bind to the extracellular matrix, presumably involving the predicted seven-bladed β-propeller domain (13). Binding affinities for both collagen type 1 and fibronectin (FN) have been reported (5, 14), and a major function of the DPPIV/FN adhesion has been disclosed in the colonization of the lungs by blood-borne cancer cells (6, 15–18). The third major function is that of a T-cell co-stimulatory protein (reviewed in Refs. 3, 4, 9, 13, and 19). Although the exact molecular mechanisms by which DPPIV mediates T-cell activation are still unresolved, the ability of DPPIV to interact with CD45, a protein-tyrosine phosphatase (20, 21), and adenosine deaminase (22–24), each of which are capable of functioning in distinct signal transduction pathways, combined with its enzyme- and FN-binding qualities, may hold the keys to its mode of action in T-cell activation.

The involvement of DPPIV in the colonization of the lungs by blood-borne cancer cells was first discovered when we screened cancer cells with different organ colonization preferences for binding to endothelial cells from various tissue sources. Rather than relying on tissue culture-isolated endothelial cells, screening was performed with outside-out membrane vesicles freshly harvested from the luminal membrane of endothelial cells by in situ vascular perfusion of rat lungs with a formaldehyde/dithothreitol solution to induce endothelial cell-surface vesiculation (6, 15). Consistently, lung endothelial vesicles adhered in large numbers to lung-metastatic cancer cells only, but not to liver-metastatic or non-metastatic cancer cells, whereas control vesicles prepared from a rarely metastasized organ (e.g. leg muscle) adhered at background levels to lung- and liver-metastatic and non-metastatic tumor cells. Using a passive/active immunization schedule involving emulsified leg endothelial vesicles for active immunization and lung endothelial vesicles for passive immunization, we identified a monoclonal antibody that totally blocked the adhesion of lung endothelial vesicles to lung-metastatic cancer cells. The molecule blocked by the antibody was identified as DPPIV (6). Subsequently, we showed that vascular arrest of lung-metastatic cancer cells was mediated by DPPIV adhesion to cancer cell-surface-associated FN (16–18). In contrast to the poor ability of DPPIV to bind to soluble plasma FN, the binding to cell-surface-associated FN was of high avidity, suggesting that cell surface-associated FN presents itself in a different configuration than soluble plasma FN (25–27). Indeed, detailed biochemical analyses of cancer cell surface-associated FN revealed that FN is assembled into large fibrillar polymers (poly-FN) that are dispersed over the cancer cell surface in multiple globular aggregates, apparently exposing multiple DPPIV-binding sites (16, 17). Participation...
of the DPPIV/poly-FN adhesion in lung metastasis was substantiated by the findings that (a) a soluble DPPIV polypeptide representing the entire extracellular domain totally abolishes adhesion of lung-metastatic breast cancer cells to DPPIV and, accordingly, prevents lung colonization (17); (b) the Fischer 344/CRJ rat substrain, which harbors a G633R substitution in DPPIV that leads to retention and degradation of much of the mutant protein in the endoplasmic reticulum and the lung, but not in C57BL/6 mice,2 respectively; (c) lung-metastatic tumor cell lines derived from various human, mouse, and rat cancers invariably carry a strong message for endogenous FN and are able to assemble poly-FN on their surfaces; (d) FN surface expression in clones derived from a rhabdomyosarcoma correlates with lung metastasis (29); and (e) human and mouse melanoma cell lines selected for enhanced lung colonization overexpress FN (30, 31). Together, these data suggest that the DPPIV/poly-FN adhesion might be a quite common mechanism in the colonization of the lungs by blood-borne cancer cells, where it may operate alone or in cooperation with other adhesion molecules, including FN ligands other than DPPIV (e.g. integrins, heparan sulfate proteoglycans, and CD44), to mediate vascular arrest (32–35).

In this study, we authenticate and refine the role played by the DPPIV/poly-FN adhesion in pulmonary metastasis, identifying DPPIV-binding sites within FN and generating synthetic peptides of these sites that, by their anti-metastatic effect, clearly distinguish DPPIV/poly-FN adhesion from other FN adhesions. The binding sites were found by generating and screening a series of successively shorter FN sequences for DPPIV binding. The sites are located in type III repeats 13, 14, and 15 (FNIII13, -14, and -15, respectively) and represent a common consensus motif. Their DPPIV-binding specificity is confirmed in biochemical, mutational, adhesion, and lung colony assays.

**EXPERIMENTAL PROCEDURES**

**Cell Lines, Antibodies, and Reagents—**Lung-metastatic MTF7 rat mammary carcinoma cells were obtained from Dr. D. R. Welch (Pennsylvania State University, Hershey, PA); B16-F10 mouse melanoma cells were from Dr. J. I. Fidler (M. D. Anderson Cancer Center, Houston, TX); and MDA-MB-231 cells were from Dr. J. Price (M. D. Anderson Cancer Center). MTF7 cells were grown in α-minimal essential medium containing 5% fetal bovine serum; MDA-MB-231 and B16-F10 cells were grown in Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum. Monoclonal antibody (mAb) 6A3 and polyclonal antibody (pAb) CU31 were generated against rat DPPIV (6). pAbs against mouse FN were from Invitrogen; and pAbs against the heparin-binding (HA) tag (YPYDVPDYA) and glutathione S-transferase (GST) were from Sigma. FN proteolytic fragments (29, 45, and 70 kDa) and MBP-FNIII fusion proteins (New England Biolabs Inc.) were purified according to the manufacturers’ instructions. Briefly, 2 liters of Escherichia coli culture were spun at a 2-h isopropanol-β-thiogalactopyranoside (0.3 mM) induction. Cell pellets were sonicated in 100 ml of column buffer (20 mM Tris-HCl (pH 7.4), 200 mM NaCl, and 1 mM EDTA) and then spun at 19,000 rpm for 20 min at 4 °C. Supernatants were diluted 1:3 with column buffer and passed through an amylose resin column. Columns were typically washed with 10 volumes of column buffer and eluted with 10 mM maltose in column buffer. The purity of the elutes was evaluated by densitometric scanning of SDS-polyacrylamide gels and Western blotting with anti-MBP pAb. Protein concentrations were measured by the Bradford method (Bio-Rad). DPPIV was immunopurified from rat lungs using mAb 6A3 (16–18). HA-tagged GST fusion proteins were purified with anti-HA mAb-conjugated agarose beads.

**Plasmid Constructs and PROTOMAT Search—**Rat FNIII1–6, FNIII8–15, and FNIII12–15 were obtained as MBP fusion proteins from Dr. J. E. Schwarzbauer (Princeton University, Princeton, NJ) (36). To generate MBP-FN fusion proteins, FNIII8–11 (amino acids 1441–1702), FNIII12–14 (amino acids 1811–2081), FNIII12–13 (amino acids 1811–1991), FNIII13–14 (amino acids 1903–2081), FNIII14–15 (amino acids 1992–2081 and 2202–2286), FNIII11 (amino acids 1811–1902), FNIII12 (amino acids 1903–1991), FNIII13 (amino acids 1992–2081), and FNIII15 (amino acids 2202–2286) were PCR-amplified from a vector expressing the HA tag (YPYDVPDYA) and was inserted into the EcoRI/HindIII sites of the pGEX-KG vector (38).

**PROTOMAT** was used to search for conserved motifs in FNIII13, -14, and -15. The MOTIF program (H. O. Smith, Institute for Genomic Research, Rockville, MD) was first run to provide candidate blocks in theFNIII repeats. It was followed by an automated implementation of the GIBBS sampler program (C. E. Lawrence, Department of Statistics, Harvard University, Cambridge, MA) for reality checking (39). A data base of blocks was constructed by successive application of the automated PROTOMAT system to individual entries in the PROSITE catalog (40) of FNIII repeats keyed to the Swiss Protein Sequence Database (41). If sequences truly have motifs in common, then both runs typically yield similar or, more significantly, identical sets of blocks (39).

**Purification of Fusion Proteins and Rat DPPIV—**MBP-FN fusion proteins (New England Biolabs Inc.) were purified according to the manufacturer’s instructions. Briefly, 2 liters of Escherichia coli culture were spun at a 2-h isopropanol-β-thiogalactopyranoside (0.3 mM) induction. Cell pellets were sonicated in 100 ml of column buffer (20 mM Tris-HCl (pH 7.4), 200 mM NaCl, and 1 mM EDTA) and then spun at 19,000 rpm for 20 min at 4 °C. Supernatants were diluted 1:3 with column buffer and passed through an amylose resin column. Columns were washed with 10 volumes of column buffer and eluted with 10 mM maltose in column buffer. The purity of the elutes was evaluated by densitometric scanning of SDS-polyacrylamide gels and Western blotting with anti-MBP pAb. Protein concentrations were measured by the Bradford method (Bio-Rad). DPPIV was immunopurified from rat lungs using mAb 6A3 (16–18). HA-tagged GST fusion proteins were purified with anti-HA mAb-conjugated agarose beads.

**Gel Overlay Assay (Far-Western)—**Equal amounts of N-terminal FN proteolytic fragments (29, 45, and 70 kDa) and MBP-FNIII fusion proteins were subjected to SDS-PAGE under nonreducing conditions.

**FIG. 1. FN peptide chart.** The functional domains and their relative positions in FN are shown. HEP I–HEP III, first, second, and third heparin-binding domains, respectively; RGD, cell binding domain (Arg-Gly-Asp); SS, C-terminal intermolecular disulfide bonds. Asterisks indicate the positions of alternatively spliced repeats (not included in any of the FN constructs). The three types of FN repeats and MBP are shown in the inset. The 29-, 45-, and 70-kDa polypeptides are N-terminal proteolytic fragments of FN. All other fragments are MBP-FN fusion proteins.

**FNIII8–15** and inserted into the EcoRI and XbaI sites of the pMAL-c2 vector (New England Biolabs Inc.). All plasmids were verified by double-stranded sequencing. Overlap extension PCR was used to swap the consensus motif of FNIII13 (amino acids 1959–1968); FNIII14 (amino acids 2049–2058), or FNIII15 (amino acids 2247–2257) with the corresponding region of FNIII12 (amino acids 1868–1877) (37). The same methods were used to replace amino acids 1868–1877 in FNIII12 with amino acids 2049–2058 in FNIII14. GST-PEPl4-HA and GST-PEPcon-HA fusion proteins were generated by PCR amplification of FNIII14 (2405–2062) and FNIII15 (1774–1791). The amplified sequence included the HA tag (YPYDVPDYA) and was inserted into the EcoRI/HindIII sites of the pGEX-KG vector (38).

* H.-C. Cheng, M. Abdel-Ghany, and B. U. Pauli, unpublished data.

* Available at www.blocks.fhcrc.org.
Gel-separated proteins were transferred to nitrocellulose membranes and denatured with 4 M guanidine HCl in Tris-buffered saline containing 0.1% Tween 20 (TTBS) for 2 h. Proteins were then slowly renatured in TTBS. After blocking with 5% skim milk in TTBS for 2 h at room temperature, membranes were incubated overnight with 2 μg/ml DPPIV in 5% skim milk at 4 °C, followed by extensive washing with TTBS and incubation with anti-DPPIV pAb CU31. Bound anti-DPPIV antibodies were detected with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody and visualized by ECL™ (Amersham Biosciences).

**Other Biochemical Methods**—A modified enzyme-linked immunosorbent assay (ELISA) was used to measure the binding of DPPIV to FN fragments.

**Fig. 2. DPPIV binding to FN fragments.** A. Far-Western assay. Affinity-purified FN fragments resolved by SDS-PAGE under nonreducing conditions were examined for DPPIV binding by Far-Western blotting as described under “Experimental Procedures.” Note that DPPIV bound only to full-length (F.L.) FN, MBP-FNIII8–15, and MBP-FNIII12–15. B, pull-down assay. DPPIV-conjugated beads were incubated with soluble MBP, MBP-FNIII1–6, MBP-FNIII8–15, MBP-FNIII8–11, and MBP-FNIII12–15, and the pulled down material was analyzed by Western blotting using anti-MBP pAb. C, binding ELISA. Means ± S.D. of DPPIV binding to various FN fragments coated onto 96-well microtiter plates at 180 nM were determined by ELISA using anti-DPPIV pAb CU31 (triplicate experiments). *, p < 0.01, relative to DPPIV binding to MBP. D, dose-response curve for DPPIV binding to FN fragments. Various concentrations of full-length FN, MBP-FNIII12–15, and MBP-FNIII8–11 were coated onto microtiter plates overnight at 4 °C, blocked with 1% BSA in PBS for 2 h at room temperature, and examined for DPPIV (10 μg/ml) binding by ELISA using anti-DPPIV pAb CU31. Means ± S.D. of triplicate measurements in three separate experiments are shown. *, p < 0.01, relative to DPPIV binding to FNIII8–11.

**Fig. 3. Inhibition of DPPIV binding of cancer cells by MBP-FNIII12–15.** A, flow cytometric quantification of the cell-surface expression of FN is depicted for lung-metastatic (MTF7, B16-F10, and MDA-MB-231) and non-metastatic (MCF7) cancer cell lines. Solid histograms, rabbit nonimmune serum; open histograms, rabbit anti-FN pAb; insets, endogenous FN message (control, 28 S). B, adhesion inhibition assay. DPPIV was bound to bare plastic dishes (bars 1), dishes coated with MBP-FNIII12–15 (160 nM) overnight at 4 °C (bars 2), and dishes coated with poly-L-lysine (30 μg/ml) overnight at 4 °C (bars 3) following blocking with 1% BSA in PBS for 2 h at room temperature (DPPIV was used at different concentrations to ensure equal binding to the three substrates as determined by ELISA binding data). Lung-metastatic tumor cells (MTF7, B16-F10, and MDA-MB-231) strongly adhered to DPPIV bound to either plastic (bars 1) or poly-L-lysine (bars 3), but not to DPPIV bound to MBP-FNIII12–15 (bars 2). Non-metastatic MCF7 cells did not adhere to DPPIV under any of the test conditions. Means ± S.D. of triplicate determinations in three independent experiments are depicted. *, p ≤ 0.01, relative to tumor cells bound to DPPIV-coated plastic dishes.

(36, 42). Gel-separated proteins were transferred to nitrocellulose membranes and denatured with 4 M guanidine HCl in Tris-buffered saline containing 0.1% Tween 20 (TTBS) for 2 h. Proteins were then slowly renatured in TTBS. After blocking with 5% skim milk in TTBS for 2 h at room temperature, membranes were incubated overnight with 2 μg/ml DPPIV in 5% skim milk at 4 °C, followed by extensive washing with TTBS and incubation with anti-DPPIV pAb CU31. Bound anti-DPPIV antibodies were detected with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody and visualized by ECL™ (Amersham Biosciences).
fusion proteins. FN fusion proteins were coated at the indicated concentrations onto 96-well microtitration plates overnight at 4°C. The plates were washed three times with phosphate-buffered saline (PBS) and then incubated overnight with 5 µg/ml immunopurified DPPIV at 4°C. DPPIV binding was detected with anti-DPPIV pAb CU31, horseradish peroxidase-conjugated goat anti-rabbit antibody, and ECL. DPPIV affinity precipitation (pull-down assays) was carried out with DPPIV-conjugated Affi-Gel 10 beads (16). FN fusion proteins (5 µg/ml in 300 µl of PBS containing 0.1% β-mercaptoethanol) were incubated overnight with DPPIV beads at 4°C. The beads were washed with 0.1% β-mercaptoethanol-containing PBS and then boiled for 10 min in SDS sample buffer. Bound SDS-PAGE-separated proteins were Western-blotted with anti-MBP pAb. For the co-immunoprecipitation of DPPIV and MBP-FNIII repeats, DPPIV (5 µg/ml in 300 µl of 0.5% β-mercaptoethanol-glucose-containing PBS) was incubated with the same concentrations of wild-type or mutant MBP-FNIII single repeats for 6 h at 4°C. Complexes were then incubated overnight with anti-MBP pAb-conjugated protein A-agarose beads at 4°C. After washing with 0.5% β-mercaptoethanol-glucose-containing PBS, immunoprecipitates were subjected to SDS-PAGE and immunoblotting with anti-DPPIV pAb CU31 or anti-MBP pAb.

Peptide Binding to Purified DPPIV and DPPIV-expressing Cells—Immunopurified DPPIV (10 µg/ml) was coated onto 96-well microtitration plates overnight at 4°C. After blocking with 1% bovine serum albumin (BSA), the coated plates were incubated with biotinylated PEP14 or PEPcon (or GST-PEP14 or GST-PEPcon) for 2 h at room temperature. Bound peptide was detected by ELISA using horseradish peroxidase-streptavidin or anti-GST pAb. HEK293 cells were transfected with either rat DPPIV cDNA or vector (pRC-CMV) alone (18). Surface expression of DPPIV was evaluated by fluorescence-activated cell sorting using anti-DPPIV mAb 6A3. To determine the ability of biotinylated PEP14 and PEPcon to bind to cell-surface-expressed DPPIV, transfected cells were incubated with 0.1 mg/ml biotinylated peptide for 1 h at room temperature. The cells were then washed with PBS, and bound peptide was detected by staining with Texas Red-conjugated streptavidin for 30 min at 4°C. Staining patterns were observed by fluorescence microscopy.

Cell Adhesion and Lung Colony Inhibition Assays—Cell adhesion and lung colony assays were described previously (16, 42). For adhesion inhibition experiments, DPPIV-binding MBP-FNIII fusion proteins (160 µg/ml) were coated onto the wells of microtitration plates overnight at 4°C (control, 30 µg/ml poly-L-lysine) and then blocked with 1% BSA in PBS and conjugated with DPPIV at a concentration selected to equate the amount of DPPIV bound to plastic as determined by ELISA with anti-DPPIV pAb CU31. The wells were then seeded with tumor cells (3–5 x 10^4 cells/well) and incubated for 30 min at 37°C. The percent tumor cell adhesion was determined as described above. Alternatively, DPPIV (10 µg/ml) was coated onto the microtitration plate directly overnight at 4°C, blocked with 1% BSA in PBS, and incubated with GST-PEP14 (control, GST-PEPcon) for 2 h at room temperature. After washing with PBS, tumor cells were added, and adhesion was determined as described above. For lung colony inhibition assays, GST-PEP14 or GST-PEPcon (1 mg/mouse) was injected intravenously 15 min prior to tumor cells (1 x 10^6 cells/0.2 ml of Dulbecco’s modified Eagle’s medium/mouse). To exclude adverse reactions of GST-PEP14 with DPPIV-expressing T-cells, lung colony assays were performed in T-cell-deficient female 4-week-old Scid/beige mice (Charles River Laboratories, Wilmington, MA).

RESULTS

FNIII12–15 Harbors the DPPIV-binding Site and Mediates Adhesion of Cancer Cells to DPPIV—Coarse FN fragments that together span the entire length of the FN molecule were screened for DPPIV-binding sites using gel overlay (Far-Western) assays (36, 42). Fragments included 29-, 45-, and 70-kDa N-terminal proteolytic cleavage products as well as MBP fusion proteins of FNIII1–6 and FNIII8–15 (Fig. 1). DPPIV binding was mediated by full-length FN and MBP-FNIII15, but not by the three proteolytic fragments, MBP-FNIII1–6, and MBP (Fig. 2A). Further dissection of FNIII8–15 into FNIII8–11 and FNIII12–15 confined the DPPIV-binding site to FNIII12–15 (Fig. 2A). To confirm our Far-Western results, pull-down assays and ELISAs were performed. DPPIV-conjugated Affi-Gel 10 beads previously employed to “pull down” cancer cell surface-associated FN aggregates from tumor cell extracts (16) readily pulled down amylose-affinity-purified MBP-FNIII15–15 and MBP-FNIII12–15, but not MBP, MBP-FNIII1–6, and MBP-FNIII11–15 (Fig. 2B). The same result was achieved when binding of DPPIV to immobilized FN fragments was analyzed by ELISA using anti-DPPIV pAb CU31 to detect bound DPPIV (Fig. 2C). Binding of DPPIV to full-length FN and FNIII12–15 was dose-dependent, reaching a plateau at 160 nM (Fig. 2D), whereas DPPIV failed to bind FNIII8–11 at any of the test concentrations.

The functional significance of the DPPIV/FNIII12–15 adhesion was substantiated in adhesion inhibition assays with four cancer cell lines. Three of these cell lines, MTF7, B16-F10, and MDA-MB-231, consistently colonized the lungs of rats and mice upon tail vein injection, expressed strong endogenous messages for FN, and accumulated significant amounts of aggregated FN on their surfaces (Fig. 3A). In contrast, MCF7 tumor cells did
not colonize the lungs and were negative for endogenous message and surface accumulation of FN. Accordingly, MTF7, B16-F10, and MDA-MB-231 cells strongly adhered to DPPIV-coated dishes as well as to DPPIV bound to dishes coated with polystyrene, but failed to adhere to DPPIV immobilized on dishes coated with FNIII12–15 (Fig. 3B). MCF7 cells did not adhere to FNIII12–15 was done toDPPIV-binding polypeptide MBP-FNIII12–15 (Fig. 3B). In these experiments, the DPPIV coating concentration was selected such that equal binding to the three substrates (plastic, MBP-FNIII12–15, and polystyrene) was achieved as determined by ELISA.

**DPPIV Binds to FNIII13, -14, and -15**—Further dissection of the DPPIV-binding polypeptide MBP-FNIII12–15 was done to yield dual (MBP-FNIII12–13, MBP-FNIII13–14, and MBP-FNIII14–15) and single (FNIII12, -13, -14, and -15) type III repeats to narrow the DPPIV-binding site to a single repeat (Fig. 1). Among these constructs, only the single repeat FNIII12 lost DPPIV-binding ability; all other fragments strongly bound DPPIV as observed by Far-Western and pull-down analyses (Fig. 4A, single repeats shown only). These binding interactions were specific because DPPIV bound only to wild-type or mutant FNIII with the corresponding loop sequence in FNIII12 revealed that the DPPIV binding-site is distinct. E and F, FNIII12 gained DPPIV binding by placing the consensus sequence of FNIII14 in lieu of the corresponding site in FNIII12 (FNIII12(14)). Binding of DPPIV to wild-type FNIII12 or FNIII12(14) was examined by DPPIV pull-down assay (E, lower panel) and anti-MBP co-immunoprecipitation and Western blotting with anti-DPPIV pAb (F, lower panels). The presence of equal amounts of wild-type and mutant FNIII12 used in the assays is shown in the upper panel of E, IB, immunoblot.

**Consensus DPPIV-binding Motif in Fibronectin**

A consensus motif in FNIII13, -14, and -15 is essential for DPPIV binding. A, amino acid sequences of the consensus DPPIV-binding motif encompassing the last amino acids of the E β-strand and the EF loop of FNIII13, -14, and -15 are contrasted with the sequence of the corresponding region in FNIII12. B, shown is the scheme for the construction of MBP-FNIII fusion protein mutants generated by swapping the consensus motif in FNIII13, -14, and -15 with the corresponding loop sequence in FNIII12 (FNIII13(12), FNIII14(12), FNIII15(12), and FNIII12(14)). C, binding of DPPIV and heparin to wild-type or mutant FNIII single repeats is shown by DPPIV and heparin pull-down assays (middle and lower panels, respectively) (see “Experimental Procedures”). Bound and loading amounts of the MBP fusion proteins were revealed by Western blotting with anti-MBP pAb. D, anti-MBP co-immunoprecipitates (IP) of wild-type or mutant FNIII single repeats and DPPIV were Western-probed with anti-DPPIV pAb CU31 (upper panels) and anti-MBP pAb (lower panels). Note that DPPIV failed to pull down and co-immunoprecipitate the mutant constructs, whereas heparin binding was unaffected by the mutant, indicating that the DPPIV- and heparin-binding sites are distinct. E and F, FNIII12 gained DPPIV binding by placing the consensus sequence of FNIII14 in lieu of the corresponding site of FNIII12 (FNIII12(14)). Binding of DPPIV to wild-type FNIII12 or FNIII12(14) was examined by DPPIV pull-down assay (E, lower panel) and anti-MBP co-immunoprecipitation and Western blotting with anti-DPPIV pAb CU31 (F, upper panel) and anti-MBP pAb (F, lower panel). The presence of equal amounts of wild-type and mutant FNIII12 used in the assays is shown in the upper panel of E, IB, immunoblot.
their abilities to mediate heparin binding. Both wild-type and mutant FNIII13 and FNIII14 were equally competent to mediate heparin binding (Fig. 5C, lower panels). The same DPPIV-binding behavior for wild-type and mutant FNIII13, -14, and -15 was also observed in co-immunoprecipitation experiments. DPPIV co-immunoprecipitated with wild-type (but not mutant) FNIII13, -14, and -15 using anti-MBP antibodies (Fig. 5D). Although the data presented so far clearly show that the identified consensus motif is essential for DPPIV binding to FNIII13, -14, and -15, the ultimate test for its serving as the DPPIV-binding domain was obtained when we introduced the corresponding sequence from the same loop in FNIIIEDA (the second alternatively spliced repeat) as the control peptide, which displayed similar charge and hydrophobicity characteristics as PEP14, but not PEPcon, bound to DPPIV-transfected HEK293 cells as shown by staining with Texas Red-conjugated streptavidin (left and middle panels, respectively). Mock-transfected HEK cells served as a negative control (right panel). IB, immunoblot.

Peptides Harboring the DPPIV-binding Site of FNIII14 Impede Tumor Cell Adhesion to DPPIV and Pulmonary Metastasis—To examine whether the consensus motif by itself is sufficient for DPPIV binding, we synthesized biotinylated peptides based on the consensus motif of FNIII14 (PEP14). Because the corresponding peptide from FNIII12 was insoluble, we used a sequence from the same loop in FNIII13 (the second alternatively spliced repeat) as the control peptide, which displayed similar charge and hydrophobicity characteristics as PEP14, i.e. EDTAELQLRPGEYTVS (PEPcon). First, we measured the binding abilities of these biotinylated peptides to immobilized DPPIV by ELISA using streptavidin detection. PEP14 bound to DPPIV in a dose-dependent manner, whereas PEPcon failed to bind DPPIV at all test concentrations (Fig. 6A). Second, we performed peptide affinity precipitation assays in which biotinylated peptides were bound to streptavidin-conjugated beads before being exposed to immunopurified soluble DPPIV (Fig. 6B, upper panel). Only PEP14 was able to precipitate DPPIV (Fig. 6B, middle panel), whereas beads alone or PEPcon-conjugated beads left DPPIV in the flow-through (Fig. 6B, lower panel). To test whether PEP14 also binds to DPPIV protein in its native transmembrane state, we transfected HEK293 cells with rat DPPIV and measured the ability of the transfected cells to bind the biotinylated peptides by Texas Red-conjugated streptavidin staining. Again, PEP14 bound selectively to the DPPIV-expressing HEK293 cells, but not to the mock-transfected HEK293 cells, whereas PEPcon bound to neither cell type (Fig. 6C).

Finally, we examined PEP14 in tumor cell adhesion and lung colony assays. For economical reasons and to guarantee longer survival in serum, we conducted our experiments with a GST-PEP14 fusion protein rather than with synthetic PEP14. GST-PEP14 blocked adhesion of MTF7 cancer cells to DPPIV in a dose-dependent manner, causing a 50% inhibition of adhesion at a concentration of 3.0 μM (Fig. 7A). The control fusion protein GST-PEPcon had no adhesion inhibitory effect at any of the test concentrations. In lung colony assays, GST-PEP14 consistently reduced the colonization of the lungs by MTF7 cells, allowing the generation of fewer and smaller tumor colonies than GST-PEPcon (Fig. 7B).

**DISCUSSION**

Fibronectin is a “pro-metastatic” gene that is overexpressed in cancer cells selected for enhanced lung colonization (16–18, 28–30). This pro-metastatic property of FN has been associated with the ability of many lung-metastatic cancer cells to assemble FN on their surfaces into multiple, randomly dispersed aggregates generated by FN self-association (16, 17). FN polymers facilitate adhesion to DPPIV, a distinct pulmonary vascular address that promotes homing of blood-borne cancer cells to the lungs (6, 16–18). Binding to FN does not engage DPPIV enzymatic activity (16) and is therefore distinct from the binding of a non-FN lung-targeting peptide to membrane dipeptidase, a second pulmonary dipeptidase-type vascular address (46). To learn more about the DPPIV/poly-FN “lung homing” mechanism and to identify “tumor homing” peptides for future...
Fig. 7. PEP14 inhibits DPPIV adhesion and metastasis of MTF7 tumor cells. A, dishes coated with DPPIV (10 µg/ml) overnight at 4°C were treated with GST-PEP14 or control GST-PEPcon as described in the legend to Fig. 6A, seeded with MTF7 (3 × 10⁵ cells/well), and incubated for 30 min at 37°C, and the percent specific adhesion was determined (18). Data represent means ± S.D. of triplicate measurements in three independent experiments. *, p < 0.01, relative to GST-PEPcon binding to DPPIV. B, GST-PEP14 (1 mg/mouse) injected intravenously 15 min prior into MTF7 tumor cells (1 × 10⁶ cells/0.2 ml of PBS/mouse) caused a dramatic reduction in the number and size of MTF7 lung colonies relative to GST-PEPcon. #, means ± S.D. are from two independent experiments, each involving eight female 6-week-old Scid/beige mice; †, p < 0.01, relative to MTF7 binding inhibition (Inh.) by GST-PEPcon.

therapeutic application (47), we screened a series of FN fragments that together span the length of the FN molecule for their abilities to bind DPPIV using various molecular, biochemical, and functional assays. We have shown that FN contains multiple DPPIV-binding sites, one in each of FNIII13–14, and -15. The binding sites are located in the N-terminal portion of a common consensus motif identified by the PROTOMAT algorithm (39). Comparison of the identified DPPIV-binding motif with sequences of the EF regions in the other FNIII repeats showed that the sequence Thr-(Leu/Ile)-Thr located at the end of the E-strand and prior to the hydrophobic core residue Leu is the most conserved sequence in FNIII13, -14, and -15 (44). Preliminary data show that replacement of Thr-Ile-Thr in FNIII14 with Ile-Val-Ser in FNIII12 with Thr-Ile-Thr from FNIII14 did not restore DPPIV binding in FNIII12, suggesting that the Thr-Ile-Thr tripeptide acts as a DPPIV-binding site only in the context of the identified consensus motif. This notion is supported by the observation that FNIII9, the only FNIII repeat other than FNIII13, -14, and -15 harboring Thr-Leu-Thr in the same region, was also unable to confer DPPIV binding to FNIII13–15.

The three DPPIV-binding domains are located within an FN region (FNIII13–15) that is involved in multiple biological functions (34, 48). For example, FNIII13–15 is important in FN fibrillogenesis (49); binds human immunodeficiency virus type 1 gp120/160 to reduce virus infectivity (50); and harbors adhesion sites for integrins α3β1 and αvβ3 (33, 48), the chondroitin sulfate proteoglycan CD44 (35), and heparin (second heparin-binding domain) (34, 44, 48, 51–53). The integrin- and heparin-binding domains are located in areas distinct from the DPPIV-binding sites, and each is characterized by a unique sequence (45, 48), suggesting that they may operate in an independent manner. However, studies by Sharma et al. (44) showed that the integrin α3β1- and heparin-binding domains located on opposite faces of FNIII13–14 may affect binding of one binding domain by loading or unloading the other. This type of interaction between distinct binding domains has also been observed for the heparin- and DPPIV-binding domains, where occupation of the heparin-binding site with its ligand totally inhibited DPPIV binding to MTF7 cells in vitro and abolished lung colonization of these tumor cells in vivo. Although we have no precise explanation for this preliminary result, it is likely that heparin binding acting in an allosteric manner perturbs FNIII13, -14, and -15 binding to DPPIV. Full disclosure of the dynamic interplay between ligand-bound and -unbound cell adhesion domains in FNIII13–15 may therefore provide new insights into the conditions that regulate FN binding engagement with integrins, heparan sulfate proteoglycans, and DPPIV and may explain the different binding behavior of FN under different microenvironmental conditions (33–35, 44, 48–51).

Functional analysis with a peptide representing the DPPIV-binding domain of FNIII14 showed competitive blocking of the DPPIV/poly-FN adhesion and a powerful anti-metastatic effect. The extent of these anti-adhesive and anti-metastatic effects was similar to that reported for adhesion-blocking antibodies and the soluble extracellular domain of DPPIV (17). A similar anti-metastatic effect by a 22-mer synthetic peptide harboring the complete DPPIV-binding motif of FNIII14 has also been reported for T lymphoma cells colonizing liver and spleen (52). Although various integrins (for which the 22-mer peptide had no binding affinity) were implicated in the anti-metastatic effect, it is likely that the peptide blocked the binding interaction between hepatic and splenic sinusoidal FN and DPPIV, which has been shown to be prominently expressed on the T lymphoma cells used (53).

Here, we have identified and characterized a novel cell-binding domain on FN and have presented data showing that binding of endothelial DPPIV to FN this domain plays an important role in pulmonary metastasis. However, at this time, it is unclear whether the DPPIV/poly-FN adhesion acts alone in mediating vascular arrest and metastasis or whether it requires the cooperation of a secondary adhesion event, as reported for the adhesion interaction between immune/inflammatory cells and endothelial cells (reviewed in Refs. 54 and 55). Because all lung-metastatic cancer cells tested in our laboratory, including MDA-MB-231, B16-F10, 4T1, MTF7, LLC1, and K7M2, exhibit high message and protein levels for both FN and integrin β4 and adhere to both of the respective pulmonary addressins (DPPIV and CLCA), it is conceivable that pulmonary vascular arrest of blood-borne cancer cells is initiated by the DPPIV/poly-FN adhesion and is stabilized by the CLCA/integrin β4 adhesion discovered in this laboratory (42, 56). Circumstantial support for this notion came from studies of the CLCA/integrin β4 adhesion under flow conditions (57). These experiments showed that integrin β4-expressing tumor cells adhere to CLCA-coated dishes only at low shear stresses. However, once adhesion is established, high shear stress levels are
able to break it, suggesting that, for engagement of the CLCA/integrin β4 adhesion, a “slowdown” of tumor cells may be beneficial. We predict that such a slowdown is mediated by the DPPIV/poly-FN adhesion, which is facilitated by the prominence of tumor cell-surface FN polymers that easily engage in binding interactions with endothelial cells. The necessity for a dual tumor cell/endothelial cell adhesion principle in lung metastasis is also supported by our finding that lung-metastatic, integrin β4-expressing tumor cells colonize the lungs of DPPIV−/− mice at reduced levels relative to DPPIV+/- mice.2 Finally, the expression of DPPIV on cells other than lung endothelial cells could extend the importance of our findings beyond metastasis. A principal DPPIV expresser cell is the T lymphocyte, which shares a dual tumor cell/endothelial cell adhesion principle in lung metastasis. Adenosine deaminase have been implicated in this activating collaboration of DPPIV with other molecules such as CD45 and integrins (α/β) in T lymphocytes, which is the missing link in the activation mechanism of T lymphocytes. If this is indeed the case, then the DPPIV/poly-FN adhesion could also be involved in T-cell-mediated cytolyis of tumor cells. 

Acknowledgments—We thank Dr. T. L. Shen for assistance in preparing some of the plasmid constructs and Lin Yu for expert technical assistance.

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