A Novel CD44-binding Peptide from the Pro-Matrix Metalloproteinase-9 Hemopexin Domain Impairs Adhesion and Migration of Chronic Lymphocytic Leukemia (CLL) Cells*

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**Background:** proMMP-9 binds to CLL cells through the hemopexin domain (PEX9), contributing to disease progression. **Results:** A 20-residue sequence (P6) within PEX9 inhibits CD44-mediated CLL-proMMP-9 interaction and cell migration. P6 cooperates with the previously identified sequence P3, also located in PEX9. **Conclusion:** P6 is a CD44-binding site and impairs proMMP-9 effects on CLL. **Significance:** P6 may be a novel therapeutic target in CLL.

(pro)MMP-9 binds to CLL cells through the PEX9 domain and contributes to CLL progression. To biochemically characterize this interaction and identify potential therapeutic targets, we prepared GST-PEX9 forms containing structural blades B1B2 or B3B4. We recently described a sequence in blade B4 (P3 sequence) that bound α4β1 integrin and partially impaired cell adhesion and migration. We have now studied the possible contribution of the B1B2 region to cell interaction with PEX9. CLL cells bound to GST-B1B2 and CD44 was the primary receptor. GST-B1B2 inhibited CLL cell migration as effectively as GST-B3B4. Overlapping synthetic peptides spanning the B1B2 region identified the sequence FDAIEIGNQYLFKDGKYW, present in B1 and contained in peptide P6, as the most effective site. P6 inhibited cell adhesion to PEX9 in a dose-dependent manner and with an IC50 value of 90 μM. P6 also inhibited cell adhesion to hyaluronic but had no effect on adhesion to VCAM-1 (α4β1 integrin ligand), confirming its specific interaction with CD44. Spatial localization analyses mapped P6 to the central cavity of PEX9, in close proximity to the previously identified P3 sequence. Both P6 and P3 equally impaired cell adhesion to (pro)MMP-9. Moreover, P6 synergistically cooperated with P3, resulting in complete inhibition of CLL cell binding to PEX9, chemotaxis, and transendothelial migration. Thus, P6 is a novel sequence in PEX9 involved in cell-PEX9/(pro)MMP-9 binding by interacting with CD44. Targeting both sites, P6 and P3, should efficiently prevent (pro)MMP-9 binding to CLL cells and its pathological consequences.

Chronic lymphocytic leukemia (CLL)§ consists of the accumulation in peripheral blood of CD5+ B lymphocytes that infiltrate the bone marrow and secondary lymphoid tissues (1, 2). CLL cells differ from normal B cells in many genetic and functional aspects, including overexpression of pro/mature matrix metalloproteinase-9 (pro)MMP-9, a member of the MMP protein family (3–5). Indeed, elevated intracellular levels of (pro)MMP-9 correlate with advanced CLL stage and poor patient survival (6). Moreover, and although a mostly secreted MMP (7), we have shown that (pro)MMP-9 is consistently present at the CLL cell surface, due to the binding to a docking complex formed by α4β1 integrin and a 190-kDa CD44 variant (8). (pro)MMP-9 binding to this complex results in *in vitro* and *in vivo* cell arrest and induction of a cell survival pathway consisting in Lyn/STAT3 activation and Mcl-1 up-regulation (8–10). The latter effect did not involve the MMP-9 catalytic activity but required the hemopexin domain (PEX9), as a recombinant mutant lacking PEX9 did not bind to cells (8). Although this survival pathway was mainly triggered by α4β1 (9), CD44 can also up-regulate Mcl-1 and promote CLL cell survival upon interaction with its ligand hyaluronan (11, 12). Accordingly, Zhang et al. (13) recently used a humanized anti-CD44 monoclonal antibody and identified this molecule as a target in CLL.

These previous reports indicate that (pro)MMP-9 localization at the cell surface contributes to CLL pathology by multiple mechanisms and that targeting PEX9-cell interaction may represent a therapeutic advantage. PEX9 consists of four-bladed β-propeller structure (blades 1–4) (14), and Dufour et al. (15) applied a genetic approach in fibrosarcoma and carcinoma cells to identify two sequences in the outermost β-strand of blade 1 (SRPQGPFL) and blade 4 (NQVDQVGY), which affected

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4 The abbreviations used are: CLL, chronic lymphocytic leukemia; HUVEC, human umbilical vein endothelial cell(s); Ab, antibody; VCAM-1, vascular cell adhesion molecule-1; B-CECF-AM, 2’,7’-bis(carboxyethyl)-5(6’)-carboxyfluorescein-acetoxyethyl ester.
endothelial cells (HUVEC) were purchased from Lonza and cultured as reported (4, 8).

**Antibodies, Reagents, Proteins, and Peptides**—mAbs HP2/1 (anti-α4 integrin subunit, function-blocking), HP1/7 (anti-α4 integrin subunit, non-blocking), and HP2/9 (anti-CD44, function blocking) were obtained from Dr. Francisco Sánchez-Madrid (Hospital de la Princesa, Madrid, Spain). Rabbit polyclonal antibodies to glutathione S-transferase (GST, sc-459) and MMP-9 (sc-6841-R) were from Santa Cruz Biotechnology (Santa Cruz, CA). HRP-labeled Abs to rabbit or mouse IgG were from Dako (Glostrup, Denmark). Alexa Fluor 488-labeled Ab was from Molecular Probes (Eugene, OR). Hyaluronan was from Sigma-Aldrich (catalog no. 63357). Vascular cell adhesion molecule-1 (VCAM-1), CXCL12, and TNF-α were from R&D Systems (Minneapolis, MN). (pro)MMP-9 was isolated from THP-1 cell cultures as described (18) or purchased from Calibochem (Darmstadt, Germany). GST fusion proteins corresponding to the entire PEX9 domain (GST-PEX9), or truncated forms lacking blades 3–4 (GST-B1B2) or blades 1–2 (GST-B3B4) (Fig. 1A) were prepared exactly as described (16). PEX9-derived peptides (Table 2) were synthesized on an automated multiple peptide synthesizer (AMS 422, ABIMED Analysentechnik GmbH, Langenfeld, Germany). Spatial localization of peptides within PEX9 was determined using the Chimera Program (version 1.5.3, Resource Biocomputing Visualization and Informatics, University of California at San Francisco, San Francisco, CA).

**Cell Adhesion and Soluble Binding Assays**—Adhesion assays were performed on 96-well plates coated with 0.5% BSA or various concentrations of appropriate proteins. Except when indicated, GST-PEX9 concentrations of 0.2 and 0.4 μM were chosen for adhesion and soluble binding assays, respectively. 2 × 10⁵ CLL cells were incubated with 1.4 ng/ml 2′,7′-bis(carboxyethyl)-5(6′)-carboxyfluorescein-acetoxyethyl ester (BCECF-AM, Molecular Probes) for 20 min, suspended in RPMI 1640/0.5% BSA (adhesion medium) and added to the coated wells. After 60 min at 37 °C, attached cells were lysed with PBS/0.1% SDS and quantified using a fluorescence analyzer (BMG Labtechnologies, Offenburg, Germany). For inhibition experiments, cells were incubated (30 min, 37 °C) with appropriate Abs (10 μg/ml) or peptides (500 μg/ml, 197–270 μM) prior to adding to the wells. For binding assays in solution, 1 × 10⁵ cells were incubated in 100 μl adhesion medium (for constitutive (pro)MMP-9 expression) or medium containing the various proteins or peptides for 30 min at room tempera-

**TABLE 2**

| Synthetic peptides prepared in this study |
|------------------------------------------|
| Blades B3B4-derived peptide |
| P3 (654–674) | PGVPLDTHDVFQYREKAYFC |
| Blades B1B2-derived peptides |
| P6 (521–541) | FDAIAEIQGLYLYLFKDKYYW |
| P7 (539–559) | KNYRFSQGGSGRFQGPLIA |
| P8 (556–576) | LLIADQPLPRKLDGVFER |
| P9 (574–590) | EERLKSLKFLFSGRQWVVYT |
| P10 (588–608) | VTYGASVLGPRLDKLQG |
| Reversed P6 peptide |
| P6rv | WYKGDKFLYLQNG1EAADF |

**TABLE 1**

| Clinical characteristics of CLL patients |
|-----------------------------------------|
| Patient | Sex/Age (years) | Stage | α4 (%) | CD38/ZAP70 | Ig status |
|---------|-----------------|-------|--------|------------|-----------|
| 1       | M/69            | C/IV  | 74     | −/+        | ND        |
| 2       | M/70            | B/II  | 75     | +/+        | ND        |
| 3       | M/79            | B/II  | 43     | +/−        | Unmutated |
| 4       | M/68            | B/II  | 12     | −/ND       | Unmutated |
| 5       | M/59            | C/IV  | 30     | +/−        | Mutated   |
| 6       | M/64            | A/1   | 35     | +/ND       | Mutated   |
| 7       | M/48            | B/I   | 10     | +/−        | ND        |
| 8       | F/54            | A/0   | 93     | +/ND       | Unmutated |
| 9       | M/68            | ND    | 36     | +/ND       | ND        |
| 10      | ND              | ND    | 99     | +/ND       | ND        |
| 11      | F/70            | C/IV  | 80     | +/ND       | ND        |
| 12      | M/66            | B/II  | 91     | +/−        | ND        |
| 13      | M/63            | B/II  | 61     | +/ND       | ND        |
| 14      | F/69            | C/IV  | 99     | +/ND       | ND        |
| 15      | M/62            | ND    | 90     | +/ND       | ND        |

*Clinical CLL staging was determined according to established classifications (1–2). Elevated expression of CD38, ZAP70, and an unmutated Ig status are considered bad prognostic markers (1–2).*
nature. After washing with ice-cold medium, cells were incubated with anti-MMP-9 rabbit polyclonal antibodies or control Abs (30 min, 4 °C), washed with cold PBS, and incubated (30 min, 4 °C) with Alexa Fluor 488-labeled secondary Abs. Surface-bound proteins were analyzed by flow cytometry. To calculate IC50 values, cells were incubated with increasing concentrations of peptides (average range, 2–270 μM), prior to the adhesion or soluble binding assays. Values were determined using the SigmaPlot program (Systat Software, Inc., San José, CA).

For cooperativity analyses between P6 and P3 peptides, CLL cells were incubated with a given concentration of one peptide (20 μM) and increasing concentrations of the other peptide (30 min, 37 °C) prior to the adhesion or soluble binding assays. To determine whether the combination of both peptides was synergistic or additive the CompuSyn software (BioSoft, Cambridge, UK) was used. This program allows the calculation of the combination index based on the algorithm of Chou and Talalay (19). Combination index values <1 indicate synergism, whereas values = 1 indicate an additive effect.

Cell Migration Assays—For chemotaxis assays, 5 × 105 CLL cells with or without previous incubation with the appropriate proteins or peptides were added to Transwell Filters (Costar, New York, NY) and allowed to migrate toward medium alone or containing 150 ng/ml CXCL12 in the lower chamber. After 24 h at 37 °C, transmigrated cells were counted by flow cytometry. For transendothelial migration, 0.75 × 105 HUVEC were plated on fibronectin-coated (10 g/ml) Transwell filters and expression of VCAM-1 (α4β1 integrin ligand, involved in transendothelial migration) on confluent monolayers was stimulated with 15 ng/ml TNF-α, for 16 h prior to the assay. 5 × 105 CLL cells, with or without previous incubation with the appropriate proteins or peptides, were added to the HUVEC monolayer, and, after 24 h, migration was quantitated as explained. In both types of assays, migrated cells were expressed as the percentage of the total number of cells added, also counted by flow cytometry.

Statistical Analyses—Statistical significance of the data were determined with a repeated measures analysis of variance using General Linear Model procedures of SAS 9.3 (SAS Institute, Cary NC). A p value of ≤ 0.05 was considered significant. Mean pairwise comparisons were computed with the two-tailed Student’s t test (α = 0.05) with the GraphPad InStat software (version 3.05, GraphPad Software, San Diego, CA). All values are expressed as means ± S.D.

RESULTS

Blades 1–2 in PEX9 Constitute a Novel Cell Adhesion-mediating Region and Primarily Interact with CD44 in CLL Cells—We recently reported that blades 3–4 of PEX9 (B3B4, Fig. 1A) supports CLL cell adhesion via α4β1 integrin and that the P3 sequence, contained in blade 4 (see Table 2), partially inhibits cell interaction with PEX9 and (pro)MMP-9 (17). To determine whether additional sites outside B3B4 were also involved in cell adhesion, we prepared GST fusion proteins containing either the entire PEX9 domain (GST-PEX9) or deletions of blades 3–4 (GST-B1B2, proMMP-9 residues 508–613) or blades 1–2 (GST-B3B4, proMMP-9 residues 609–707) (Fig. 1, A and B) and tested them in cell adhesion assays. All values represent the percentage of the total number of cells added. *, p < 0.05; **, p ≤ 0.01; ***, p ≤ 0.001.
age adhesion at the maximal concentration tested. Interestingly, GST-B3B4 and GST-B1B2 also mediated cell adhesion in a dose-dependent manner, with similar maximum values for both proteins (average 63 and 57% for GST-B3B4 and GST-B1B2, respectively). Thus, although the maximum % of cell attachment attained by these forms was lower than for the parental GST-PEX9 (p ≤ 0.05 for GST-B3B4 and p ≤ 0.01 for GST-B1B2), likely due to their truncated nature, GST-B1B2 was as efficient as GST-B3B4 in promoting cell adhesion. Cells did not attach to GST alone at any of the concentrations tested (Fig. 1C).

To identify the receptor recognizing GST-B1B2, we tested the effect of blocking α4β1 integrin or CD44 (both (pro)MMP-9 receptors (8)) function with antibodies on cell adhesion to this substrate. Cell preincubation with the function-blocking anti-α4 mAb HP2/1 had a mild effect (19% inhibition), whereas the anti-CD44 mAb HP2/9 was highly effective, reducing adhesion by 68% (Fig. 1D). The control HP1/7 antibody had no effect. This was clearly opposite to the inhibition pattern observed for adhesion to GST-B3B4 (Fig. 1D), where blocking CD44 had a minor effect (20% inhibition), and α4β1 was the primary receptor as we reported previously (17).

The PEX9 B1B2 Region Inhibits CLL Cell Chemotaxis and Transendothelial Migration—Constitutive (pro)MMP-9 is important for CLL cell migration and invasion (4, 6), and we previously showed that PEX9 or its B3B4 region inhibit this migration (17). We now studied whether the B1B2 PEX9 region modulated CLL cell chemotaxis and transendothelial migration. Cells from three different samples were incubated with 0.4 μM GST, GST-B1B2, or, for comparison, GST-B3B4 and GST-PEX9, and their migratory ability analyzed after 24 h using Transwell chambers. In chemotaxis assays, the average value for control cell migration in response to CXCL12 was 12%, and this was normalized to 100. Cell preincubation with GST had no effect (data not shown), but preincubation with GST-B1B2 inhibited chemotaxis by 72% and thus was as effective as the positive inhibitory controls GST-B3B4 (70% inhibition) or GST-PEX9 (77% inhibition) (Fig. 2A). The effect of GST-B1B2 was also analyzed in transendothelial migration assays. In these experiments, 18% control cells migrated in response to CXCL12, and this was normalized to 100. GST-B1B2 also significantly inhibited this migration by 62%, again comparable with the effect of GST-B3B4 (66%) or GST-PEX9 (69%) (Fig. 2B). Altogether, these results indicated that B1B2 was a functional region with the ability to modulate CLL cell adhesion and migration.

Identification of Functional Amino Acid Sequences within the PEX9 B1B2 Region—To identify the specific cell-binding sequence(s) within PEX9 blades 1–2, we prepared five overlapping synthetic peptides (P6–P10) spanning residues 521–608 of proMMP-9 (Table 2 and Fig. 3A) and tested their ability to block cell adhesion to GST-PEX9 (0.2 μM). In these initial experiments, peptides were used at 500 μg/ml, equivalent to 209 μM (P6 and P8), 212 μM (P7), 198 μM (P9), and 244 μM (P10) concentrations, all in a similar molar range. In the absence of peptides, 44% of cells (average, n = 8 different samples) attached to GST-PEX9, and this was normalized to 100. Fig. 3B shows that peptide P6, representing the FDIAEAIGNQLYLF-DGKYW sequence located in blade 1, was the most effective, impairing cell adhesion to GST-PEX9 by 59%. The adjacent peptide P7, containing a sequence (SRPQGPFL) shown previously to be involved in CD44 interactions in carcinoma cells (14), also inhibited adhesion although to a lesser extent (42% inhibition). Peptides P8, P9, and P10 did not significantly affect cell adhesion.

We next analyzed whether these peptides also affected cell binding of soluble GST-PEX9. CLL cells from four different patients were treated with peptides P6–P10 at the mentioned μM concentration, followed by incubation with GST-PEX9 and analysis by flow cytometry. Fig. 3C shows for a representative sample that, as observed for cell adhesion, P6 was the most effective peptide, impairing binding of soluble GST-PEX9 to CLL cells by 62%, whereas P7 diminished adhesion by 44% (average of seven different samples). Peptides P8, P9, and P10 had no effect.

Having identified P6 and P7 as the most effective inhibitory sequences in PEX9 blades 1–2, we further compared the effect of both peptides by performing dose-dependent inhibition analyses. After initial tests, the chosen coating concentration for GST-PEX9 in these experiments was 0.1 μM. 41% of control CLL cells attached to this concentration, and this was normalized to 100. The previously identified P3 peptide located in
blade 4 (17) was also included in these assays for comparison. Additionally, and as a control for specificity, we prepared a peptide containing the reverse sequence present in P6 (peptide P6rv, Table 2). Fig. 3D shows that both P6 and P7, but not the control peptide P6rv, inhibited cell adhesion to GST-PEX9 in a dose-dependent manner, with average IC$_{50}$ values of 90 µM (P6) and 280 µM (P7), thus confirming the above results on the different activity of both peptides. The IC$_{50}$ for P3 in these analyses was 35 µM. The reverse peptide P6rv did not inhibit adhesion at any concentration, confirming the specificity of the
sequences tested. Peptide inhibition of soluble GST-PEx9 cell binding was also dose-dependent, rendering IC_{50} values of 75, 220, and 40 μM, respectively, for P6, P7, and P3 (Fig. 3E). Altogether, these results established that peptides P6 and P7 were functional sequences in PEx9 blade 1 and that P6 was more effective than P7 in inhibiting CLL cell interaction with PEx9.

The P6 Sequence Interferes with CD44-Ligand Interaction—The results shown in Fig. 1B indicated that the B1B2 region primarily interacted with CD44. We therefore studied whether the two most active peptides from this region, P6 and P7, interfered with CD44-ligand binding. CLL cells, preincubated or not with P6 (209 μM), P7 (212 μM), or the control peptide P6rv (209 μM), were tested for adhesion to the natural CD44 ligand hyaluronan. 48% of control cells attached to hyaluronan, and this was normalized to 100. As observed for B1B2 in Fig. 1D, cell preincubation with P6 significantly reduced cell adhesion to hyaluronan by 58%, whereas preincubation with P7 produced a minor effect (25% inhibition), and P6rv did not inhibit adhesion (Fig. 4A). In contrast to this P6 activity, the P3 peptide (blade 4, Table 2), shown previously to impair α4β1 function (17), was a poor inhibitor of cell adhesion to hyaluronan (18% inhibition, Fig. 4A). Peptides P6, P7, and P3, displayed similar activities for inhibition of cell adhesion to proMMP-9 (42–48% inhibition), whereas the control P6rv peptide had no effect (Fig. 4B). To further establish the specificity of these peptides in interfering with (pro)MMP-9 cell binding, we studied their effect on CLL cell adhesion to VCAM-1, a well known ligand for α4β1 integrin. Fig. 4C shows that none of these peptides affected cell adhesion to VCAM-1, confirming the specificity of their effect on CLL cell interaction with (pro)MMP-9.

Cooperative Effect of Peptides P6 and P3 in Inhibiting CLL Cell Adhesion and Migration—We next studied whether the newly identified active site P6, present in blade 1, cooperated with the previously described P3 sequence located in blade 4, in inhibiting cell adhesion and migration. For comparison, the P7 peptide alone or combined with P3 was also included in these experiments. In initial experiments, CLL cells were preincubated with or without P3 (198 μM), P6 (209 μM), or both peptides together and tested for adhesion to GST-PEx9. Peptide P6rv (209 μM) alone or combined with P3 or P6 was also included as control in these experiments. Fig. 5A shows that P3 and P6 inhibited adhesion to this substrate by 65 and 66%, respectively, confirming our previous results. Importantly, the combination of both peptides significantly increased this inhibition to 81%, thus reducing adhesion to the basal levels induced by the control GST (13%) and indicating a cooperative effect between both sites. In contrast, combining P7 with P3 did not increase the individual effect of either peptide, confirming that only the P6 sequence specifically cooperated with P3. Additionally, combinations of these peptides with the control P6rv did not modify the effect of P6, P7, or P3 alone, further confirming the specificity of the P6-P3 combination.

A cooperative effect between P6 and P3 was also observed when analyzing soluble GST-PEx9 binding to CLL cells. In this case, the combination of P6 and P3 increased the inhibitory effect of P6 (70%) or P3 (76%) to 85%, again reducing binding to the basal levels attained by GST (13%) (Fig. 5B). All other peptide combinations had no effect (Fig. 5B).

Having established the specific cooperation of the P6 and P3 sequences in inhibiting cell adhesion, we next determined whether this cooperation was synergistic or additive. To this purpose, we measured the effect of increasing concentrations of one peptide (P3 or P6) in the absence or presence of a low concentration (20 μM) of the other peptide. Fig. 5, C and D, shows that the combination of P6 and P3 significantly increased the inhibitory effect of the individual peptides at all concentrations tested. The calculated combination index in these assays was <1 in both cases and for all concentrations tested (Fig. 5, C and D), indicating that the cooperation of peptides P6 and P3 was synergistic.

We next tested the effect of these peptides, alone or combined, on CLL cell migration. Preincubation of CLL cells (five different samples) with P6 or P3 reduced CXCL12-induced cell chemotaxis (average control, 12%; normalized to 100) by 48 and 54%, respectively, and the combination of both peptides reduced migration to basal levels in the absence of chemokine (Fig. 6A). Likewise, P6 and P3 impaired CLL transendothelial migration (average control, 16%; normalized to 100) by 55 and 66%, respectively, and the combination of both peptides completely inhibited chemokine-induced cell migration (Fig. 6B). Combinations of P6 or P3 with the control P6rv did not increase the inhibition attained by P6 or P3 alone (Fig. 6, A and B). In agreement with the observed P3-P6 cooperation, spatial localization...
analyses indicated that these two sequences were located in close proximity within the PEX9 central pocket (Fig. 6C).

DISCUSSION

We have studied whether the B1B2 region (blades 1–2) of PEX9 had a functional role in (pro)MMP-9 interaction with CLL cells. Our major findings are as follows: 1) the B1B2 region mediates cell adhesion by interacting primarily with CD44; 2) B1B2 is a good inhibitor of CLL cell migration; 3) the most active sequence in B1B2 is FDAIAEIGNQLYLFKDGKYW, located in blade 1 and represented by the synthetic peptide P6; and 4) P6 synergistically cooperates with the previously described sequence P3 (FPGVPLDTHVFQYREKAYFC, blade 4) to increase inhibition of cell adhesion and migration.

The GST–B1B2 fusion protein studied here effectively mediated CLL cell adhesion and inhibited cell migration. This
behavior was similar to the previously described functions for the GST-B3B4 protein, containing blades 3–4 of PEX9 (17). However, there were major differences regarding the receptors involved in the recognition of both proteins. Although we previously identified \(\alpha_4\beta_1\) integrin as the main receptor for the B3B4 region (17), our present results clearly indicate that cell interaction with the B1B2 region primarily involved CD44. This is in agreement with the known role for CD44 as a docking receptor for (pro)MMP-9 in many cell systems and with its ability to bind to the PEX9 domain (7, 8, 20, 21). Moreover, blade 1 was shown to be required for CD44 binding to PEX9 in transfected COS-1 cells (15) and for binding to the PEX domain of MT1-MMP (MMP-14) in several carcinoma cell lines (22), also in line with the CD44 recognition of the B1B2 region reported here. In our previous report (17), the HP2/9 anti-\(\alpha_4\beta_1\) antibody did not impair cell adhesion to GST-PEX9 and silencing CD44 had a limited effect (19% inhibition). Therefore, one possible explanation for our current results on the activity of GST-B1B2/CD44 interaction is that the site recognized by CD44 in B1B2 may be totally or partially cryptic within the immobilized PEX9 domain. An alternative explanation is that the interaction of PEX9 with \(\alpha_4\beta_1\) integrin (via binding to B3B4) is of higher affinity than the interaction with CD44, thus prevailing in adhesion to the entire PEX9 domain. In support of this, soluble GST-B3B4 was able to bind to CLL cells while soluble GST-B1B2 only bound poorly (17).

Using a series of overlapping peptides we have identified the FDAIAEIGNQLYLFKDGYKW sequence in blade 1 as a novel CD44 binding site in PEX9. The synthetic peptide P6 containing this sequence effectively inhibited CLL cell interaction with PEX9, as well as chemotaxis and transendothelial migration. This suggests that P6 and the HP2/9 antibody may bind to different sites in CD44 and thus affect its properties differentially. Importantly, P6 also inhibited CLL cell adhesion to hyaluronan, the natural ligand for CD44, further confirming that the P6 sequence bound to CD44 and blocked its subsequent functions. In a previous report (15), Dufour et al. identified a different site in PEX9, SRPQGPFL, located in the outermost \(\beta\)-strand of blade 1, that when presented as a synthetic peptide inhibited (pro)MMP-9-CD44 interaction and COS-1 and carcinoma cell migration. In our present study, this sequence was contained in peptide P7 (KYWRFSEGRGSRPQGPFLIA) and also inhibited CLL cell interaction with PEX9. However, there were several differences between the P6 and P7 peptides: 1) the inhibitory effect of P7 was weaker than the effect of P6, with IC\(_{50}\) values of 280 and 90 \(\mu\)M, respectively; 2) P7 was a poor inhibitor of cell adhesion to hyaluronan. Thus, our results do not contradict the report by Dufour et al. (15) but reveals a new sequence within blade 1, which is more active in CLL cells than SRPQGPFL. Because peptide P6 was not tested in the study of Dufour et al. (15), it is not known if the P6 sequence is also active in other cell systems.

Besides displaying different inhibitory activity, peptides P6 and P7 also differ in their spatial location within blade 1 of PEX9. Although P7 localizes in the outermost \(\beta\)-strand, P6 is located within the central cavity (Figs. 3A and 5E). The central localization of P6 is similar to that of peptide P3 (PGVPLDTH-DVFQYREKAYFC), which we reported previously to bind to \(\alpha_4\beta_1\) integrin and impair \(\alpha_4\beta_1\)-MMP-9-induced functions in CLL cells (17). Interestingly, two recent studies have identified...
small molecules that bind to PEX9 or to the PEX domain of MMP-14 and inhibit carcinoma cell migration, proliferation, and tumor growth (16, 23). The binding site of these compounds was mapped by in silico approaches to the central cavity in PEX9, further supporting that this is the main cell-binding region in the PEX domain and thus constitutes a target. Moreover, and in agreement with the spatial proximity of P6 and P3, our study shows that P6 synergistically cooperated with P3, resulting in significantly increased inhibition of cell adhesion and migration. The fact that a similar cooperation between P7 and P3 was not observed, further supports that the P6 sequence is the most active site in blade 1 and specifically coordinates with P3 to ensure a stronger effect.

Our present report therefore establishes that PEX9 binding to CLL cells involves the cooperative interaction of the P6 sequence (blade 1) with CD44 and the P3 sequence (blade 4) with α4β1 integrin. Numerous studies have addressed the role of α4β1 in CLL and revealed its importance in CLL aggressiveness, in vitro and in vivo migration, cell survival, and drug resistance (9, 24–29). Indeed, some of these functions are induced by α4β1 upon binding to (pro)MMP-9/PEX9 (9). The role of CD44 in CLL has been less studied, but we previously showed that blocking CD44 interaction with (pro)MMP-9 prevented the (pro)MMP-9-induced survival effect in CLL cells (9). Additionally, several reports have shown that CD44 interaction with hyaluronan (30–32) or (pro)MMP-9/PEX9 (8) and contribute to CLL pathology, targeting the identified PEX9 sequences that bind to these receptors should constitute a good therapeutic approach to prevent CLL progression.

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