Identification of a Glycoprotein Ligand for E-selectin on Mouse Myeloid Cells

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Abstract. E-selectin is an inducible endothelial cell adhesion molecule for neutrophils which functions as a Ca\textsuperscript{2+}-dependent lectin. Using a recombinant, antibody-like form of mouse E-selectin, we have searched for glycoprotein ligands on mouse neutrophils and the neutrophil progenitor cell line 32D cl 3. We have identified a 150-kD glycoprotein as the only protein which could be affinity-isolated with soluble E-selectin from \[^{35}S\]methionine/\[^{35}S\]cysteine-labeled 32D cl 3 cells. Binding of this protein was strictly Ca\textsuperscript{2+}-dependent, was blocked by a cell adhesion-blocking mAb against mouse E-selectin, and required the presence of sialic acid on the 150-kD ligand. This glycoprotein was also affinity-isolated from mature neutrophils, in addition to a minor component at 250 kD, but could not be isolated from several other non-myeloid cell lines. The 150-kD glycoprotein was the only protein from 32D cl 3 cells, which was detectable by silverstaining after a one-step affinity-isolation.

The selectins form a class of three cell adhesion molecules which mediate the binding of leukocytes to endothelial cells (McEver, 1991; Vestweber, 1992). One of them, L-selectin (LAM-1, MEL-14-antigen), is expressed on the surface of most leukocyte types (Gallatin et al., 1986; Siegelman et al., 1989; Lasky et al., 1989; Tedder et al., 1990; Camerini et al., 1989) and is involved in the homing of lymphocytes into peripheral lymph nodes (Gallatin et al., 1983) as well as in the migration of neutrophils into sites of inflammation (Lewinsohn et al., 1987; Watson et al., 1991). The other two selectins, E-selectin (ELAM-1) and P-selectin (GMP-140, PADGEM, CD62), are expressed on endothelial cells (Bevilacqua et al., 1987; McEver et al., 1989) and bind to neutrophils, monocytic cells, and certain subsets of lymphocytes (Bevilacqua et al., 1989; Picker et al., 1991a; Shimizu et al., 1991; Geng et al., 1990; Damle et al., 1992).

In contrast to the vast majority of all known cell adhesion molecules, the selectins function as carbohydrate lectins. They share a highly homologous (60–65%) NH\textsubscript{2}-terminal lectin domain which resembles the Ca\textsuperscript{2+}-dependent lectin domains of animal lectins (Drickamer, 1988). Binding of the same tetrasaccharide structure, sialyl Lewis x, to E-, P-, and L-selectin has been demonstrated (Phillips et al., 1990; Polley et al., 1991; Foxall et al., 1992). However, the stereoisomeric form, sialyl Lewis a, can also bind to all three selectins (Berg et al., 1991a; Handa et al., 1991; Tyrrell et al., 1991; Berg et al., 1992), and lately also sulphated Lewis x and sulphated Lewis a carried by glycolipids, and lacking sialic acid have been reported to bind L- and E-selectin (Yuen et al., 1992; Green et al., 1992). To what extent the physiological ligands resemble these structures is still unknown. Also, whether different selectins share the same physiological ligands or have different ones, is an important question to answer.

This is especially interesting for the two endothelial selectins, E- and P-selectin, since they can interact with the same types of leukocytes. Differences in the regulation of the cell surface expression of the two selectins suggest that both can support leukocyte adhesion to endothelium in different situations. Only P-selectin is stored in intracellular granula, from which it can rapidly be mobilized to the cell surface within minutes upon induction with preinflammatory agents such as thrombin or histamine (Geng et al., 1990). However, both selectins can also be induced by the same stimuli and expressed simultaneously on the cell surface. The TNF-\alpha induced synthesis of E-selectin, which leads to a transient increase in the expression of the protein on the cell surface with maximal expression after 3–4 h of induction, could also be demonstrated for P-selectin on mouse endothelial cells (Weller et al., 1992; Hahne et al., 1993). Since both selectins can be expressed on the cell surface simultaneously and since both interact with neutrophils, the physiological ligands for both selectins on neutrophils need to be identified to understand how E- and P-selectin cooperate in the process of neutrophil extravasation.

Some glycoprotein ligands for the selectins have been identified in the past. Best characterized is the L-selectin ligand GlyCAM-1. This is a 50-kD mucin-like molecule which was affinity-isolated from mesenteric lymph nodes using an L-selectin–IgG chimeric protein (Imai et al., 1991; Lasky et al., 1992). GlyCAM-1 is also recognized by the mAb MECA 79 which reacts with several glycoproteins on endothelial cells of peripheral lymph nodes (Berg et al., 1992).
purified P-selectin membrane protein as an affinity-probe, been described as a specific glycoprotein ligand on neutrophils, which functions as a presenter of sialyl Lewis x to the two endothelial selectins (Kishimoto et al., 1991; Picker et al., 1991b). However, in a direct biochemical approach using purified P-selectin membrane protein as an affinity-probe, L-selectin could not be demonstrated to bind P-selectin. Instead, a 120-kD glycoprotein which can form a disulfide-linked dimeric structure was identified as a ligand for P-selectin on human neutrophils (Moore et al., 1992). For E-selectin, a direct biochemical approach to identify a glycoprotein ligand has not yet been reported.

To search for a glycoprotein ligand of E-selectin on mouse myeloid cells, we chose a biochemical approach similar to that used to identify the L-selectin ligand GlyCAM-1. We constructed an E-selectin–IgG chimeric protein containing the first four NH₂-terminal domains of mouse E-selectin, fused to the constant region of human IgG. With this affinity-probe, a single 150-kD glycoprotein ligand was isolated from a mouse neutrophil progenitor cell line, which was also present on mature neutrophils. This protein could not be detected on a number of nonmyeloid cells. Binding of this glycoprotein to E-selectin–IgG is dependent on the presence of Ca²⁺, is inhibited by a mAb against mouse E-selectin, and requires sialic acid on the glycoprotein ligand.

Materials and Methods

**Cells**

The neutrophilic progenitor 32D cl 3 (Valtieri et al., 1987; Migliaccio et al., 1989) provided by Dr. Rovera (Wistar Institute, Philadelphia) was grown in DMEM with 20% FCS, supplemented with 10% conditioned medium of WEHI-3B cells (as source for IL-3). The following cell lines were grown in DMEM with 10% FCS: the monocytic cell line WEHI-3B (obtained from Dr. Klemm), mouse endothelioma bEnd.3 (obtained from Dr. Risau), mouse fibroblastic L-cells (obtained from Dr. Kemler), and mouse myeloma J558L (obtained from Dr. Roth). The mouse B lymphoma L1-2 (obtained from Dr. Holzmann) and the mouse lymphoma EL₄ F15 (obtained from Dr. Simon) were grown in RPMI with 10% FCS. J558L cells secreting either the E-selectin–IgG fusion protein or CD4-IgG were grown under serum-free conditions in IMDM supplemented with 5 μg/ml insulin, 0.3% of Primontone (Basel Institute for Immunology, Basel, Switzerland), and 50 μM β-mercaptoethanol. Polymorphonuclear granulocytes (PMNs) were freshly isolated from the femurs of 10-wk-old NMRI mice by flushing out the bone marrow cells with cold PBS, using a syringe and a 23-g needle. The cell suspension was filtered through a nylon tissue. In agreement with Lewinsohn et al. (1987), we found that 75–80% of such cell preparations are mature PMNs as analyzed by staining the cell nuclei of cytopsin preparations and by FACS analysis with the granulocyte marker RB6-8C5 (Dianova, Hamburg, FRG).

**Antibodies**

The rat IgM mAb 2K120 against mouse E-selectin was recently described (Hahne et al., 1993). Briefly, rat mAbs had been raised against TNF-α-induced bEnd.3 endothelioma cells and screened in a cell-surface ELISA for binding to intact, TNF-α-induced cells. The mAb 2K120 only detected its antigen on endothelioma cells if these cells had been pretreated with TNF-α and the antibody specifically recognized the mouse E-selectin–IgG but not an analogous mouse P-selectin–IgG chimeric protein (Hahne et al., 1993). For rat IgM mAb 28AG6 was raised against the E-selectin–IgG chimeric protein and recognizes the C-region of human IgG₁. The mAb against mouse L-selectin was the rat mAb MEL-14 (Gallatin et al., 1985).

**E-selectin–IgG Chimeric Protein**

The construction of the E-selectin–IgG chimera has been described (Hahne et al., 1993). Briefly, the cDNA sequence for the first four NH₂-terminal domains of E-selectin (lectin, EGF, first two complement binding domains) was fused to the C-part ( hinge, C1 and C2 domains) of human IgG. The chimeric protein was expressed in J558L myeloma cells using the expression vector pCD4-Hgl CEI (provided by Dr. Klaus Karjalainen, Basel; Traunecker et al., 1991) and purified from culture supernatant by affinity chromatography with protein A-Sepharose. The fusion protein CD4-IgG was purified in a similar way from transfected J558L cells which were kindly provided by Dr. Klaus Karjalainen.

**Affinity Isolation of the Metabolically Labeled E-selectin Ligand and Immunoprecipitations**

32D cl 3 cells were labeled overnight with 200 μCi [³⁵S]methionine and 200 μCi [³⁵S]cysteine in 1 ml medium (5 × 10⁶ cells/ml) in MEM without methionine and cysteine, supplemented with 10% FCS and 10% conditioned medium (from WEHI 3B cells), both dialyzed against PBS. Other nonmyeloid cell lines were labeled similarly, but without the conditioned medium. For sialylated PMNs from mouse bone marrow were labeled in 500 μl of the same medium (without the conditioned medium supplemented with 400 μCi [³⁵S]methionine and 400 μCi [³⁵S]cysteine for 3 h.

Labeled cells were lysed at a density of 0.5–1 × 10⁷ cells/ml in lysis buffer (0.5% Triton X-100; 50 mM Tris-Cl, pH 7.5; 150 mM NaCl, 1 mM CaCl₂, 1 mg/ml Ovalbumin, 1 mM PMSF) for 10 min, insoluble material was pelleted at 10,000 g for 10 min; these and all subsequent steps were performed at 4°C. The lysate was incubated for 30 min with 50 μl of packed protein A-Sepharose. After removal of the sepharose beads, carrying unspecifically bound proteins, the cell extract was aliquoted and a fraction routinely corresponding to 1 × 10⁶ cells was incubated with 10–20 μl of protein A-Sepharose preloaded overnight with 7.5–8 μg of E-selectin–IgG or CD4-IgG. In cases where antigens were immunoprecipitated by mAbs, 20 μl of protein A-Sepharose preloaded with 20 μg rabbit anti-rat IgG and 20 μg of rat mAb were used. After 3-h incubation, the resin was washed five times with washing buffer (0.05% Triton X-100; 50 mM Tris-Cl, pH 8.5; 50 mM NaCl). In cases where EDTA washed resin was eluted with SDS-sample buffer, the resin was washed five times with washing-buffer containing 5 mM EDTA instead of 1 mM CaCl₂. To test the inhibitory effect of the anti E-selectin mAb 2K120 on the interaction of E-selectin–IgG with the E-selectin–ligand, 20 μl protein A-Sepharose loaded with 10 μg of E-selectin–IgG was incubated overnight with 200 μg of the IgM mAb 2K120 and in controls with 200 μg of the IgM mAb 28AG6 which recognizes the C-part of human IgG₁.

**Sialidase and Endoglycosidase F Treatment of the E-selectin Ligand**

The 150-kD E-selectin ligand was affinity isolated from [³⁵S]methionine/ [³⁵S]cysteine–labeled 32D cl 3 cells as described above and eluted with EDTA. 40 μl EDTA-eluate corresponding to 10⁶ cells was supplemented with 0.05% Triton X-100 and digested overnight at 37°C without (control) or with 0.5 U (10 μl) of Endoglycosidase F (Boehringer, Mannheim, FRG).

For sialidase treatment of the ligand, elution of the ligand from the affinity matrix was done in 3 mM EDTA, 25 mM Na acetate pH 5.5; 50 mM NaCl; and 0.025% Triton X-100. 50 μl EDTA-eluate corresponding to 3 × 10⁶ labeled cells was digested with 1 U of neuraminidase from Arthrobacter ureafaciens (Calbiochem-Novabiochem Corp., La Jolla, CA) for 6 h at 37°C. Mock treatment was done under identical conditions in the absence of the enzyme. Half of the digest was set aside for PAGE while the other half was again incubated with E-selectin–IgG protein A-Sepharose in the presence of 6 mM CaCl₂ for 3 h, and then washed and eluted as described above.
Partial Purification of the E-selectin Ligand

The fusion protein was covalently conjugated to the matrix using the dimethylpimelidate orientation kit (Pierce, Rockford, IL). The extract was incubated with the affinity matrix for 6 h at 4°C and the matrix was washed five times with the same buffer (each wash with 50 ml buffer). In the final wash, CaCl₂ was omitted. The matrix was eluted with 80 μl 20 mM Tris-HCl, pH 7.0; 10 mM EDTA. Half of the eluted material was electrophoresed on a polyacrylamide gel and detected by silverstaining. As estimated using a BSA standard, 20 ng of the E-selectin-ligand was obtained from 2.5 × 10⁶ cells.

Cell Adhesion Assay with Plastic-coated E-selectin-IgG

Flow Cytometry

32D cl 3 cells were incubated in PBS containing 10% normal mouse serum, then with E-selectin-IgG (25 μg/ml) or CD4-IgG (25 μg/ml), and finally with DTAF-conjugated goat anti-human IgG (6 μg/ml, Dianova, Hamburg, FRG). All incubations were performed for 30 min on ice in PBS containing 1 mM CaCl₂, 1 mM MgCl₂, 2% FCS (heat-inactivated), 0.02% sodium azide. Between incubations, cells were washed twice in the same buffer. To analyze if the binding of E-selectin-IgG to 32D cl 3 cells was Ca²⁺-dependent, cells were incubated and washed in a buffer containing 1 mM EDTA, and no divalent cations. After the last wash, cells were analyzed in a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Results

E-selectin-IgG Specifically Binds to the Mouse Neutrophil Progenitor Cell 32D cl 3

To identify a ligand for E-selectin on mouse myeloid cells, we used a chimeric protein consisting of the first four NH₂-terminal domains of murine E-selectin fused to the hinge domain and the two constant domains of the Fe part of human IgG (Hahne et al., 1993). Using protein A-Sepharose, the chimeric protein was affinity-purified from culture supernatant of transfected myeloma cells. A Coomassie stained polyacrylamide gel with 1 μg of the purified chimeric protein, electrophoresed under reducing and nonreducing conditions, is shown in Fig. 1. Since it has been reported that L-selectin on human neutrophils binds to human E-selectin, we tested whether the anti-mouse L-selectin mAb MEL-14 could inhibit the binding of 32D cl 3 cells to E-selectin-IgG. Even at high concentrations (200 μg/ml), MEL-14 did not inhibit cell attachment in our assay (Fig. 2 B). This suggests that binding of 32D cl 3 cells to E-selectin-IgG is not mediated by L-selectin.

Qualitatively similar results were obtained by FACS analysis with 32D cl 3 cells and the soluble E-selectin-IgG fusion protein. No binding above background was detectable for CD4-IgG, while E-selectin-IgG specifically bound to the cells. Binding was undetectable when E-selectin-IgG was incubated with the cells in the presence of 1 mM EDTA (see Fig. 3).

E-selectin-IgG Specifically Binds a Single 150-kD Glycoprotein from 32D cl 3 Cells

The E-selectin-IgG fusion protein, bound to protein A-Sepharose, was used as an affinity matrix to identify and isolate glycoprotein ligand(s) from detergent extracts of 32D cl 3 cells. Cells were metabolically labeled with [³⁵S]methionine and [³⁵S] cysteine and aliquots of the detergent extract were incubated with E-selectin-IgG or CD4-IgG bound to protein A-Sepharose. Proteins which were still bound to the affinity matrix beads after washing in the presence of 1 mM Ca²⁺ were eluted with SDS-containing PAGE-loading buffer and separated by gel electrophoresis (see Fig. 4, lanes 1 and 2). Among many proteins binding to both matrices, one protein at 150 kD was detected which was eluted from the E-selectin-IgG matrix (Fig. 4, lane 2), but not from the CD4-IgG matrix (Fig. 4, lane 1). This was the only protein which could be completely removed from a separate aliquot of the E-selectin-IgG matrix by washing the beads first in the pres-
Figure 2. 32D cl 3 cells specifically bind to E-selectin-IgG. (A) 32D cl 3 cells were allowed to bind to 96 well plates coated either with CD4-IgG, or E-selectin-IgG, as indicated. Binding was for 20 min at 7°C under mild rotation (50 rpm) as described under Materials and Methods. Bar, 50 μm. (B) Quantitation of cell binding of 32D cl 3 cells to CD4-IgG (left bar) or E-selectin-IgG (all other bars)-coated 96 well plates. Before the addition of cells, the E-selectin-IgG-coated wells were incubated for 1 h at 7°C either with HBSS (−) or with HBSS containing 1mM EDTA (EDTA) or with the indicated concentrations of the mAb 21KC10 (anti-mouse E-selectin) or the class type matched mAb 28AG6 (anti-human IgG, Fc-part). The inhibitory effect of the anti-mouse L-selectin mAb MEL-14 was tested by preincubating 32D cl 3 cells with the indicated concentrations of MEL-14 mAb for 1 h at 7°C. Bound cells were quantitated by counting the cells under the microscope in 10 randomly chosen areas of defined size in each well. Each bar represents the mean ± SD for four wells. The depicted experiment represents one out of three similar experiments.
Figure 3. Flow cytometry with 32D cl 3 cells. 32D cl 3 cells were analyzed by flow cytometry with CD4-IgG and E-selectin-IgG, both in the presence of 1 mM CaCl₂, and E-selectin-IgG, in the presence of 1 mM EDTA (as indicated). IgG fusion proteins were detected with DTAF-conjugated goat anti-human IgG. The background signal with the second antibody is shown as a negative control.

Figure 4. Affinity isolation of a 150-kD protein from metabolically labeled 32D cl 3 cells using E-selectin-IgG. 32D cl 3 cells were metabolically labeled with [³⁵S]methionine and [³⁵S]cysteine and detergent extracts were incubated with protein A–Sepharose loaded with CD4-IgG (lane 1), E-selectin-IgG (lanes 2–4), mAb MEL-14 (lane 5), or an isotype matched control mAb (lane 6). The affinity matrices were washed in the presence of 1 mM CaCl₂ and eluted with PAGE-loading buffer (lanes 1, 2, 5, and 6). Alternatively, the E-selectin-IgG matrix was washed in the presence of 3 mM EDTA and eluted with PAGE-loading buffer (lane 3). When another aliquot of the E-selectin-IgG matrix was washed with a Ca²⁺-containing buffer and eluted with EDTA instead of SDS, the 150-kD protein was the only detectable protein in this eluate (Fig. 4, lane 4). Thus, a single protein of 150 kD from the detergent extract of [³⁵S]methionine/[³⁵S]cysteine–labeled 32D cl binds in a Ca²⁺-dependent fashion to E-selectin-IgG. This protein is clearly distinct from L-selectin which was immunoprecipitated with the mAb MEL-14 (Fig. 4, lane 5). The Ca²⁺-dependent binding of the 150-kD protein to E-selectin-IgG and the lack of binding to CD4-IgG suggest that the selectin part in the E-selectin–IgG fusion protein is a distinct protein from L-selectin.
Figure 6. The 150-kD E-selectin ligand contains intramolecular disulfide bridges and N-linked carbohydrate side chains. The 150-kD E-selectin ligand was affinity isolated from [³⁵S]methionine/[³⁵S]cysteine-labeled 32D cl 3 cells using E-selectin-IgG bound to protein A-Sepharose and was eluted with EDTA. The eluted material was either directly electrophoresed under nonreducing and reducing conditions (left panel) or incubated without (-) or with (+) 0.5 U of Endoglycosidase F for 12 h at 37°C, and then electrophoresed on an 8% polyacrylamide gel and visualized by fluorography (right panel). Molecular mass markers (in kD) are indicated on the left.

Sialic Acid on the 150-kD Glycoprotein Ligand Is Involved in the Binding to E-selectin-IgG

The carbohydrate ligand(s) for E-selectin on intact cells have been described as sialic acid containing structures (Walz et al., 1990; Phillips et al., 1990). To determine whether sialic acid would also be required for the specific binding of the 150-kD glycoprotein ligand to E-selectin-IgG, we treated the [³⁵S]methionine/[³⁵S]cysteine-labeled ligand which had been eluted from the E-selectin-IgG matrix with EDTA and electrophoresed under nonreducing and reducing conditions. The apparent molecular mass under nonreducing conditions is 130 kD. Treatment of the EDTA eluted E-selectin-ligand with the Endoglycosidase F decreases the apparent molecular mass from 150 to 135 kD (Fig. 6, right panel) suggesting that the 150-kD ligand is a glycoprotein.

Among all the cell lines that we have analyzed in such affinity-isolation experiments with the E-selectin-IgG fusion protein, the 150-kD ligand was only detected in the 32D cl 3 cells. Results are shown in Fig. 7 for the neutrophilic progenitor 32D cl 3, the B-lymphoma L1-2, the lymphoma EL4, FL5, the endothelioma bEnd.3, and the fibroblastic L-cells. This indicates that the 150-kD glycoprotein or the structural motif of this glycoprotein, which allows the specific binding to E-selectin, is specifically expressed on myeloid cells.

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The 150-kD E-selectin ligand is only found on the neutrophil progenitor 32D cl 3. Five different cell lines, all labeled with [35S]methionine and [35S]cysteine were analyzed by affinity-isolation experiments with E-selectin-IgG or CD4-IgG bound to protein A-Sepharose (as indicated). The analyzed cell lines were 32D cl 3 (neutrophil progenitor), L1-2 (B-lymphoma), EL4 F15 (lymphoma), bEnd.3 (endothelioma), and L-cells (fibroblast). All five cell lysates applied to the affinity matrix contained equivalent amounts of incorporated radioactivity. Specifically bound, labeled proteins were eluted with EDTA, electrophoresed under reducing conditions on an 8% polyacrylamide gel and visualized by fluorography. Molecular mass markers (in kD) are indicated on the left.

of the neuraminidase and the mock-treated samples were reprecipitated with E-selectin-IgG, only 20% of what was reprecipitable from the mock-treated sample was reprecipitated from the neuraminidase-treated sample. Thus, removal of sialic acid from the 150-kD glycoprotein ligand impairs the interaction with E-selectin-IgG.

**The 150-kD Glycoprotein Ligand Is Present on PMNs**

We tested whether the 150-kD glycoprotein ligand could also be detected on freshly isolated PMNs. To this end, PMNs were isolated from mouse bone marrow and metabolically labeled for 3 h with [35S]methionine and [35S]cysteine. Detergent extracts were incubated with protein A-Sepharose loaded either with CD4-IgG or E-selectin-IgG and bound proteins were eluted with SDS and electrophoresed (see Fig. 9 A, left panel). The E-selectin-IgG fusion protein specifically bound a protein from the PMN-lysate, which had the same molecular mass as the 150-kD glycoprotein ligand on 32D cl 3 cells (Fig. 9 A, left panel).

Elution of the E-selectin-IgG matrix with EDTA instead of SDS reduced the level of unspecifically bound proteins in the eluate and allowed more sensitive analysis of the eluted material. Under these conditions a second minor ligand at ~250 kD was in addition to the 150-kD ligand detectable (Fig. 9 B).

Similar to the results with the 32D cl 3 neutrophilic progenitor, L-selectin could not be affinity-isolated with the E-selectin-IgG fusion protein from cell lysates of PMNs, although L-selectin was metabolically labeled and present in the cell detergent extract and was clearly detectable by the mAb MEL-14 (Fig. 9 A, right panel).

This lack of an interaction between L-selectin and the E-selectin-IgG fusion protein in such affinity-isolation experiments prompted us to test the effect of the mAb MEL-14 on the binding of PMNs to the E-selectin-IgG fusion protein coated onto plastic. The cell binding assay was performed under mild rotation (50 rpm) at 7°C. PMNs bound at a density of 1250 ± 230 cells/mm² to E-selectin-IgG and background binding to CD4-IgG was as low as 10 ± 8 cells/mm². Inhibition with MEL-14 was tried with 10, 100, and 200 μg/ml mAb. Even at the highest concentration cell attachment was only inhibited by ~20-30% indicating a minor role for L-selectin in these assays.

**Partial Purification of the E-selectin-Ligand**

We used the E-selectin-IgG chimeric protein to purify the 150-kD glycoprotein ligand from 32D cl 3 cells. To this end, the fusion protein was bound to protein A-Agarose and covalently crosslinked using dimethyl pimelidate. This affinity matrix was incubated with a Triton X-100 lysate of 32D cl
Figure 10. Partial purification of the 150-kD E-selectin ligand. The detergent extract of $5 \times 10^6$ 32D cl 3 cells was split and half of it was incubated with 15 $\mu$g E-selectin-IgG crosslinked with dimethyl pimelidate to protein A-Agarose (middle lane) and the other half was incubated with protein A-Agarose alone (right lane). Bound proteins were eluted with EDTA. As a control for proteins which bleed from the affinity matrix, protein A-Agarose crosslinked to E-selectin-IgG was EDTA eluted, without having been incubated with detergent extract from 32D cl 3 cells (left lane). Note that the 150-kD protein (indicated by an arrow) is the only protein in the partially purified material which originates from the 32D cl 3 cell extract. Proteins were electrophoresed on a 10% polyacrylamide gel under reducing conditions and visualized by silverstaining. Molecular mass markers (in kD) are indicated on the left.

3 cells and specifically bound proteins were eluted with EDTA. Fig. 10 (lane 2) shows the purified material from $2.5 \times 10^6$ cells electrophoresed on a 10% polyacrylamide gel and detected by silver-staining. The 150-kD ligand represented the major protein in this purification. The additional bands in the gel originated from proteins which were bleeding from the affinity matrix since these proteins were also eluted with EDTA from the same amount of affinity matrix beads which had not been incubated with the 32D cl 3 cell lysate (Fig. 10, lane J). No proteins were detected when protein A-Agarose beads were eluted with EDTA after incubation with the same amount of 32D cl 3 cell lysate as had been added to the E-selectin-IgG affinity matrix (Fig. 10, lane J). The amount of purified 150-kD ligand was roughly estimated by comparison with BSA standards to be 20 ng of protein per $2.5 \times 10^6$ cells.

Figure 9. The 150-kD E-selectin ligand can be detected on isolated mouse PMNs. (A) 32D cl 3 cells and freshly isolated PMNs from mouse bone marrow (as indicated) were metabolically labeled with [$^{35}$S]methionine/$^{35}$S)cytsteine and detergent extracts were incubated with E-selectin-IgG, CD4-IgG, mAb MEL-14, or an isotype matched control mAb bound to protein A-Sepharose (as indicated). After washing in the presence of Ca$^{2+}$, the beads were eluted with PAGE-loading buffer. The 150-kD ligand, affinity isolated by E-selectin-IgG from 32D cl 3 cells, was also isolated from PMNs and is indicated by an arrow head. Mouse L-selectin was only detectable by the mAb MEL-14 (right panel). (B) If labeled proteins from PMNs, bound to E-selectin-IgG, were eluted with EDTA, the 150-kD E-selectin ligand was clearly visible as well as a second, minor ligand at ~250 kD. Labeled proteins were electrophoresed under reducing conditions on 8% polyacrylamide gels and visualized by fluorography. Molecular mass markers (in kD) are indicated on the left.
**Discussion**

We have identified a 150-kD glycoprotein ligand for E-selectin on mouse neutrophils and a mouse myeloid cell line using immobilized E-selectin-IgG fusion protein as affinity matrix. The specific requirements for the binding of this ligand to E-selectin-IgG are similar to those known for the binding of neutrophils to E-selectin.

Selectin-IgG fusion proteins have been successfully used in the past as valuable tools to analyze the binding of selectins to cells and to carbohydrate ligands. An L-selectin-IgG fusion protein was shown to inhibit lymphocyte binding to lymph node high endothelial venules (Watson et al., 1990) and neutrophil influx into sites of inflammation (Watson et al., 1991), and was successfully used to identify and clone the L-selectin ligand GlyCAM-1 (Imai et al., 1991; Lasky et al., 1992). Analogous fusion proteins for human E- and P-selectin have also been used in various carbohydrate-ligand binding studies (Walz et al., 1990; Aruffo et al., 1991; Erbe et al., 1992), and in immunohistology experiments on tumor tissue (Aruffo et al., 1992).

We find that the mouse E-selectin-IgG fusion protein described here binds in a selectin-specific manner to mouse myeloid cells as demonstrated by flow cytometry and in cell substrate adhesion assays. No specific binding was observed with CD4-IgG. Specific cell-binding to E-selectin-IgG was absolutely dependent on the presence of calcium and was blocked by the mAb 21KC10 against mouse E-selectin but not by a control antibody to the Fc-part of the fusion protein.

The E-selectin-IgG fusion protein specifically binds to only one single 150-kD glycoprotein in detergent extracts of the neutrophil progenitor 32D cl 3, labeled with [35S]methionine and [35S]cysteine. This binding is selectin-specific by the following criteria: First, no binding of the protein occurs to CD4-IgG. Specific cell-binding to E-selectin-IgG was absolutely dependent on the presence of calcium and was blocked by the mAb 21KC10 against mouse E-selectin but not by a control antibody to the Fc-part of the fusion protein. Second, the binding in selectin-specific by the following criteria: First, no binding of the protein occurs to CD4-IgG. Specific cell-binding to E-selectin-IgG was absolutely dependent on the presence of calcium and was blocked by the mAb 21KC10 against mouse E-selectin but not by a control antibody to the Fc-part of the fusion protein. Third, the binding is partially inhibited by the anti-mouse E-selectin mAb 21KC10, but not by an anti-human IgG (Fc-part) specific mAb. Fourth, the binding is drastically reduced (by 80%), if sialic acid is removed from the glycoprotein ligand by treatment with sialidase. Furthermore, the 150-kD ligand could not be affinity-isolated from several nonmyeloid cell lines indicating that either the whole protein or its selectin-specific binding motif is specific for myeloid cells. Also, mature neutrophils isolated from mouse bone marrow expressed the 150-kD glycoprotein. In addition, a minor, not further characterized ligand at ~250 kD was observed.

Interestingly, the major ligand for P-selectin on human myeloid cells seems to be different from the 150-kD ligand for E-selectin. As was shown by Moore et al. (1992), human P-selectin binds to a 120-kD glycoprotein expressed by human neutrophils as well as by the human monocytic cell line HL60. This protein forms a 250-kD dimeric structure under nonreducing conditions. In contrast, the 150-kD protein, which we identified as a ligand for mouse E-selectin, does not form a disulfide-linked dimer and exhibits an apparent molecular mass of 130 kD under nonreducing conditions. Furthermore, treatment of the P-selectin ligand with sialidase decreases its electrophoretic mobility (Moore et al., 1992), while this treatment increases the electrophoretic mobility of the E-selectin ligand. However, a minor ligand on neutrophils at ~250 kD apparent molecular mass (under reducing conditions) that we observed for E-selectin was also reported for P-selectin. Since both, E- and P-selectin have been demonstrated to bind to the same tetrasaccharide structure sialyl Lewis x, it will be important to directly compare the physiological ligands of these two selectins in the future.

L-selectin on neutrophils has been described to bind to E- and P-selectin (Kishimoto et al., 1991; Picker et al., 1991b). Binding of E- and P-selectin transfected cells to neutrophils could be inhibited by antibodies to L-selectin and the transfected cells bound to purified L-selectin. However, direct binding of L-selectin to the isolated E- and P-selectin proteins in biochemical experiments has not been demonstrated. Moore et al. (1992) did not detect binding of iodinated P-selectin to L-selectin in Western blots with neutrophil membrane proteins. Also, we were unable to affinity-isolate L-selectin with E-selectin-IgG from detergent extracts of neutrophils and 32D cl 3 cells after metabolically labeling the cells with [35S]methionine and [35S]cysteine. Accordingly, in cell adhesion assays the anti-mouse L-selectin mAb MEL-14 had no effect on the binding of 32D cl 3 cells to E-selectin-IgG. Thus, the 150-kD glycoprotein ligand of E-selectin is the only glycoprotein candidate for a cell adhesion ligand on 32D cl 3 cells. Adhesion of neutrophils to E-selectin-IgG was weakly (by ~20-30%) inhibited by mAb MEL-14. The more prominent role that was reported for L-selectin in the binding of human neutrophils to E-selectin—expressing cells (Kishimoto et al., 1991; Picker et al., 1991b) might be due to differences in the assay conditions (e.g., different temperature). Our data argue for a minor role of L-selectin in the binding of mouse neutrophils to E-selectin. If L-selectin directly binds to E-selectin, the affinity of this interaction is not sufficient to allow affinity-isolation from cell detergent extracts. However, a concentration of L-selectin on surface microvilli of human neutrophils (Picker et al., 1991b) might compensate for such a low binding affinity. It is conceivable that multiple ligands on neutrophils mediate the binding to E-selectin. Which of them plays the predominant role in vivo still needs to be analyzed.

The tetrasaccharide sialyl Lewis x (sLe\(^x\)) can bind to all three selectins, yet the complete structure of the carbohydrate part of the physiological ligands for all three selectins is not known. The L-selectin ligand GlyCAM-1 has been demonstrated to be a strongly O-glycosylated mucin like protein whose O-linked carbohydrate side chains mediate the binding to L-selectin (Lasky et al., 1992). Since O-glycosylation but not N-glycosylation of cell surface structures has also been shown to be involved in binding to E-selectin (Kojima et al., 1992; Leeuwenberg et al., 1991), it is possible that also the 150-kD E-selectin ligand contains mucin-like structural elements. This needs to be directly analyzed in the future. It is interesting that the binding of \(^{125}I\)-labeled purified P-selectin to human neutrophils could not be blocked by sLe\(^x\)-containing neo-glycoproteins (Moore et al., 1991). Furthermore, several sLe\(^x\)-containing glycoproteins, including L-selectin, were not detected by \(^{125}I\)-labeled P-selectin in Western blots. This suggests that structural features in addition to the tetrasaccharide sLe\(^x\) are involved in high affinity binding of the physiological ligand to P-selectin. Which carbohydrate structure, in addition to sialic acid, is relevant for the binding of the identified 150 kD glycoprotein ligand to mouse E-selectin, will be important to analyze.
Other potential candidates for glycoprotein-ligands of E-selectin are the carcinoembryonic antigen-like non-specific crossreacting antigens. A mAb against human E-selectin inhibited the binding of soluble carcinoembryonic antigens (prepared from perchloric acid extracts of human colonic adenocarcinomas) to cytokine activated human endothelial cells, suggesting a possible role for NCA-160 and NCA-90 as presenters of sialyl Lewis x to E-selectin (Kuijpers et al., 1992). However, the electrophoretic migration of these antigens as relatively broad bands in polyacrylamide gel argues against a possible relation to the 150-kD glycoprotein ligand which we describe here.

Among all proteins extracted with detergent from the neutrophilic progenitor 32D cl 3, only a single 150-kD glycoprotein binds specifically to the E-selectin--IgG fusion protein. It will be of major importance to define the binding motif on this glycoprotein which is responsible for this strong and highly specific binding to E-selectin. This knowledge may be valuable to construct highly specific E-selectin ligands which could block the putative role of E-selectin in inflammation.

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