Journal of Cell Science 110, 687-694 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
JCS9531

The E-selectin-ligand ESL-1 is located in the Golgi as well as on microvilli on the cell surface

Martin Steegmaier[1], Eric Borges[1], Jürgen Berger[2], Heinz Schwarz[2] and Dietmar Vestweber[1,*]

[1]Institute of Cell Biology, ZMBE, University of Münster, Germany
[2]Max-Planck-Institute of Developmental Biology, Tübingen, Germany

*Author for correspondence

SUMMARY

Neutrophils and subsets of lymphocytes bind to E-selectin, a cytokine inducible adhesion molecule on endothelial cells. The E-selectin-ligand-1 (ESL-1) is a high affinity glycoprotein ligand which participates in the binding of mouse myeloid cells to E-selectin. The sequence of mouse ESL-1 is highly homologous to the cysteine rich FGF receptor (CFR) in chicken and the rat Golgi protein MG160. We have analysed the subcellular distribution of ESL-1 by indirect immunofluorescence, flow cytometry, various biochemical techniques and by immunogold scanning electron microscopy. We could localize ESL-1 in the Golgi as well as on the cell surface of 32Dcl3 cells and neutrophils. Cell surface staining was confirmed by cell surface biotinylation and by cell surface immunoprecipitations in which antibodies only had access to surface proteins on intact cells. In addition, ESL-1^{high} and ESL-1^{low} expressing cells, sorted by flow cytometry, gave rise to high and low immunoprecipitation signals for ESL-1, respectively. Based on immunogold labeling of intact cells, we localized ESL-1 on microvilli of 32Dcl3 cells and of the lymphoma cell line K46. Quantitative evaluation determined 80% of the total labeling for ESL-1 on microvilli of K46 cells while 69% of the labeling for the control antigen B220 was found on the planar cell surface. These data indicate that ESL-1 occurs at sites on the leukocyte cell surface which are destined for the initiation of cell contacts to the endothelium.

Key words: Selectin, Selectin-ligand, Leukocyte microvilli

INTRODUCTION

Leukocyte extravasation into tissue is regulated by specific leukocyte-endothelial cell interactions with several families of cell adhesion molecules involved in that process (Butcher, 1991; Springer, 1994). The selectins are a group of three Ca^{2+}-dependent lectins that mediate the initial attachment and rolling of leukocytes on the vessel wall (Lasky, 1995; Vestweber, 1994). Selectin-mediated rolling is followed by an activation of leukocyte-integrins, which promotes stable attachment and, finally, leads to leukocyte-migration across the blood vessel wall. Two of the three selectins, E- and P-selectin (CD62E and CD62P), are expressed on endothelial cells upon stimulation with inflammatory mediators; L-selectin (CD62-L) is found on most types of leukocytes.

The E-selectin-ligand ESL-1 is a 150 kDa glycoprotein which was identified and isolated by using a recombinant antibody-like form of mouse E-selectin as an affinity probe (Levinovitz et al., 1993; Lenter et al., 1994). ESL-1 is a major ligand for E-selectin on mouse myeloid cells and mediates binding of these cells to E-selectin (Steegmaier et al., 1995). Although the protein is broadly distributed, only ESL-1 expressed by myeloid cells and some lymphoid cells (E. Borges and D. Vestweber, unpublished data) occurs in the correct glycoform which is capable of binding to E-selectin. Fucosylation of ESL-1 is essential for the generation of this glycoform (Steegmaier et al., 1995). For all known selectin ligands the correct posttranslational modification is imperative for selectin-binding. The P-selectin-ligand PSGL-1 needs to be fucosylated (Sako et al., 1993) as well as tyrosine-sulfated (Sako et al., 1995; Pouyani and Seed, 1995; Moore et al., 1995) in order to bind to P-selectin. Sulphation-requirements have also been shown for the L-selectin-ligand GlyCAM-1 (Imai et al., 1993), which carries sulfated carbohydrate side chains (Hemmerich and Rosen, 1994).

In addition to its posttranslational modifications, the topographic distribution of an adhesion receptor on the cell surface of leukocytes is a major determinant of their ability to mediate initial contacts to the endothelium under flow. The presentation of adhesion receptors on microvilli has been shown to facilitate the establishment of primary interactions between leukocytes and the vascular lining under physiologic shear forces (von Andrian et al., 1995). L-selectin, PSGL-1 and the leukocyte integrin $\alpha_4\beta_7$ which are all capable of initiating tethering and rolling of leukocytes, were all localized on microvilli of neutrophils or lymphocytes (Moore et al., 1995; Picker et al., 1991; Erlandsen et al., 1993; Berlin et al., 1993).

Molecular cloning of ESL-1 revealed the primary structure of a type-I transmembrane protein of 1,148 amino acids with a 1,114 amino acid extracellular domain containing 16 conserved cysteine-rich repeats (Steegmaier et al., 1995). Surprisingly, the sequence displayed a strong homology to a recently identified chicken cysteine-rich fibroblast growth

factor receptor (CFR) (Steegmaier et al., 1995; Burrus et al., 1992). The amino acid sequences of both proteins were found to be 94% identical, except for a unique 70 amino acid N-terminal domain of ESL-1 which has no correlate in CFR. CFR had been identified and isolated by ligand affinity chromatography from membranes of 7-day-old chicken embryos (Burrus et al., 1992; Burrus and Olwin, 1989) and was reported to bind specifically to FGF-1, FGF-2, FGF-4 and FGF-7 (Zhou and Olwin, 1994). CFR is not related to the family of tyrosine kinase FGF receptors or to FGF binding proteoglycans and the capacity of CFR to transduce signals upon binding to FGF has not yet been demonstrated.

Unexpectedly, ESL-1 also shares a high degree of sequence identity with a recently cloned membrane sialoglycoprotein of the rat Golgi apparatus (Gonatas et al., 1989, 1995). This rat glycoprotein, termed MG160, has also been shown to bind basic FGF (FGF-2) in ligand blotting experiments, however, in contrast to ESL-1 and CFR, MG160 has not been detected on the cell surface. Its biological function is not yet known.

In this study we have analysed the subcellular distribution of ESL-1 in leukocytes by indirect immunofluorescence, flow cytometry and biochemical detection methods. We found that ESL-1 is located in the Golgi as well as on the surface of leukocytes. A more detailed analysis of the ultrastructural distribution of ESL-1 using immunogold scanning electron microscopy, revealed preferential labeling for ESL-1 on microvilli on the leukocyte surface.

MATERIALS AND METHODS

Cells

The neutrophilic progenitor 32Dcl3 and the monocytic cell line WEHI-3B were cultured as described (Levinovitz et al., 1993). The mouse B lymphoma K46 (provided by Dr Reth, Max-Planck-Institute for Immunobiology, Freiburg, Germany) was grown in RPMI with 10% FCS and 50 μM β-mercaptoethanol. Mouse neutrophils (polymorphonuclear granulocytes) were freshly isolated from the femurs of 10-week-old NMRI mice as described (Levinovitz et al., 1993).

Antibodies

The antibodies from rabbit antiserum 89060 against ESL-IgG, affinity-purified on ESL-IgG were recently described (Steegmaier et al., 1995). Rabbit antiserum 96 was raised against a peptide covering the intracellular C-terminal 13 amino acids of ESL-1 (Steegmaier et al., 1995) and was affinity-purified on the same peptide by a method described before (Weller et al., 1992). Total rabbit IgG was purified from a nonimmune serum with Protein A-Sepharose. The following monoclonal antibodies (mAbs) were used: Mel-14 (rat IgG_{2a}; Gallatin et al., 1983) against mouse L-selectin; 10E9 (rat IgG_{2a}; Bosse and Vestweber, 1994) against mouse E-selectin; EA-3 (rat IgG_3; Piala et al., 1993) against mouse PECAM-1 (CD31), which was kindly provided by Dr Beat Imhof (Basel Inst. for Immunology, Basel, Switzerland); G1/133 (mouse IgG_1; Linstedt and Hauri, 1993) against the Golgi protein giantin, which was kindly provided by Dr Hans-Peter Hauri (Biocenter, Basel, Switzerland); RA3-6B2 (rat IgG_{2a}) against the mouse surface antigen B220, obtained from Dr Lamers (Max-Planck-Institute for Immunobiology, Freiburg, Germany) and B-5-1-2 (mouse IgG_1) against α-Tubulin, purchased from Sigma (St Louis, MO).

Immunofluorescence

32Dcl3 cells were allowed to settle on Bio-Rad adhesion slides (Bio-Rad, Hercules, CA) and subsequently fixed with 4% paraformaldehyde. The fixed cells were then permeabilized with 0.05% Triton X-100 in HBSS. Antibody incubations were done for 1 hour at room temperature in a humid chamber. As first antibodies, either affinity-purified anti-ESL-1 antibodies, total rabbit IgG or anti-giantin mouse mAb were used. All antibodies were used at 10 μg/ml in DMEM containing 10% FCS. As second stage antibodies either fluorescein-conjugated goat anti-rabbit IgG or rhodamine-conjugated rabbit anti-mouse IgG (Dianova, Hamburg, Germany) in DMEM containing 10% FCS were used. The cells were microscopically examined using an Axioskop 20 (Zeiss, Oberkochen, Germany) with filters selective for fluorescein or rhodamine.

Flow cytometry

32Dcl3 cells or PMNs were first incubated with affinity-purified rabbit antibodies, total rabbit IgG (30 μg/ml) or the mAbs Mel-14, EA-3 or 10E9 (10 μg/ml). FITC-conjugated goat anti-rabbit IgG or rabbit anti-rat IgG were used as second-stage reagents. All incubations were performed for 20 minutes on ice in PBS containing 3% FCS and 0.02% sodium azide. Between incubations, cells were washed with the same buffer. Finally, cells were analyzed using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Cell surface biotinylation

Surface biotinylation was done as described (Kim et al., 1994). In brief, 2×10^7 cells were washed twice with PBS and resuspended in 1 ml of PBS containing 0.5 mg/ml of *N*-hydroxysulfosuccinimide-Biotin (Pierce). Free succinimide groups were then blocked by the addition of 1 ml non-supplemented DMEM on ice for 5 minutes. After centrifugation this step was repeated and the cells were then washed 3 times with PBS and resuspended in 1 ml Triton X-100 lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$, 1% Triton X-100, 1 mM PMSF, 1 mM benzamidine). After 12 minutes of lysis on ice, insoluble material was removed by centrifugation for 15 minutes at 13,000 ***g***. The supernatant was precleared for subsequent immunoprecipitations by incubation with 50 μl of Protein A-Sepharose (Pharmacia, Freiburg, Germany).

Immunoprecipitation and immunoblots

Immunoprecipitations and immunoblots were performed as described previously (Weller et al., 1992) with some modifications. Samples of the precleared detergent extracts of surface biotinylated cells were incubated for 4 hours with 20 μl of Protein A-Sepharose loaded with either 3 μg of rabbit IgG or 3 μg of mAb. To load Protein A-Sepharose with rat mAbs, the beads were preincubated with 5 μg rabbit anti-rat IgG. Anti-α-tubulin mouse mAb was immobilized onto an Affi-Gel 10 matrix (Bio-Rad, Hercules, CA). The immunoprecipitate was washed 5 times with washing buffer (50 mM Tris-HCl, pH 8.4, 400 mM NaCl, 0.05% Triton X-100, 1 mM CaCl, 1 mg/ml ovalbumin) and subjected to 6% SDS-PAGE and immunoblotting.

Electrophoresed immunoprecipitates of surface biotinylated cells were blotted onto nitrocellulose and biotinylated proteins were detected with the enhanced chemiluminescence (ECL) western blotting detection system (Amersham, Braunschweig, Germany) after incubating the membrane with peroxidase-conjugated streptavidin (Dianova, Hamburg, Germany). To perform western blot analysis of whole cell extracts, 2.5×10^7 32Dcl3 cells or 7.5×10^7 freshly isolated PMNs (see above) were first resuspended in 1 ml Laemmli's sample buffer with 50 mM DTT and then boiled for 5 minutes. A 40 μl sample of extract was loaded onto one lane of a 10% SDS-PAGE gel and after electrophoresis the separated proteins were blotted onto a nitrocellulose membrane as described above. Primary antibodies were detected by using alkaline phosphatase-conjugated goat anti-rabbit IgG (Dianova, Hamburg, Germany) as secondary antibodies, and enzyme reactions were monitored by a color reaction with nitro blue tetrazolium/5-bromo-4-chloro-3-indoyl-phosphate (Promega, Madison, WI); 1/50 diluted nonimmune serum from rabbit 89060 was used as negative control. Blocking the filters, all antibody incubations and all

washes were done in Tris-buffered saline (10 mM Tris-HCl, pH 7.4, 140 mM NaCl) containing 4% (w/v) defatted milk powder. For all western blots detected with enhanced chemiluminescence 0.1% Tween-20 was added to the Tris-buffered saline.

Cell surface immunoprecipitation

Cell surface immunoprecipitations were carried out in a modified form as previously described by Weller et al. (1992) with the exception that instead of metabolically labeled cells, surface labeled cells were used. 3×10^7 32Dcl3 or K46 cells were surface biotinylated as described above. Half of the cells were used for conventional immuno-precipitation while the remaining cells were split into three aliquots and incubated for 45 minutes at 4°C with either total rabbit IgG (40 μg/ml), affinity-purified rabbit antibodies (40 μg/ml) against ESL-IgG or with affinity-purified antibodies against the cytoplasmic tail of ESL-1. Incubations were done in PBS containing 3% FCS and 0.02% sodium azide. Unbound antibodies were removed by washing the cells three times with PBS. Cells were lysed in 500 μl lysis buffer (see above) that already contained the extracted cell proteins of a fivefold excess of nonbiotinylated cells. The lysates were incubated with goat anti-rabbit IgG antibodies immobilized onto CNBr-Sepharose (7.5 μg on 20 μl matrix). After 45 minutes at 4°C, the beads were washed five times with washing buffer. Bound proteins were removed from CNBr-Sepharose by incubating the beads for 30 minutes at 56°C with Laemmli's sample buffer and were then separated on 6% polyacrylamide gels. For conventional immunoprecipitations the same antibodies were used as described for cell surface immunoprecipitations. For each immunoprecipitation, 3 μg affinity-purified antibodies or total rabbit IgG were used, bound to goat anti-rabbit IgG antibodies which had been covalently linked to CNBr-Sepharose.

Sorting of cells

32Dcl3 cells were surface biotinylated as described above, washed with sorting buffer (PBS containing 2% FCS and 10% conditioned medium of WEHI-3B cells) and incubated with affinity-purified antibodies (30 μg/ml) against ESL-IgG. FITC-conjugated goat anti-rabbit IgG (Dianova, Hamburg, Germany) was used as second stage reagent. All washes and antibody incubations were done in sorting buffer. Cells were selected for low and high expression of ESL-1 by fluorescence activated cell sorting using a FACStar PLUS cell sorter (Becton Dickinson, Mountain View, CA). 1.2×10^6 sorted cells were lysed and subjected to immunoprecipitatons with affinity-purified anti-ESL-IgG antibodies as described above. K46 cells were selected for high expression of ESL-1 in the same way. The sorting buffer for K46 cells did not contain conditioned medium of WEHI-3B cells. Two cycles of fluorescence activated cell sorting resulted in a population of K46 cells which had a threefold higher expression level of ESL-1. These cells were used for ultrastructural immunolocalization as described below.

Immunogold scanning electronmicroscopy

K46 cells or 32Dcl3 cells were incubated with either affinity-purified anti-ESL-1 (50 μg/ml) or with biotin-conjugated RA3-6B2 mAb (against B220) for 30 minutes on ice in PBS with 0.5% BSA (Sigma, St Louis, MO) and 0.02% sodium azide. Nonspecific rabbit IgG (50 μg/ml) or PBS with 0.5% BSA and 0.02% sodium azide were used as controls in place of specific antibodies. Cells were washed three times in PBS contaning 0.5% BSA, 0.2% gelatin (Gibco, Eggenstein, Germany) and 0.02% sodium azide (PBSG). Rabbit anti-biotin-IgG (Enzo Diagnostics, New York, NY) was used to detect primary biotinylated antibodies. Cells were fixed briefly for 10 minutes with 4% paraformaldehyde in PBS, washed in PBSG and then incubated with Protein A conjugated to 15 nm colloidal gold. Labelled cells were fixed with 1% glutaraldehyde, mounted on polylysin-coated coverslips, postfixed with 1% osmium tetroxide in phosphate-buffered saline, dehydrated in ethanol and critical-point-dried from CO_2. The samples were sputter-coated with 1 nm chromium and examined at 10 kV accelerating voltage in a Hitachi S-800 field emission scanning electron microscope equipped with a detector for backscattered electrons (BSE) of the YAG type (Autrata et al., 1992). Micrographs were taken at primary magnifications of ×40,000 and ×60,000.

RESULTS

ESL-1 can be stained by indirect immunofluorescence in the Golgi as well as on the cell surface

To determine the subcellular distribution of ESL-1 we examined permeabilized 32Dcl3 cells by indirect immunofluorescence microscopy. Affinity-purified antibodies from the antiserum 89060 against an ESL-IgG fusion protein produced a distinct perinuclear staining (Fig. 1A). The staining-pattern appeared identical to the pattern observed with the mouse mAb G1/133 (Fig. 1B) which recognizes the 400 kDa Golgi-protein giantin (Linstedt and Hauri, 1993). No such pattern was observed for cells stained with control antibodies (Fig. 1C,D). The same observations were made with freshly isolated mouse PMNs stained with anti-ESL-1 and anti-giantin antibodies (not shown). Since the anti-ESL-1 pattern was indistinguishable from that of giantin we conclude that in leukocytes ESL-1 is concentrated within the Golgi apparatus.

The same affinity-purified antibodies against ESL-1 were also used for FACS analysis of 32Dcl3 cells and freshly

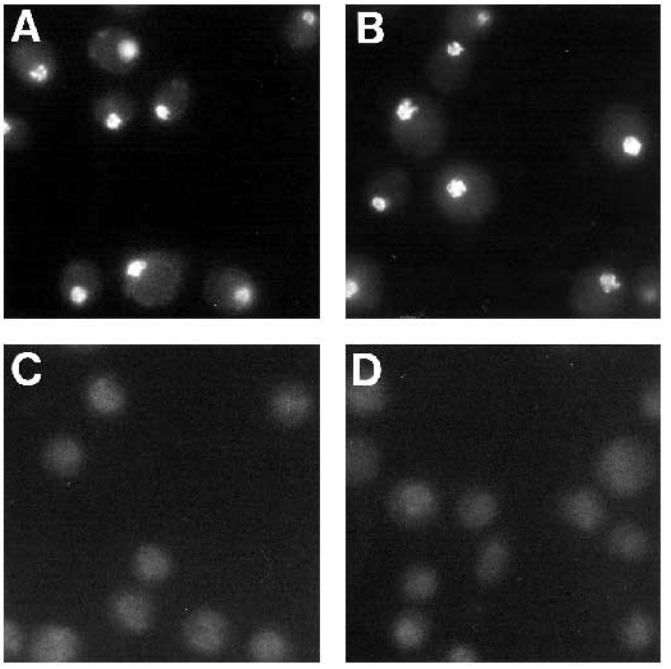

Fig. 1. ESL-1 is detectable by indirect immunofluorescence in the Golgi of neutrophilic cells. 32Dcl3 cells were fixed onto coverslips, permeabilized with detergent and analysed by indirect immunofluorescence staining with affinity-purified rabbit antibodies against ESL-1 (A), non-immune rabbit IgG (C), the mouse mAb G1/133 against the Golgi marker giantin (B) and an isotype matched control mAb (D). First antibodies were detected either with fluorescein-conjugated goat anti-rabbit IgG (A and B) or with fluorescein-conjugated rabbit anti-mouse IgG (C and D). Bar, 30 μm.

isolated mouse PMNs. In both cases the cells were specifically stained for ESL-1 (Fig. 2A). The FACS signals for ESL-1 were compared to the signals obtained with the mAb Mel-14 against mouse L-selectin and the mAb EA-3 against mouse PECAM-1 (Fig. 2A). L-Selectin was found to be heterogenously expressed on 32Dcl3 cells, with the highest levels similar to what was seen for PMNs. In contrast, PECAM-1 was strongly expressed on the neutrophilic precursor cell line 32Dcl3 but only weekly expressed on PMNs. The FACS signal for ESL-1 on PMNs was about 4-6 times weaker than that for L-selectin and three times stronger than that for PECAM-1.

To control the specificity of our affinity-purified anti-ESL-1 antibodies, we analysed their reactivity in immunoblots on whole cell lysates of 32Dcl3 cells and mouse PMNs. Only a single specific band at 150 kDa was recognized by these antibodies (Fig. 2B). Weakly detected additional signals were based on reactivities of the secondary antibody as demonstrated in the controls using nonimmune rabbit IgG as primary antibody (Fig. 2B). We conclude, that the affinity-purified anti-ESL-1 antibodies are specific for ESL-1.

ESL-1 is accessible on the cell surface for membrane impermeable reagents

To verify the results obtained by flow cytometry, we analysed, whether ESL-1 could be specifically labeled by a cell surface biotinylation technique. To this end, immunoprecipitation experiments were carried out with lysates of surface-biotinylated 32Dcl3 cells and mouse PMNs. Intact cells were incubated with a membrane impermeable, charged biotin derivative (*N*-hydroxysulfosuccinimide-biotin), which was then blocked and carefully removed before lysing the cells. Samples of the detergent extracts were then immunoprecipitated with affinity-purified anti-ESL-1 antibodies and for controls with mAbs against PECAM-1, L-selectin, and the intracellular protein α-tubulin. Strong signals for surface labeled ESL-1 were obtained with both cell types (Fig. 3A and B). On 32Dcl3 cells the signal for ESL-1 was weaker than that for PECAM-1. With PMNs the result was more variable, in some cases the signal for ESL-1 was similar to the PECAM-1 signal (Fig. 3B) in others the ESL-1 signal was stronger (not shown). This was in good agreement with the FACS-signals for both proteins on these cells (Fig. 2A). L-selectin was well labeled by surface biotinylation on PMNs but more weakly detectable on 32Dcl3 cells. This weak detectability might have been due to the heterogeneous expression of L-selectin on these cells, as demonstrated by FACS analysis (Fig. 2A). Alternatively, surface labeled L-selectin could have been lost by shedding, possibly activated during the labeling procedure. For mouse PMNs, the L-selectin signal was also variable in different immunoprecipitation experiments, possibly due to variable degrees of shedding. The result depicted in Fig. 3B for L-selectin on PMNs was the strongest obtained from five independent experiments. In contrast to ESL-1, PECAM-1 and L-selectin, α-tubulin was not detectable after cell surface labeling (Fig. 3A and B), demonstrating that intracellular proteins were not labeled by this technique. In immunoprecipitations of metabolically labeled cells the same antibody clearly allowed the detection of α-tubulin, demonstrating that the antibody was intact (data not shown).

We further analysed the cell surface expression of ESL-1 by testing whether antibodies would have access to ESL-1 on intact

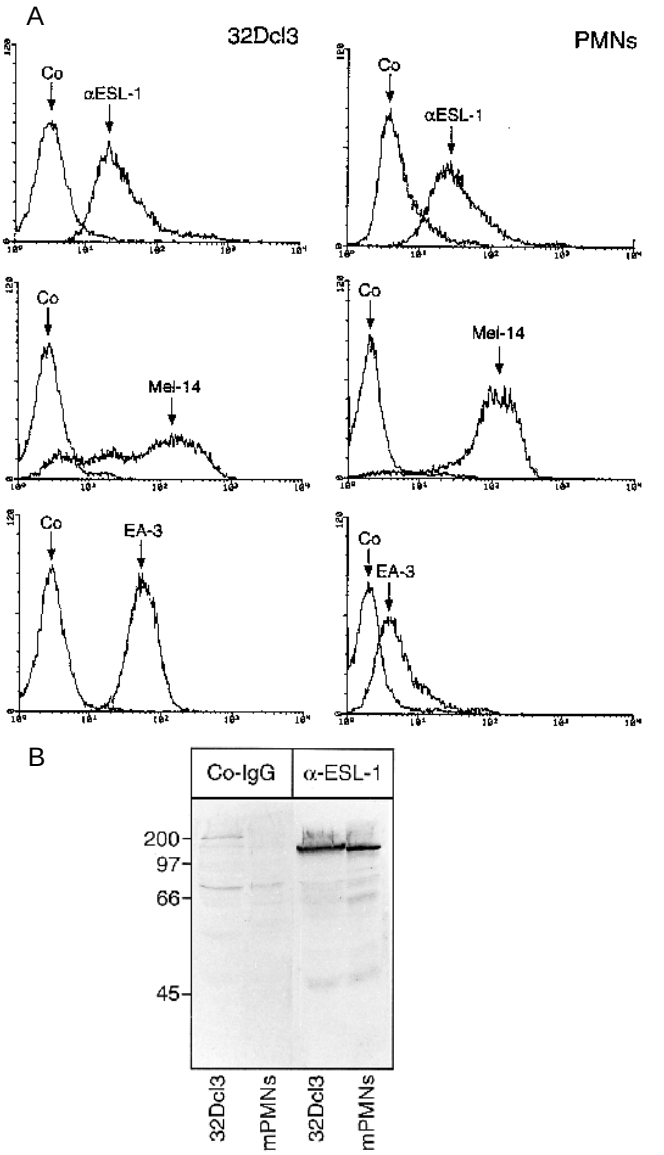

Fig. 2. ESL-1 is detectable by flow cytometry on the surface of 32Dcl3 cells and mouse PMNs. (A) 32Dcl3 cells (left chart) and mouse PMNs (right chart) were analysed by flow cytometry using affinity-purified anti-ESL-1 antibodies, the rat mAb Mel-14 against mouse L-selectin or the rat mAb EA-3 against PECAM-1 (as indicated). Total rabbit IgG and the rat mAb10E9 against mouse E-selectin were used for negative controls. First antibodies were detected either with FITC-conjugated goat anti-rabbit IgG or rabbit anti-rat IgG. (B) Total cell extracts of 32Dcl3 cells or mouse PMNs were electrophoresed on a 10% polyacrylamide gel under reducing conditions blotted onto nitrocellulose and filters were incubated with either total rabbit IgG (Co-IgG) or the same affinity-purified anti-ESL-1 antibodies (α-ESL-1) as used in (A). First antibodies were detected with alkaline phosphatase-conjugated goat anti-rabbit IgG and visualized by color reaction. Molecular mass markers (in kDa) are indicated on the left.

cells prior to detergent-extraction. To this end we performed so-called 'cell-surface immunoprecipitations' with surface biotinylated cells, where labeled, intact cells were first incubated with anti-ESL-1 antibodies, then carefully removed from unbound antibodies by washing and finally lysed in a Triton X-100 buffer

which already contained the total cellular proteins of a five times larger amount of unlabelled cells. This excess of unlabelled cellular proteins was used to compete antibodies which could possibly exchange their bound cell surface antigen with intracellular antigens that become exposed after cell lysis. In order to control that by this technique intracellular epitopes were not accessible for antibodies, parallel experiments were done either with affinity-purified antibodies against the extracellular part or with antibodies against the cytoplasmic tail of ESL-1. The results of such immunoprecipitations were compared with those of 'normal' immunoprecipitations where antibodies were added to the detergent extracts of identically labeled cells. These experiments were done with 32Dcl3 cells and K46 cells, a lymphoma cell line which was found to express slightly higher levels of ESL-1 on the cell surface as determined by FACS analysis (data not shown). As shown in Fig. 4, antibodies against the extracellular part of ESL-1 precipitated the antigen with similar efficiency in conventional as well as in cell surface immunoprecipitations. In contrast, antibodies against the cytoplasmic tail of ESL-1 only had access to the antigen in conventional immunoprecipitations, demonstrating that intracellular epitopes were not detectable in cell surface immunoprecipitations. Similar results were obtained for both cell lines. We conclude that the ESL-1 glycoprotein is accessible for antibodies on the surface of intact cells.

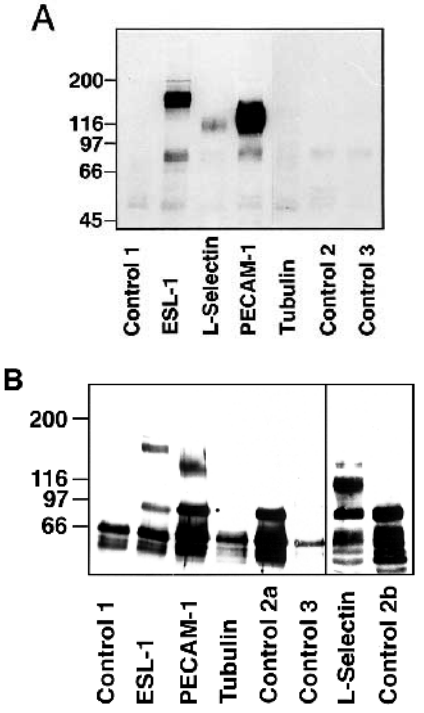

Fig. 3. ESL-1 can be labeled on intact 32Dcl3 cells and PMNs by a membrane impermeable biotin-reagent. Intact 32Dcl3 cells (A) or mouse PMNs (B) were surface biotinylated and cell lysates were immunoprecipitated either with total rabbit IgG (Control 1), affinity-purified anti-ESL-1 antibodies (ESL-1), rat mAb EA-3 (PECAM-1), mouse mAb B-5-1-2 (Tubulin) or rabbit anti-rat IgG (Control 2a and 2b), unloaded Affi-Gel 10 (Control 3), rat mAb Mel-14 (L-Selectin). The last two lanes in B are from a different experiment than the first six lanes. Rabbit antibodies were precipitated with Protein A-Sepharose, rat antibodies with rabbit anti-rat IgG-loaded Protein A-Sepharose and the mouse mAb was directly conjugated to Affi-Gel 10. Immunoprecipitates were electrophoresed (8% SDS-PAGE) and immunoblotted and filters were incubated with peroxidase-conjugated Streptavidin and analysed by enhanced chemiluminescence. Molecular mass markers (in kDa) are indicated on the left.

32Dcl3 cells can be sorted for low and high cell surface expression of ESL-1

To demonstrate the correlation of the FACS signal obtained with affinity-purified anti-ESL-1 antibodies with the immunoprecipitation signal for ESL-1 from surface biotinylated leukocytes we carried out the following experiment. 32Dcl3 cells were first surface biotinylated and subsequently sorted for low and high expression of ESL-1 by flow cytometry. The two sorted cell populations were then lysed and subjected to conventional immunoprecipitations with anti-ESL-1 antibodies. Samples of both sorted cell populations were re-analysed by flow cytometry for ESL-1 expression (Fig. 5A). Immunoprecipitations were performed with identical cell numbers of both subpopulations. Cells sorted for high expression of ESL-1 showed a much stronger signal for the 150 kDa ligand than the low expressing cell population (Fig. 5B). We conclude that the FACS signal which is generated by affinity-purified anti-ESL-1 antibodies is due to the detection of ESL-1 on the cell surface.

ESL-1 on the cell surface is presented by microvilli

At the electron microscope level, leukocytes in the blood display a complex surface architecture with prominent microvillus-like membrane protrusions. These microvilli are

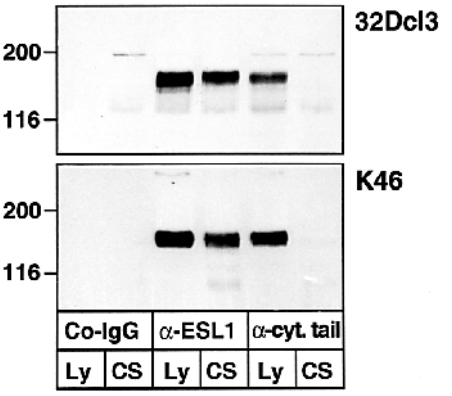

Fig. 4. ESL-1 is detectable by cell surface immunoprecipitations. 32Dcl3 cells (upper panel) and K46 cells (lower panel) were surface biotinylated and either detergent extracts (lysate; Ly) or intact cells (cell surface; CS) were incubated with total rabbit IgG (Co-IgG), affinity-purified antibodies against the extracellular part of ESL-1 (α-ESL-1) or affinity-purified antibodies against the C-terminal 13-amino acid peptide of ESL-1 (α-cyt. tail). For cell surface immunoprecipitations, intact cells were washed after antibody incubations and lysed in detergent buffer containing 5-fold excess of unbiotinylated cellular protein. Antigen-antibody complexes were pelleted with CNBr-Sepharose loaded with goat anti-rabbit IgG, electrophoresed on 6% polyacryamide gels under reducing conditions and immunoblotted. Biotinylated antigens were visualized with peroxidase-conjugated Streptavidin and enhanced chemiluminescence. Note that only antibodies against the extracellular part of ESL-1, but not antibodies against its cytoplasmic tail had access to their epitope in cell surface immunoprecipitations, demonstrating the specificity of this technique to detect only cell surface antigens. Molecular mass markers (in kDa) are indicated on the left.

thought to represent the principal sites of initial contact with the vascular endothelium under flow. To investigate whether ESL-1 would be located on such surface protrusions, we analysed 32Dcl3 cells and K46 cells by immunogold scanning electron microscopy with affinity-purified antibodies against ESL-1. Although immunogold labeling for ESL-1 was observed on microvilli of 32Dcl3 cells, labeling was too weak for a significant evaluation of the signal ratio between microvilli and the planar cell surface. Therefore, we analysed the lymphoma cell line K46 which expresses ESL-1 at about three times higher levels than 32Dcl3 cells, as was found by FACS analysis (not shown). Although K46 cells do not carry ESL-1 in a glycoform which is able to bind to E-selectin, we have found recently that lymphoid cells can indeed express an E-selectin binding form of ESL-1 if they are activated (E. Borges and D. Vestweber, unpublished data).

As shown in Fig. 6B,E the immunogold label for ESL-1 on K46 cells was conspiciously concentrated on microvillous processes, with 80% of gold particles found on microvilli (*n*=6 cells; 254 gold particles analysed). Control experiments performed with rabbit IgG of non-immune serum revealed no specific gold label (Fig. 6A,D). In contrast to ESL-1, labeling for B220 was enriched on the planar cell surface (Fig. 6C,F) with 69% of gold particles (*n*=6 cells; 625 gold particles analysed) found on the cell body. Negative control experiments performed without primary antibodies, but with rabbit anti-biotin and Protein A gold conjugate did not reveal any specific gold label on the cell surface (data not shown).

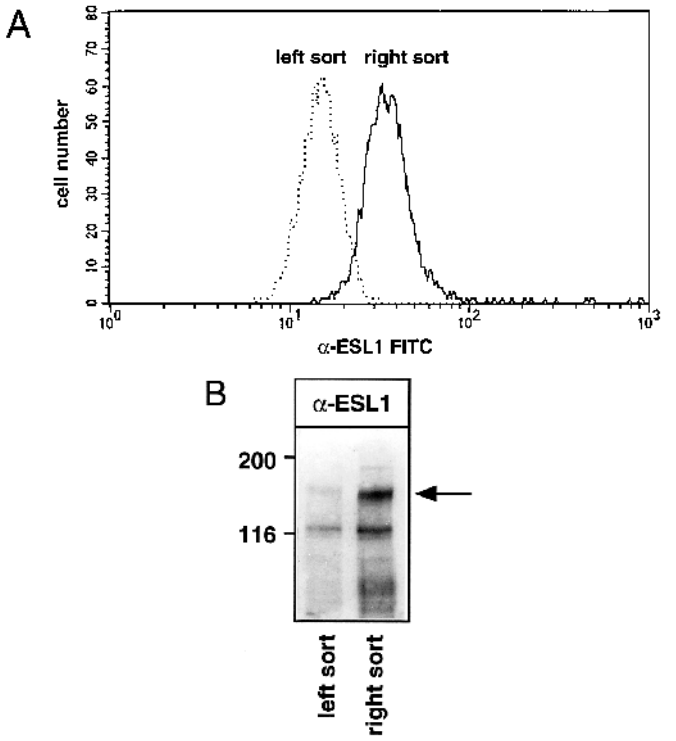

Fig. 5. Stronger FACS signals for ESL-1 correlate with stronger immunoprecipitation signals. 32Dcl3 cells were cell surface biotinylated and subsequently sorted by flow cytometry with affinity-purified antibodies against ESL-1. (A) Aliquots of the low expressing cells (left sort) and high expressing cells (right sort) were re-analysed by flow cytometry with anti-ESL-1 antibodies. (B) Equal numbers of ESL-1 low and high expressing cells were lysed and subjected to immunoprecipitations with affinity-purified anti-ESL-1 antibodies. Immunoprecipitates were electrophoresed on a 6% polyacryamide gel under reducing conditions, immunoblotted and cell surface labeled ESL-1 (indicated by the arrow) was detected by enhanced chemiluminescence as described. Molecular mass markers (in kDa) are indicated on the left.

DISCUSSION

We have analysed the subcellular distribution of the mouse E-selectin ligand ESL-1 in leukocytes. Based on indirect immunofluorescence staining techniques and various bio-

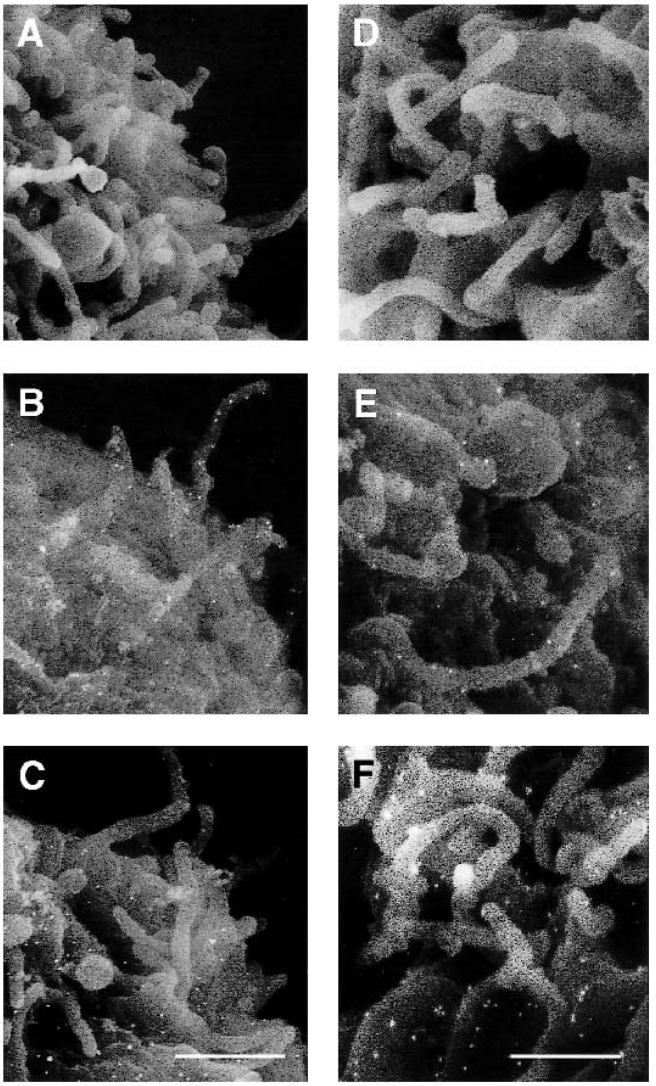

Fig. 6. Immunolocalization of ESL-1 on microvilli by scanning electron microscopy. Immunogold staining (the bright white dots) of K46 cells is shown for total IgG from non-immune serum (A and D), affinity-purified anti-ESL-1 antibodies (B and E) or a mAb against B220 (C and F). Typical staining patterns are shown at low magnification (A,B,C; bar, 0.75 μm) and at high magnification (D,E,F; bar, 0.50 μm). Note the conspicuous staining of microvilli with anti-ESL-1 antibodies, whereas colloidal gold on anti-B220 stained cells was localized to the cell body.

chemical techniques, we could detect ESL-1 in the Golgi of permeabilized leukocytes and on the surface of intact cells. The more detailed ultrastructural immunolocalization revealed that surface-located ESL-1 is preferentially expressed on microvillous processes while it is largely excluded from the planar cell body. The microvillous presentation argues for the participation of ESL-1 in early interactions between leukocytes and endothelial cells during leukocyte extravasation.

The conclusion that the FACS signals which we obtained with affinity-purified anti-ESL-1 antibodies were indeed due to the presence of ESL-1 molecules on the cell surface is supported by the following biochemical evidence: first, ESL-1 could be labeled by surface-biotinylation of intact cells, a technique that did not allow the labeling of highly abundant intracellular proteins. Second, ESL-1 could be precipitated in cell surface immunoprecipitations where antibodies only had access to cell surface located antigens on intact cells. In these experiments, only antibodies against the extracellular part of ESL-1 could precipitate their antigen while antibodies against the cytoplasmic tail of ESL-1 could not. Third, cells which had been sorted for higher surface expression of ESL-1 by flow cytometry gave rise to stronger immunoprecipitation signals for ESL-1 than cells sorted for low expression. Fourth, the affinity-purified anti-ESL-1 antibodies, which were used in this study, did not recognize other glycoproteins in immunoblots (this report) or immunoprecipitations (Steegmaier et al., 1995) of total cellular detergent extracts. We conclude that ESL-1, although detectable in the Golgi, is readily accessible on the cell surface of leukocytes.

The sequences of chicken CFR and rat MG160 are very closely related to that of mouse ESL-1. CFR binds to FGF-1, FGF-2, FGF-4 and FGF-7 and has been postulated to function as a novel type of FGF receptor with no structural relation to the classical types of FGF receptors (Burrus and Olwin, 1989). It is not yet known whether FGF-binding transduces intracellular signals. The biological function for MG160, which was reported to be a protein of the Golgi apparatus, is also still unknown. Based on its ubiquitous and early appearence it has been suggested that MG160 may function as an important structural component of the Golgi apparatus during embryonic development and in adult stages (Stieber et al., 1995).

The apparent discrepancy between the close structural relatedness of ESL-1, CFR and MG160 and the unrelatedness of their suggested functions may be explained in several ways. First of all, it needs to be considered that the ESL-1 protein backbone is broadly distributed on different cell types, and only on myeloid cells and on subsets of activated lymphoid cells (E. Borges and D. Vestweber, unpublished data) does it exist in its correct glycoform which is capable of binding to E-selectin. Expression of 'non-functional' isoforms of selectin ligands in cells where they don't function as ligands is also characteristic for the P-selectin ligand PSGL1 (Sako et al., 1993) or the L-selectin ligands GlyCAM-1 and CD34 (Lasky et al., 1992; Baumhueter et al., 1993). It is conceivable that, in addition to its function as a cell adhesion mediating E-selectin-ligand on subsets of leukocytes, the ESL-1 protein may fulfill additional functions in other cells. Such functions may be related to those that were suggested for CFR or MG160.

Second, splicing variants of ESL-1, CFR and MG160 may exist with different subcellular and tissue distribution. The ESL-1 protein backbone, although for most of its sequence 94% identical to that of CFR, contains a peculiar proline-glutamine-rich 70 amino acid domain at the N terminus of the mature form, which is unique to ESL-1 and for which no correlate was found in CFR. Recently, we have indeed found a splice variant of ESL-1 in brain tissue of mouse embryos which lacks exactly this peculiar domain (J. E. Blanks and D. Vestweber, unpublished data). The function and localization of this variant is currently under investigation.

A third possibility to explain the relation between ESL-1, CFR and MG160 would be the concept of 'gene sharing' (Piatigorsky and Wistow, 1989). This describes the use of one gene encoding a protein that has two entirely different functions and subcellular locations. This implies that a gene may acquire and maintain a second function without duplication and without the loss of the primary function. Several crystallines are striking examples for this concept (Piatigorsky and Wistow, 1989). They have structural roles in the refractive properties of the lens but they can also function as housekeeping enzymes inside a cell such as lactate dehydrogenase B4/ε-crystallin, α-enolase/τ-crystallin, or argininosuccinate lysate/δ2-crystallin (Wistow et al., 1987, 1988; Piatigorsky et al., 1988). Another example for one protein which can function in two distinct biological processes depending on its location is the cation-independent mannose 6-phosphate receptor/insulinlike growth factor II receptor (Kornfeld, 1992).

Another example for a cell surface protein which is strongly expressed in the Golgi is the 55 kDa tumor necrosis factor (TNF) receptor. In human endothelial cells it was predominantly found in the Golgi (Bradley et al., 1995), although it is well known to be the essential receptor on these cells for mediating proinflammatory and cytotoxic activities of TNF (Tartaglia and Goeddel, 1992; Vandenabeele et al., 1995). The Golgi-located receptor molecules could not be chased to the cell surface (Bradley et al., 1995) and it is not known what regulates their distribution between the Golgi and the cell surface, or for what purpose a large amount of this TNF receptor stays in the Golgi.

Although leukocytes in the blood stream are non polar cells, two different surface areas can be distinguished: the microvillous processes and the planar cell surface. That these surface domains indeed differ in their molecular composition of glycoproteins was very elegantly shown in a recent study (von Andrian et al., 1995), which analysed the distribution of L-selectin and CD44 on transfected lymphoid cells. While L-selectin was concentrated on microvilli, CD44 was restricted to the planar cell surface. Using chimeric molecules it was demonstrated that the transmembrane and intracellular domains of CD44 targeted the extracellular part of L-selectin to the planar body. Analogously, the extracellular part of CD44 was directed to microvilli when fused to the transmembrane and intracellular domain of L-selectin. These experiments established a mechanism for the specific targeting or anchoring of surface proteins to the two cell surface domains on leukocytes. In addition, this study demonstrated, that the expression of L-selectin on microvilli strongly improved its ability to initiate contacts of the transfected cells under flow to ligand bearing substrates. L-selectin-CD44 chimeric molecules which were excluded from microvilli, initiated leukocyte rolling under flow only very inefficiently. In agreement with these findings other adhesion molecules which have been demonstrated to mediate cell contact formation under flow are also found to be enriched on microvillous processes. This was

shown for the P-selectin ligand PSGL-1 (Moore et al., 1995) and for the integrin $\alpha_4\beta_7$. In contrast, β2-integrins which are essential for leukocyte adhesion to endothelium but which are not able to initiate contacts under flow conditions, are excluded from microvillous processes. In this context the localization of ESL-1 on microvilli is a strong indication for its possible role in early steps of leukocyte endothelial contact formation. It will be important to elucidate the molecular details of the machinery which targets and/or anchors L-selectin, PSGL-1 and ESL-1 to the surface of microvillous processes.

We thank Dr Peter Hauri for the mAb against giantin, Dr Beat Imhof for the mAb against PECAM-1, Hubertus Kohler for his expert technical assistance with flow cytometry and Lore Lay for the art work.

REFERENCES

Autrata, R., Hermann, R. and Müller, M. (1992). An efficient single crystal BSE detector in SEM. *Scanning* **14**, 127-135.

Baumhueter, S., Singer, M. S., Henzel, W., Hemmerich, S., Renz, M., Rosen, S. D. and Lasky, L. A. (1993). Binding of L-selectin to the vascular sialomucin CD34. *Science* **262**, 436-438.

Berlin, C., Berg, E. L., Briskin, M. J., Andrew, D., Kilshaw, P., Holzmann, B., Weissman, I. L., Hamann, A. and Butcher, E. C. (1993). α4β7 integrin mediates lymphocyte binding to the mucosal vascular addressin MAdCAM-1. *Cell* **74**, 185-195.

Bosse, R. and Vestweber, D. (1994). Only simultaneous blocking of the L- and P-selectin completely inhibits neutrophil migration into mouse peritoneum. *Eur. J. Immunol.* **24**, 3019-3024.

Bradley, J. R., Thiru, S. and Pober, J. S. (1995). Disparate localization of 55-kd and 75-kd tumor necrosis factor receptors in human endothelial cells. *Am. J. Pathol.* **146**, 27-32.

Butcher, E. C. (1991). Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. *Cell* **67**, 1033-1036.

Burrus, L. W. and Olwin, B. B. (1989). Isolation of a receptor for acidic and basic fibroblast growth factor from embryonic chick. *J. Biol. Chem.* **264**, 18647-18653.

Burrus, L. W., Zuber, M. E., Lueddecke, B. A. and Olwin, B. B. (1992). Identification of a cysteine-rich receptor for fibroblast growth factors. *Mol. Cell. Biol.* **12**, 5600-5609.

Erlandsen, S. L., Hasslen, S. R. and Nelson, R. D. (1993). Detection and spatial distribution of the β2 integrin (Mac-1) and L-selectin (LECAM-1) adherence receptors on human neutrophils by high-resolution field emission SEM. *J. Histochem. Cytochem.* **41**, 327-333.

Gallatin, W. M., Weissman, I. L. and Butcher, E. C. (1983). A cell-surface molecule involved in organ-specific homing of lymphocytes. *Nature* **304**, 30-34.

Gonatas, J. O., Mezitis, S. G. E., Stieber, A., Fleischer, B. and Gonatas, N. K. (1989). MG-160: a novel sialoglycoprotein of the medial cisternae of the Golgi apparatus. *J. Biol. Chem.* **264**, 646-653.

Gonatas, J. O., Mourelatos, Z., Stieber, A., Lane, W. S., Brosius, J. and Gonatas, N. K. (1995). MG160, a membrane sialoglycoprotein of the medial cisternae of the rat Golgi apparatus, binds basic fibroblast growth factor and exhibits a high level of sequence identity to a chicken fibroblast growth factor receptor. *J. Cell Sci.* **108**, 457-467.

Hemmerich, S. and Rosen, S. D. (1994). 6′-Sulfated sialyl Lewis x is a major capping group of GlyCAM-1. *Biochemistry* **33**, 4830-4835.

Imai, Y., Lasky, L. A. and Rosen, S. D. (1993). Sulphation requirement for GlyCAM-1, an endothelial ligand for L-selectin. *Nature* **361**, 555-557.

Kim, K.-M., Adachi, T., Nielsen, P. J., Terashima, M., Lamers, M. C., Köhler, G. and Reth, M. (1994). Two new proteins preferentially associated with membrane immunoglobulin D. *EMBO J.* **13**, 3793-3800.

Kornfeld, S. (1992). Structure and function of the mannose 6-phosphate/insulinlike growth factor II receptors. *Annu. Rev. Biochem.* **61**, 307-330.

Lasky, L. A., Singer, M. S., Dowbenko, D., Imai, Y., Henzel, W. J., Grimley, C., Fennie, C., Gillett, N., Watson, S. R. and Rosen, S. D. (1992). An endothelial ligand for L-selectin is a novel mucin-like molecule. *Cell* **69**, 927-938.

Lasky, L. A. (1995). Selectin-carbohydrate interactions and the initiation of the inflammatory response. *Annu. Rev. Biochem.* **64**, 113-139.

Lenter, M., Levinovitz, A., Isenmann, S. and Vestweber, D. (1994). Monospecific and common glycoprotein ligands for E- and P-selectin on myeloid cells. *J. Cell Biol.* **125**, 471-481.

Levinovitz, A., Mühlhoff, J., Isenmann, S. and Vestweber, D. (1993). Identification of a glycoprotein ligand for E-selectin on mouse myeloid cells. *J. Cell Biol.* **121**, 449-459.

Linstedt, A. D. and Hauri, H.-P. (1993). Giantin, a novel conserved Golgi membrane protein containing a cytoplasmic domain of at least 350 kDa. *Mol. Biol. Cell* **4**, 679-693.

Moore, K. L., Patel, K. D., Bruehl, R. E., Fugang, L., Johnson, D. A., Lichenstein, H. S., Cummings, R. D., Bainton, D. F. and McEver, R. P. (1995). P-selectin glycoprotein ligand-1 mediates rolling of human neutrophils on P-selectin. *J. Cell Biol.* **128**, 661-671.

Piala, L., Albelda, S. M., Baldwin, H. S., Hammel, P., Gisler, R. H. and Imhof, B. A. (1993). Murine platelet endothelial cell adhesion molecule (PECAM-1)/CD31 modulates β2 integrins on lymphokine-activated killer cells. *Eur. J. Immunol.* **23**, 2464-2471.

Piatigorsky, J. P., O'Brien, W. E., Norman, B. L., Kalumuck, K., Wistow, G. J., Borras, T., Nickerson, J. M. and Wawrousek, E. F. (1988). Gene sharing by d-crystallin and argininosuccinate lysate. *Proc. Nat. Acad. Sci. USA* **85**, 3479-3483.

Piatigorsky, J. and Wistow, G. J. (1989). Enzyme/crystallins: gene sharing as an evolutionary strategy. *Cell* **57**, 197-199.

Picker, L. J., Warnock, R. A., Burns, A. R., Doerschuk, C. M., Berg, E. L. and Butcher, E. C. (1991). The neutrophil selectin LECAM-1 presents carbohydrate ligands to the vascular selectins ELAM-1 and GMP-140. *Cell* **66**, 921-933.

Pouyani, T. and Seed, B. (1995). PSGL-1 recognition of P-selectin is controlled by a tyrosine sulfation consensus at the PSGL-1 amino terminus. *Cell* **83**, 333-343.

Sako, D., Chang, X. J., Barone, K. M., Vachino, G., White H. M., Shaw, G., Veldman, G. M., Bean, K. M., Ahern, T. J., Furie, B., Cumming, D. A. and Larsen, G. R. (1993). Expression cloning of a functional glycoprotein ligand for P-selectin. *Cell* **75**, 1179-1186.

Sako, D., Comess, K. M., Barone, K. M., Camphausen, R. T., Cumming, D. A. and Shaw, G. D. (1995). A sulfated peptide segment at the amino terminus of PSGL-1 is critical for P-selectin binding. *Cell* **83**, 323-331.

Springer, T. A. (1994). Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* **76**, 301-314.

Steegmaier, M., Levinovitz, A., Isenmann, S., Borges, E., Lenter, M., Kocher, H. P., Kleuser, B. and Vestweber, D. (1995). The E-selectin-ligand ESL-1 is a variant of a receptor for fibroblast growth factor. *Nature* **373**, 615-620.

Stieber, A., Mourelatos, Z., Chen, Y.-J., Le Douarin, N. and Gonatas, N. K. (1995). MG160, a membrane protein of the Golgi apparatus which is homologous to a fibroblast growth factor receptor and to a ligand for E-selectin, is found only in the Golgi apparatus and appears early in chicken embryo development. *Exp. Cell Res.* **219**, 562-570.

Tartaglia, L. A. and Goeddel, D. V. (1992). Two TNF receptors. *Immunol. Today* **13**, 151-153.

Vandenabeele, P., Declercq, W., Beyaert, R. and Fiers, W. (1995). Two tumor necrosis factor receptors: structure and function. *Trends Cell Biol.* **5**, 392-399.

Vestweber, D. (1994). Glycoprotein ligands of the two endothelial selectins. *Res. Immunol.* **144**, 704-708.

von Andrian, U. H., Hasslen, S. R., Nelson, R. D., Erlandsen, S. L. and Butcher, E. C. (1995). A central role for microvillous receptor presentation in leukocyte adhesion under flow. *Cell* **82**, 989-999.

Weller, A., Isenmann, S. and Vestweber, D. (1992). Cloning of the mouse endothelial selectins: Expression of both E and P-selectin is inducible by tumor necrosis factor-α. *J. Biol. Chem.* **267**, 15176-15183.

Wistow, G. J., Mulders, J. W. M. and de Jong, W.W. (1987). The enzyme lactate dehyrogenase as a structural protein in avian and crocodilian lenses. *Nature* **326**, 622-624.

Wistow, G. J., Lietman, T., Williams, L. A., Stapel, S. O., de Jong, W. W., Horwitz, J. and Piatigorsky, J. (1988). t-Crystallin/α-enolase: one gene encodes both an enzyme and a lens structural protein. *J. Cell Biol.* **107**, 2729-2736.

Zhou, Z. and Olwin, B. B. (1994). Characterization of the FGF binding domain of the cysteine-rich FGF receptor. *Mol. Biol. Cell* **5**, 136a (abstract).

(Received 5 August 1996 – Accepted, in revised form, 14 January 1997)