RESEARCH COMMUNICATION

Identification of nucleolin as a new L-selectin ligand

Gesche HARMS*, Regine KRAFT†, Gerlinde GRELL†, Bärbel VOLZ*, Jens DERNEDDE* and Rudolf TAUBER*¹

*Institut für Klinische Chemie und Pathobiochemie, Universitätsklinikum Benjamin Franklin, Freie Universität Berlin, Hindenburgdamm 30, 12200 Berlin, Germany, and

†Max-Delbrueck Zentrum für Molekulare Medizin, Robert-Rössle-Str. 10, 13125 Berlin, Germany

Apart from leucocyte–endothelial interactions, the adhesion molecule L-selectin mediates the homotypic adhesion of leucocytes during recruitment at sites of acute inflammation, as well as intercellular adhesion of haematopoietic progenitor cells during haematopoiesis. There is evidence that, in addition to P-selectin glycoprotein ligand-1, other as-yet-unidentified proteins function as L-selectin ligands on human leucocytes and haematopoietic progenitor cells. In the present study, we show: (i) by affinity chromatography on L-selectin–agarose; (ii) by protein identifica-

tion using MS; and (iii) by covalent cell-surface labelling with sulphosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate that the multifunctional nuclear protein nucleolin is partly exposed on the cell surface, and is a ligand of L-selectin in human leucocytes and haematopoietic progenitor cells.

Key words: cell adhesion, CD34, myeloid leucocytes, P-selectin glycoprotein ligand 1 (PSGL-1).

INTRODUCTION

The adhesion receptor L-selectin, expressed on leucocytes and distinct types of haematopoietic progenitor cells, has crucial roles in mediating leucocyte extravasation into inflamed tissues, lymphocyte trafficking to peripheral lymph nodes and cell–cell adhesion of haematopoietic progenitor cells in the haematopoietic microenvironment.

During inflammation, leucocytes, especially neutrophils and monocytes, migrate through the vascular endothelium into inflamed tissues. This process of leucocyte extravasation is induced by cytokines, and constitutes a complex adhesion cascade of leucocyte capturing, rolling, firm adhesion and transmigration [1]. Capturing and rolling are predominantly mediated by adhesion molecules of the selectin family (E-, P- and L-selectin), and by their ligands [2]. The selectins are cell-surface, integral-membrane glycoproteins bearing a C-type lectin domain, and are expressed on activated endothelium (E- and P-selectin), activated platelets (P-selectin) or on almost all blood leucocytes (L-selectin) [3].

By virtue of L-selectin exposed on leucocyte microvilli, leucocytes are able to roll not only on activated endothelium, but also on other leucocytes already attached to the vascular wall [4]. L-selectin-mediated leucocyte–leucocyte interactions are hypothesized to accelerate the recruitment of leucocytes into the inflamed tissue [5]. On the basis of binding and inhibition assays, it has been shown that the fucosylated, sialylated and sulphated P-selectin glycoprotein ligand-1 (PSGL-1), which primarily binds to P-selectin, also has E- and L-selectin-binding properties [6], and is the major counter-receptor in L-selectin-mediated leucocyte–leucocyte adhesion [7,8]. Experimental evidence indicates, however, that, in addition to PSGL-1, further as-yet-unidentified leucocytic L-selectin ligands exist, which act synergistically with PSGL-1 [5,9,10].

Moreover, during haematopoiesis, L-selectin-mediated intercellular adhesion has been shown to influence localization, proliferation and differentiation of progenitor cells. Although still a matter of controversy, the sialomucin CD34 has been shown to function as an additional L-selectin ligand of the haematopoietic progenitor cell line KG1a [11]. Recently, a similar function has been shown for a distinct glycoform of CD44 on human haematopoietic CD34⁺ cells [12].

It was the aim of the present study to identify further, as-yet-unknown, L-selectin ligands present on cells of the myeloid lineage and on haematopoietic progenitor cells. Using affinity chromatography on immobilized L-selectin–agarose, the acidic protein nucleolin was identified as a new L-selectin ligand.

EXPERIMENTAL

Cell culture, antibodies and recombinant L-selectin

The human haematopoietic progenitor cell line KG1a (A.T.C. CCL-246.1) and the human myeloid, monohistiocytic cell line U937 (A.T.C.C. CRL-1593.2) were maintained in RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal calf serum, 5 mM glutamine and 10 mM Hepes buffer, pH 7.5.

Immunodetection was performed using the monoclonal antibodies (mAbs) anti-(PSGL-1) (clone KPL1 [13]; BD Pharmingen, San Diego, CA, U.S.A.), anti-CD34 (clone QBEND/10 [14]; CYMBUS Biotechnology, Dianova, Hamburg, Germany) and anti-nucleolin (clone 4E2; StressGen Biotechnologies Corp., Victoria, BC, Canada) at concentrations of 1 µg/ml, 10 µg/ml and 1 µg/ml respectively.

A soluble form of human L-selectin (sL-selectin), lacking the transmembrane and the cytosolic domains, was overexpressed in baby hamster kidney cells. The functional and biochemical

Abbreviations used: mAb, monoclonal antibody; PSGL-1, P-selectin glycoprotein ligand 1; sL-selectin, soluble L-selectin; sulpho-NHS-SS-biotin, sulphosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate; TBS, Tris-buffered saline.

¹ To whom correspondence should be addressed (e-mail tauber@ukbf.fu-berlin.de).

characterization of this recombinant sL-selectin has been described previously [15,16].

Furthermore, an L-selectin-Ig chimaera was constructed, consisting of the human sL-selectin fused to human Fc γ fragment (GenBank® accession number J00228) [17], kindly provided by Dr Steven Rosen (San Francisco, CA, U.S.A.). The L-selectin-Ig chimaera was produced in the human myeloid cell line K562.

Preparation of membrane fractions

As described in [18], cells (1×10^6 cells/ml) were harvested by centrifugation for 5 min at 4 °C (130 g), washed twice with PBS and resuspended in ice-cold hypotonic buffer 1 (20 mM Tris/HCl, pH 7.5, 70 mM NaCl and 0.5 mM EDTA, containing the protease inhibitors chymostatin, leupeptin and pepstatin A, each at a concentration of 10 μ g/ml). After incubation for 20 min on ice, cells were disrupted by 30 strokes with a Potter-Elvehjem-type homogenizer. Cell nuclei were sedimented by centrifugation for 5 min at 4 °C and 500 g, and then discarded; cellular membranes in the supernatant were processed to microsomes by two rounds of centrifugation (2500 g) for 5 min at 4 °C and manual homogenization of the pellets in buffer 2 [20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA and protease inhibitors (as for buffer 1)]. After a further centrifugation for 10 min at 4 °C (10000 g), the supernatant contained cytosol and microsomes that were derived particularly from the plasma membrane during the homogenization steps. By centrifugation at 100000 g for 1 h and at 4 °C, microsomes were sedimented [18], and these were then washed twice with buffer 2.

To generate a detergent extract of membrane proteins, the microsome pellet was homogenized and incubated for 10 min in buffer 2 containing 1% (w/v) Brij 58 (Merck, Darmstadt, Germany). Insoluble components were removed by centrifugation for 10 min at 4 °C (20800 g). The supernatant was diluted 1:3 with Tris-buffered saline (TBS; 20 mM Tris/HCl, 150 mM NaCl, pH 7.5) containing 2.7 mM CaCl₂, before chromatography.

To separate membrane-associated proteins from integral-membrane proteins, microsome preparations were suspended and incubated in buffer 2 containing 1 M NaCl for 1 h on ice. Detached membrane-associated proteins were separated from microsomes by centrifugation (100000 g) for 1 h at 4 °C. The supernatant containing the membrane-associated proteins was dialysed against TBS/1 mM CaCl₂. Integral-membrane proteins were detergent-extracted from the sediment in buffer 2 containing 1% (w/v) Brij 58.

Purification of L-selectin ligands

An L-selectin affinity column was prepared by immobilizing 8 mg of recombinant sL-selectin on 10 ml of MINI-LEAK divinylsulphone-activated agarose (KEM-EN-TEC; Biotrend, Cologne, Germany) following the manufacturer's instructions. A control column was generated in the same way by coupling 8 mg of asialofetuin (Sigma-Aldrich, Deisenhofen, Germany) with 10 ml of the matrix.

For affinity purification of L-selectin ligands, 15 ml of membrane-associated proteins prepared from 3×10^9 cells, with a protein concentration of 1 mg/ml in TBS containing 1 mM CaCl₂, was split into two portions, and each half was loaded on to one of the two columns equilibrated with the same buffer. The protein fractions were applied with a flow rate of 1.75 ml/min for at least three column passages. Unbound protein was washed out with equilibration buffer until absorbance at 280 nm reached the baseline. Elution of bound proteins was performed with TBS containing 25 mM EGTA. Proteins remaining bound to the

column were eluted in a second step with 1 M NaCl in 20 mM Tris/HCl, pH 7.5, and collected separately. Eluted proteins were concentrated by centrifugal filter devices (Centriplus YM-10; Millipore, Bedford, MA, U.S.A.) or precipitated with 3 volumes of ice-cold acetone.

Identification of polypeptides by in-gel cleavage and MS

Coomassie-Blue-stained gel pieces containing the 60 kDa and the 70 kDa polypeptide were excised with a scalpel, cut into small 1 mm gel cubes, washed extensively in 0.1 M Tris/acetonitrile (1:1, v/v) and dried under vacuum. After reduction and alkylation *in situ* by iodoacetamide, proteolytic digestion of the gel-immobilized protein was performed overnight at 37 °C with 0.1 μ g of porcine trypsin (Promega, Madison, WI, U.S.A.) in 50 μ l of 0.05 M Tris/HCl buffer, pH 8.5, 10% (v/v) acetonitrile, 1 mM CaCl₂. The peptides were recovered from the gel pieces by consecutive extractions with Tris buffer/acetonitrile and 5% formic acid/acetonitrile (1:1, v/v) for 10 min at 30 °C and ultrasonication for 5 min. Pooled extracts were dried in a Speed Vac. Peptide mass fragments were determined by matrix-assisted laser-desorption ionization-time-of-flight MS (ToFSpec 2E; Micromass Limited, Altrincham, Cheshire, U.K.). The identity of the two polypeptides with human nucleolin was assigned by comparison of the observed mass fingerprint with the predicted mass fingerprint of nucleolin in the database. In addition, relevant mass peaks were subjected to electrospray ionization for mass spectrometric sequencing (electrospray ionization tandem MS; QToF from Micromass, U.K.) to confirm mass fingerprint analysis.

Biotinylation and detection of cell-surface proteins

Cell-surface proteins of U937 cells were labelled with sulphosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate (sulpho-NHS-SS-biotin), essentially as described previously [19]. Cells (1×10^9) were washed gently twice with ice-cold PBS and resuspended in 50 ml of a freshly prepared solution of 0.5 mg/ml sulpho-NHS-SS-biotin (Pierce, Rockford, IL, U.S.A.) in PBS, before incubation for 40 min at 4 °C with gentle agitation. Subsequently, any unreacted biotin was quenched by incubating the cells in serum-free culture medium for an additional 10 min at 4 °C and washing them twice with TBS. Microsomes were prepared as described above, and membrane-associated proteins were extracted. As a negative control, cells were treated in the same way, but with the omission of the biotinylation reagent.

For the isolation of biotinylated proteins, samples (1.8 mg of protein in 2 ml of PBS) were applied to 1 ml of streptavidin-agarose (ImmunoPure Immobilized Streptavidin; Pierce) equilibrated with PBS, and were incubated for 2 h at 4 °C with gentle agitation. The matrices were transferred to Poly-Prep columns (0.8 cm $\times$ 4 cm; Bio-Rad Laboratories, Munich, Germany) and washed with 20 mM sodium phosphate buffer, pH 7.5/500 mM NaCl. Biotinylated proteins were eluted with 50 mM dithiothreitol in PBS, reducing the disulphide bond in the biotin-spacer group. Eluted proteins were precipitated with acetone and subjected to SDS/PAGE and Western blot analysis.

Detachment of membrane-associated proteins from intact cells

Two aliquots of washed U937 cells (1×10^8) were resuspended in either 100 μ l of PBS or 100 μ l of 1.5 $\times$ detachment buffer [final concentration: 20 mM phosphate buffer, pH 7.5, 500 mM L-proline (Biomol, Hamburg, Germany), 1 M NaCl, 0.5 mM EDTA, 10 μ g/ml each of the protease inhibitors chymostatin, leupeptin and pepstatin A], and incubated for 2 min on ice. Cells

were thereafter sedimented for 5 min at 4 °C and 130 *g*, and the supernatants containing membrane-associated proteins that had become detached from the cell surface under the respective conditions were collected. Cell integrity was controlled by monitoring lactate dehydrogenase activity in the supernatants obtained during both the washing steps and the detachment step. No leakage of cytosolic lactate dehydrogenase activity was detectable.

Binding of L-selectin to nucleolin on the surface of intact cells

KG1a cells (1×10^8) were washed in PBS, blocked with 1 % (w/v) BSA plus 20 % FcR-Blocking Reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) for 20 min on ice and incubated further (2 h at 4 °C) in PBS containing 1 mM CaCl_2 and 50 μg of L-selectin-Ig chimaera. Controls were treated similarly, but without the L-selectin-Ig chimaera. Thereafter, cells were washed twice with PBS containing 1 mM CaCl_2 , lysed by incubation in PBS containing the detergent MEGA 9 at a final concentration of 0.5 % (w/v), and insoluble material was removed by centrifugation (20800 *g*) for 10 min at 4 °C. From the detergent extracts, L-selectin-Ig chimaera and complexes of proteins bound to the chimaera were collected by binding to Protein A beads (Dyna, A/S Oslo, Norway). Protein A beads were incubated with the detergent extracts for 1 h at 4 °C, washed and eluted with SDS sample buffer without the reducing agent. Eluted proteins were separated by SDS/PAGE (7.5% gels), followed by Western blotting and immunodetection.

RESULTS

Purification of L-selectin ligands from the myeloid cell line U937

Since PSGL-1 is the only commonly accepted non-endothelial L-selectin ligand, we used a PSGL-1-containing membrane fraction of the haematopoietic progenitor cell line KG1a [20] to confirm the specific nature of the binding activity of the L-selectin affinity column. The cell line KG1a was used in preference to the cell line U937, since it contains a higher concentration of PSGL-1. As shown in Figure 1(A), PSGL-1 was almost completely bound to the L-selectin column and eluted in the EGTA fraction. The physiological homodimeric form of PSGL-1 was partially reduced to monomers under reductive SDS/PAGE conditions (Figure 1A, lane 3), which is in agreement with previous observations [21]. By subsequent high-salt elution (Figure 1B, lane 4), no immunoreactive protein was eluted in detectable amounts, reflecting the Ca^{2+} -dependent interaction between immobilized L-selectin and PSGL-1. As expected, PSGL-1 was not enriched in the EGTA eluate from the asialofetuin control column (Figure 1A, lane 6). Likewise, CD34 from the same membrane fraction was bound to L-selectin in a Ca^{2+} -dependent manner and eluted by EGTA with no detectable non-specific binding to the asialofetuin matrix (Figure 1B).

In order to detect as-yet-unidentified L-selectin ligands assumed to be present on U937 cells in addition to PSGL-1 [9], membrane proteins from U937 cells were separated by affinity chromatography on L-selectin-agarose. Prior to this, the presence of L-selectin ligands was semi-quantitatively assayed using an ELISA-based L-selectin binding assay [16], comparing membrane-associated proteins with integral-membrane proteins. Since the fraction of membrane-associated proteins exhibited approximately twice the binding activity of that of the integral-membrane proteins, membrane-associated proteins were used for chromatography. Proteins bound to the affinity matrix were eluted with EGTA and analysed by SDS/PAGE and silver staining, revealing four major polypeptide bands of 50, 60, 70

Figure 1 Specific binding of PSGL-1 and CD34 to the L-selectin affinity matrix

Membrane proteins from KG1a cells were prepared and separated by affinity chromatography on L-selectin-agarose or on asialofetuin-agarose as a control. Column flow-through, EGTA eluates and 1 M NaCl eluates were collected, separated by SDS/PAGE (7.5% gels), electrotransferred on to PVDF membranes and were immunostained. (A) Immunodetection of PSGL-1 with mAb KPL1. Since the native dimeric PSGL-1 could only be partially reduced to monomeric PSGL-1, as described in [21], both dimeric and monomeric PSGL-1 forms are detectable. PSGL-1 was only enriched by the L-selectin affinity matrix, but not by the asialofetuin control column. (B) Immunodetection of CD34 with mAb QBEND. CD34 was only enriched by the L-selectin affinity matrix, but not by the asialofetuin control column. Lanes 1, 5 μg of membrane proteins from KG1a cells; lanes 2, 5 μg of column flow-through of membrane proteins after L-selectin affinity chromatography; lanes 3, concentrated EGTA eluate; lanes 4, concentrated 1 M NaCl eluate; lanes 5, 5 μg of membrane proteins from KG1a cells; lanes 6, 5 μg of column flow-through of membrane proteins after asialofetuin control column; lanes 7, concentrated EGTA eluate from the asialofetuin control column. Molecular mass values (in kDa) are indicated on the left. The arrowheads (A, lane 4) indicate the position of the dimeric (I) and the monomeric (II) form of PSGL-1.

and 95 kDa. No proteins were bound to the asialofetuin control column in detectable amounts (Figure 2A).

Identification of the 60 kDa and the 70 kDa polypeptide band by MS

From the four polypeptide bands, the 60 kDa band (Figure 2A, lane 2) could be easily excised and separated from co-migrating minor polypeptide bands, and was chosen for further analyses. The protein band was subjected to tryptic digestion in the SDS/polyacrylamide gel, with subsequent MS. The mass fingerprint observed (Table 1) fitted the predicted mass fingerprint of human nucleolin from the SwissProt database (accession number P19338). Moreover, mass spectrometric sequencing of selected peptides proved unequivocally that the 60 kDa polypeptide band corresponds to human nucleolin (Table 1). As shown in Figure 3, each of the analysed peptides could be identified within the nucleolin sequence. No mass data of tryptic peptides were obtained that corresponded to the N-terminal part of nucleolin. This is probably due to the occurrence both of frequent tryptic cleavage sites and of clustered glutamic acid residues in the N-terminal nucleolin region preventing the formation of reasonably sized peptides for the MS analysis. Likewise, the 70 kDa polypeptide band was identified as nucleolin by MS (results not shown).

Figure 2 Isolation of L-selectin ligands from U937 cell membranes

Membrane-associated proteins from U937 cells were prepared and separated by affinity chromatography on L-selectin–agarose or on asialofetuin–agarose as a control. Proteins bound to the matrices were eluted by EGTA, as described in the Experimental section. Protein samples were analysed by SDS/PAGE (7.5% gels) and were either silver-stained (**A**) or were electrotransferred on to PVDF membranes and immunostained with mAb 4E2 (**B**). Lanes 1, membrane-associated proteins from U937 cells (**A**, 800 ng; **B**, 10 µg); lanes 2, EGTA eluate from the L-selectin affinity column; lanes 3, EGTA eluate from the asialofetuin control column. Molecular-mass marker values (in kDa) are indicated in the left.

Immunodetection

The identity of the 60 and 70 kDa polypeptide bands with nucleolin was proven further by Western blotting using the monoclonal anti-nucleolin antibody, clone 4E2 (StressGen). In addition to the 60 kDa and the 70 kDa polypeptide, the antibody reacted with 50, 95 and 105 kDa polypeptides in the L-selectin eluate (Figure 2B, lane 2). With the exception of the 105 kDa polypeptide, each of these polypeptides corresponded to one of the four major silver-stained polypeptides eluted from the L-selectin–agarose column (Figure 2A, lane 2). The same polypeptides reacting with the anti-nucleolin antibody were detectable in the fraction of membrane-associated proteins before chromato-

graphy (Figure 2B, lane 1). Since intact nucleolin has an apparent molecular mass of 105 kDa [22], these data indicate that the 50, 60, 70 and 95 kDa polypeptides represent proteolytic fragments of nucleolin. This assumption is supported by previous reports that nucleolin is proteolytically cleaved into fragments corresponding in size to the polypeptides observed in Figure 2(B) [22,23]. In addition, binding of membrane-associated nucleolin to L-selectin–agarose could also be demonstrated for myeloid HL60 cells and for the haematopoietic progenitor cell line KG1a using the same experimental approach (results not shown).

Detection and isolation of nucleolin from the cell surface

In order to examine whether cellular nucleolin is exposed on the cell surface, where it might be accessible to function as an L-selectin ligand, surface proteins of intact U937 cells were labelled with sulpho-NHS-SS-biotin, followed by preparation of microsomes. Biotinylated proteins were then isolated by binding to a streptavidin–agarose column, followed by reductive elution and analysis by immunoblotting. As shown in Figure 4, nucleolin was detectable with the 4E2 antibody only in the eluted fraction obtained from the biotinylated cells (Figure 4A, lane 1), but not from the unlabelled control cells (Figure 4A, lane 2). This demonstrates that a fraction of cellular nucleolin is exposed on the cell surface. In the fraction of biotinylated cell-surface proteins, the 105 kDa form of nucleolin prevailed, whereas the proteolytic fragments were only weakly stained.

In order to verify whether nucleolin exposed on the cell surface may function as an L-selectin ligand, the fraction of membrane-associated cell-surface proteins was prepared from intact cells by incubation with 1 M NaCl and was, thereafter, applied to the L-selectin affinity column. As shown by Western blotting, nucleolin could be dissociated from the surface of intact cells with 1 M NaCl, but not with PBS. In the protein fraction detached with 1 M NaCl, only the intact form of nucleolin was detectable (Figure 4B). When applied to L-selectin–agarose, the 105 kDa form and the fragments of 95, 70 and 50 kDa were eluted with EGTA, indicating that fragmentation of nucleolin occurs during chromatography (Figure 5). These different experiments indicate

Table 1 MS analysis of the 60 kDa protein

MS spectra were obtained from the 60 kDa gel band after *in situ* alkylation, in-gel tryptic digestion and desalting. Peptides determined by peptide mass-fingerprint analysis (matrix-assisted laser-desorption ionization–time-of-flight MS) or tandem MS (Q-time-of-flight) sequencing of relevant mass peaks, found to correspond with a high degree of certainty to human nucleolin (SwissProt database accession number P19338), are listed. Peptides derived from sequencing experiments are marked by an asterisk.

Mass [<i>M</i> -H] ⁺			
Observed	Predicted	Peptide	Position in nucleolin
2696.26	2696.405	KQKVEGTEPTTAFNLFVGNLFNFK	294–317
937.50	937.499	TGISDVFAK *	324–332
1000.46	1000.543	NDLAVVDVR *	333–341
2474.20	2474.234	FGYVDFESAEDLEKALELTGLK	348–369
2607.22	2607.331	NLPYKVTQDELKEVFEDAAEIR *	398–419
1178.72	1178.569	EVFEDAAEIR *	410–419
2319.03	2319.139	GIAYIEFKTEADAETKFEK	429–448
3175.32	3175.544	GIAYIEFKTEADAETKFEKQGTEIDGR	429–456
1160.50	1160.583	SISLYYTGEK *	457–466
2501.14	2501.266	TLVLSNLSYSATEETLQEVFEK	86–507
2199.94	2200.026	GLSEDTTEETLKESFDGSGVR *	577–596
1192.72	1192.617	IVTDRETGSSK *	599–609
2150.99	2150.952	ETGSSKGFGFVDFNSEDAK *	604–623
1561.68	1561.600	GFGFVDFNSEDAK	610–623
2102.22	2102.089	VTLDWAKPKGEGFGGRGGGR	636–656

Figure 1 consists of two Western blot panels, A and B. Panel A shows the detection of p115. It has two lanes labeled 1 and 2. Lane 1 shows a strong band at approximately 115 kDa, while lane 2 shows a significantly reduced band at the same position. Panel B shows the detection of p110. It also has two lanes labeled 1 and 2. Lane 1 shows a strong band at approximately 110 kDa, while lane 2 shows a significantly reduced band at the same position. Molecular weight markers are indicated on the left of panel A, ranging from 50 to 160 kDa.

Figure 4 Detection of nucleolin as a 105 kDa cell-surface protein

that the 105 kDa polypeptide is the predominant nucleolin form present on the cell surface, and is consistent with previous observations that nucleolin is highly sensitive for partial proteolysis [22,23].

L-selectin binds to nucleolin exposed on the surface of intact cells

In order to assess whether nucleolin present on the surface of intact cells is accessible to L-selectin from the cellular microenvironment, the binding of an L-selectin-Ig chimera to KG1a cells was examined. Cells were incubated with the chimeric protein, washed carefully and lysed in the presence of 0.5 % (w/v) non-ionic detergent. Complexes of proteins bound to the L-selectin-Ig chimera were thereafter collected by binding to Protein A beads, and these were analysed by SDS/PAGE and Western blotting. Immunodetection with different antibodies clearly showed that nucleolin was contained in the L-selectin-Ig chimera protein complexes (Figure 6B, lane 4). Polypeptides of 220 kDa and 120 kDa detectable in the complexes reacted directly with the secondary antibody, and represent the L-selectin-Ig chimera itself (220 kDa) and, most probably, a fragment of the chimera (120 kDa) (Figures 6A–6C, lane 4). In addition to nucleolin, PSGL-1 (approx. 240 kDa) could also be detected in the complex (Figure 6C, lane 4), indicating that the L-selectin-Ig chimera binds to nucleolin in a way similar to that of the well-characterized ligand PSGL-1.

Figure 5 Fragmentation of nucleolin occurs during chromatography on L-selectin–agarose

Proteins detached from the cell surface of U937 cells by high-salt extraction were separated by affinity chromatography on L-selectin–agarose. Proteins bound to the matrix were eluted with EGTA. Protein samples were analysed by SDS/PAGE (7.5% gels) and immunostaining for nucleolin with mAb 4E2. Lane 1, 2 µg of salt-extracted proteins from U937 cells before chromatography. This fraction contains solely the 105 kDa form of nucleolin. Lane 2, EGTA eluate from the L-selectin affinity matrix. The eluate contains the 105 kDa form and proteolytic fragments of nucleolin. Molecular-mass values (in kDa) are indicated on the left.

DISCUSSION

It was the aim of this study to examine whether, apart from PSGL-1, leucocytes and haematopoietic progenitor cells express additional ligands for L-selectin. The integral-membrane glycoprotein PSGL-1 is so far the only L-selectin ligand known to be expressed by human leucocytes. The presence of additional L-selectin ligands was inferred by the finding that L-selectin-mediated intercellular adhesion of U937 cells could not be completely blocked by anti-(PSGL-1) antibodies [9]. Here, we report the identification of the multifunctional protein nucleolin as a new L-selectin ligand, as determined by affinity chromatography on L-selectin–agarose and MS. The suitability of the matrix for isolating L-selectin ligands was demonstrated by

the specific binding of the L-selectin ligands PSGL-1 [20] and CD34 [11] (Figure 1). For affinity chromatography, the membrane-associated protein fraction derived from U937 cells was used, since this fraction exhibited a high L-selectin binding affinity in an L-selectin binding assay. The identification of two polypeptides of 60 kDa and 70 kDa bound to L-selectin–agarose in a Ca^{2+} -dependent fashion as being human nucleolin was assigned by MS, and was subsequently confirmed by the reactivity of the polypeptides with an anti-nucleolin mAb. Western blotting of the column eluates with the anti-nucleolin mAb showed that three additional polypeptide bands eluted from L-selectin–agarose represent other nucleolin fragments (Figure 2). Covalent labelling of surface proteins on intact cells with sulpho-NHS-SS-biotin showed that nucleolin is present as the intact 105 kDa form on the cell surface, and can be released from the cell surface by high-salt extraction (Figure 4A and 4B). The polypeptides isolated by binding to L-selectin–agarose result from fragmentation of the 105 kDa nucleolin during chromatography (Figure 5). Experiments on the binding of a recombinant L-selectin–Ig chimera to intact cells demonstrated that, similar to PSGL-1, cell-surface nucleolin may serve as a specific binding site for L-selectin from either interacting cells or the extracellular fluid.

Nucleolin has been described as a major nuclear protein, having an apparent molecular mass of 100–110 kDa, as determined by SDS/PAGE [22], and a calculated molecular mass of 76 kDa, as predicted by the amino acid sequence [24]. The difference between the calculated and observed molecular masses are most likely due to post-translational modifications, and to a high content of negatively charged amino acids in the N-terminal part of nucleolin. Numerous functions were assigned to nucleolin, reflecting the multiple sites of subcellular localization and post-translational modifications within the protein's several domains. Moreover, different forms of nucleolin have been described to result from specific proteolytic cleavage [25]. The observation that nucleolin is proteolytically cleaved during chromatography (Figure 5), and the apparent sizes of the nucleolin fragments, are in accordance with previous reports [22,23].

Figure 6 Binding of an L-selectin–Ig chimera to nucleolin on the surface of intact cells

KG1a cells were incubated with the L-selectin–Ig chimera, as detailed in the Experimental section. As a control, cells were incubated similarly, but without the L-selectin–Ig chimera. After incubation cells were washed, lysed, and cellular proteins bound to the L-selectin–Ig chimera were collected by binding to Protein A beads, separated by SDS/PAGE and analysed by immunoblotting. Lanes 1, 10 µg of total cell lysate of control cells. Lanes 2, 10 µg of total cell lysate of cells incubated with L-selectin–Ig chimera. Lanes 3, proteins of the control cells bound to Protein A beads. Lanes 4, proteins from cells complexed with L-selectin–Ig chimera retrieved by Protein A beads. Lane 5 in (A), 0.5 µg of L-selectin–Ig chimera. (A) Immunostaining with secondary antibody. As a control for non-specific binding of the secondary antibody [goat anti-(mouse IgG), horseradish-peroxidase-conjugated], PVDF membranes were stained solely with the secondary antibody. The latter reacted only with the Fcγ domain of the L-selectin–Ig chimera (arrowhead I) and with a fragment of the chimera (arrowhead II) (lanes 4 and 5). (B) Immunodetection of nucleolin with mAb 4E2. Nucleolin (arrowhead III) was detectable in the total cell lysates (lanes 1 and 2) and in the fraction of proteins bound to the L-selectin–Ig chimera retrieved by Protein A beads (lane 4). No nucleolin was detectable in the fraction of proteins from control cells bound to Protein A (lane 3). (C) Immunodetection of PSGL-1 with mAb KPL-1. PSGL-1 (arrowhead IV) was detectable in total cell lysates (lanes 1 and 2) and in the fraction of proteins bound to the L-selectin–Ig chimera (lane 4). No PSGL-1 was detectable in the proteins from control cells bound to Protein A (lane 3). Molecular mass values (in kDa) are indicated on the left.

The results of the present paper obtained by covalent labelling with the membrane-impermeant reagent sulphy-NHS-SS-biotin and by the release with 1 M NaCl show unequivocally that nucleolin is localized on the cell surface as the intact 105 kDa protein bound to the membrane by electrostatic interactions. This is in line with previous observations that, apart from its nuclear and cytoplasmic localization, nucleolin is also present on the surface of various cell types [26]. Cell-surface nucleolin has been described as a binding protein in different interactions, such as with apoB- and apoE-containing lipoproteins on HepG2 cells [27], with the urokinase receptor of human vascular-smooth-muscle cells [28] and as a leucocytic ligand of the V3 loop domain of HIV [29] and Factor J [30]. Moreover, a fructosyl-lysine-specific binding protein from cell membranes of U937 cells has been described as a 'nucleolin-like' protein [31]. Interestingly, the two peptides generated from this protein [31] were contained in the group of peptides generated from the 60 kDa and 70 kDa nucleolin fragments of our study, indicating that the fructosyl-lysine-specific binding protein is identical with nucleolin.

Apart from PSGL-1, nucleolin is the first ligand for L-selectin from CD34⁺ myeloid cells identified to date. The mechanism of binding of nucleolin to L-selectin is still unknown. While PSGL-1 binds to L-selectin via its carbohydrate moieties and its tyrosine sulphate residues within the anionic N-terminus [20], it is unknown if nucleolin on the cell surface has similar types of post-translational modification. Interestingly, nucleolin has acidic amino acid stretches; in particular, from amino acid residues 142–170, 184–208 and 233–270. It has been described that L-selectin also binds specifically to non-glycosylated anionic groups [32]. Thus it remains to be established whether these charged protein domains or post-translational modifications, such as tyrosine sulphates or carbohydrates not yet identified on nucleolin, are involved in the specific Ca²⁺-dependent binding of nucleolin to L-selectin. The proposed identity between nucleolin and the fructosyl-lysine-specific binding protein indicates that nucleolin might also interact with L-selectin in an alternate mode. Fructosyl-lysine is a physiological glucose adduct in extracellular proteins formed by a condensation between ϵ -amino groups of lysine residues and glucose, followed by an Amadori rearrangement [33]. The lectin domain of L-selectin bears 13 lysine residues, some of which could be glucosylated and might be part of the nucleolin-binding epitope.

The biological role of the interaction of L-selectin and cell-surface nucleolin is not known. In addition to PSGL-1, nucleolin could act as a counter-receptor of L-selectin in the intercellular adhesion of both leucocytes and haematopoietic progenitor cells. This assumption is supported by the finding in the present study that an L-selectin-Ig chimera binds specifically to both PSGL-1 and nucleolin on the surface of U937 cells. On the other hand, it cannot be ruled out that nucleolin might be involved in *cis*-binding to L-selectin. Recently, co-localizations of nucleolin with the urokinase receptor [28] on the one hand and of L-selectin with the urokinase receptor on the other hand [34] have been reported. These two findings might be explained by the proposal that L-selectin, nucleolin and the urokinase receptor may form an associative protein complex on the cell surface.

The understanding of the biological role of nucleolin–L-selectin binding requires the characterization of the binding mechanisms. Studies are being undertaken to identify specific domains and putative post-translational modifications of both L-selectin and cell-surface nucleolin involved in L-selectin–nucleolin binding.

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