CD38 binding to human myeloid cells is mediated by mouse and human CD31

Alberto L. HORENSTEIN*[1], Hannes STOCKINGER†, Beat A. IMHOF‡ and Fabio MALAVASI§

*Laboratory of Cell Biology, Department of Genetics, Biology and Biochemistry, University of Torino, Via Santena 19, 10126 Torino, Italy, †Institute of Immunology-VIRCC, University of Vienna, Brunnerstrasse 59, Vienna, Austria, ‡Basel Institute for Immunology, Grenzacherstrasse 487, CH-4005, Basel, Switzerland, and §Institute of Biology and Genetics, Via Brecce Bianche-Montedago, University of Ancona Medical School, 60131 Ancona, Italy

Soluble forms of membrane receptors are emerging candidates as physiological regulators of leukocyte trafficking. In the present study, we found that the soluble form of the CD38 antigen (sCD38) bears a binding domain of low affinity for a cellular receptor on U937 cells. Cross-linking and peptide-mapping studies confirmed the physical association and the identification of the U937 receptor as a 130 kDa protein. The binding of sCD38 to the receptor was differentially inhibited by several monoclonal antibodies against the CD31 cell-adhesion molecule. Thus the interaction was analysed through direct association of soluble and membrane CD38 with soluble recombinant murine CD31 with three N-terminal and with all six extracellular Ig domains. Cross-linking experiments on U937 intact cells, and ligand blot assays of the immunoprecipitated CD38 molecule, indicated that (i) the recognized epitope is determined by the tertiary structure of the molecule, and that (ii) the binding domain involved resides in the ectocellular portion of the CD31 molecule, more precisely in the first three N-terminal domains. A comparative functional activity between murine and human CD31 was also explored. The data presented suggest that (i) human CD31 bears a highly functional similarity with its murine counterpart, as it is a receptor in myeloid cells with more than one ligand (the $\alpha_v\beta_3$ integrin and the CD38 molecule), and that (ii) the activity of sCD38 as decoy molecule for CD31 may play an important role in cell–cell interactions in physiological and pathological conditions.

INTRODUCTION

Cell adhesion, activation and extracellular proteolysis are causally connected in leukocyte migration, a process essential to immunity and inflammation [1]. The complexity of this process requires concerted action and interaction of molecules underlying each of the involved mechanisms. For example, leukocyte adhesion is a regulated multi-step process mediated by specific molecular interactions between cell-surface receptors. It is initially driven by selectin-induced labile cell–cell contact leading to leukocyte rolling, and proceeds through an 'outside-in' cell-signalling step activating integrins and leading to tight adhesion of leukocytes to the endothelium [2]. Emerging candidates as physiological regulators of such cross-talk between cells are among the generation of soluble forms of the adhesion receptors [3]. These soluble receptors most commonly retain ligand-binding capacity, blocking the interactions between the membrane-bound receptor and its cell-bound or soluble ligands, thus inhibiting receptor functions [3,4]. One of these molecules seems to be the CD38 leukocyte antigen, a 45 kDa type II transmembrane receptor (mCD38), predominantly expressed by cells of lympho-monocytic lineages at discrete stages of differentiation. CD38 is not, however, an exclusive haematopoietic marker, and is expressed on other tissues, such as pancreas, brain, gut, spleen, kidney, liver and cardiac and skeletal muscles [5,6]. The function of CD38 *in vivo* remains to be defined, although a multiplicity of cellular roles *in vitro* has been attributed to this molecule. CD38 can be considered as exhibiting two principal types of behaviour: that of (i) an ectoenzyme, and that of (ii) a transmembrane cell-signalling molecule [5]. The ectocellular domain of the CD38 protein bears a significant amino-acid-sequence similarity to its murine homologue; furthermore, both behave as bifunctional enzymes that catalyse first the synthesis, from the substrate NAD^+, and then the hydrolysis of cyclic ADP-ribose, a recently identified second-messenger nucleotide that regulates the mobilization of intracellular Ca^{2+} [7,8]. The binding to mCD38 by ligand-mimicking monoclonal antibodies (mAbs) can activate intracellular signal-transduction pathways triggering (i) activation, proliferation and mobilization of intracellular Ca^{2+} in T-lymphocytes [9]; (ii) tyrosine phosphorylation of intracellular proteins in myeloid cells [10], in B-lymphocytes [11] and T-lymphocytes [12]; and (iii) inhibition of selectin-like adhesion of leukocytes to endothelium [13]. In addition to its transmembrane form, human CD38 is found in a 39 kDa soluble form (sCD38), which is detectable in biological fluids in normal conditions and is increased in selected diseases [14]. sCD38 behaves *in vitro* as a bifunctional enzyme, catalysing both the synthesis of cGDP-ribose from nicotinamide guanine dinucleotide (NGD^+) (cyclase activity) and the hydrolysis of cADP-ribose (hydrolase activity). The origin and the physiological significance of this naturally occurring form are to be defined in full. As pulse-chase experiments reveal a single polypeptide precursor, sCD38 is thought to derive from the membrane form by proteolytic cleavage occurring near the cell membrane. Moreover, the inverse correlation between surface expression and sCD38 is consistent with shedding of the surface molecule as the major source of released material [6,14].

The CD38 molecule may interact with soluble or membrane-bound molecules, since occupancy by agonist antibodies triggers several functions [9–13]. One such ligand was recently described on the surface of endothelial cells [15]. It is conceivable that sCD38 also binds to a ligand, because its molecular mass

Abbreviations used: DSS, disuccinimidylsuberate; DSP, dithio-bis-succinimidylpropionate; ECL, enhanced chemiluminescence; Gαmlg, goat antibody to mouse Ig; HRP, horseradish peroxidase; mAb, monoclonal antibody; mCD38, membrane CD38; NGD^+, nicotinamide-guanine dinucleotide; sCD38, soluble CD38; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; mu, murine; hu, human; 2-ME, 2-mercaptoethanol.

[1] To whom correspondence should be addressed.

corresponds to the entire ectocellular domain of the membrane receptor. Indeed, biochemical data show that sCD38 can bind an $\approx$ 130 kDa cell-surface protein [14]. This observation correlates with previous results showing that CD38 mediates an adhesion between leukocytes and endothelial cells, which is blocked by antibodies against the 130 kDa molecule CD31 [15,16].

CD31 is a cell-adhesion molecule of the Ig gene superfamily receptors, with six Ig-like domains expressed by monocytes, neutrophils, T-cell subsets, platelets, vascular endothelial cells and solid tumour lines [17,18]. Leukocyte trafficking and monocyte egress to inflammatory sites are highly dependent on CD31 molecules [19], which promote both homotypic and heterotypic adhesion, via binding to $\alpha_v\beta_3$ integrin [16,20]. However, not all these activities can be attributed to the interaction of CD31 with this molecule: CD31 contains multiple functional domains capable of mediating ligand–receptor cross-talk with a large number of molecules. Indeed, a T-cell protein bearing a glycosaminoglycan moiety has been postulated as an alternative ligand of CD31 [21,22]. In the present study, we tried to extend the notion of surface-adhesion molecules to interaction between soluble receptors and to define the binding parameters of CD38/CD31 co-ligation in promyelocytic cell lines.

EXPERIMENTAL

Reagents and antibodies

All reagents and tissue-culture media used were obtained from Sigma (Milan, Italy), unless otherwise indicated. The chemical cross-linkers disuccinimidylsuberate (DSS) and dithio-bis-succinimidylpropionate (DSP) were obtained from Pierce (Rockford, IL, U.S.A.). The Nonidet P-40 and Triton X-100 detergents were obtained from Fluka (Buchs, Switzerland). Carrier-free $Na^{125}I$, nitrocellulose (Hybond-C extra), and reagents for Western blotting and chemiluminescent detection of mouse and rabbit antibodies were purchased from Amersham (Milan, Italy). The silver-staining kit and prestained SDS/PAGE standards were purchased from Bio-Rad (Hercules, CA, U.S.A.), *Staphylococcus aureus* protease-V8 from Miles Laboratories (Naperville, IL, U.S.A.) and Centricon-10 from Amicon (Milan, Italy). The following mAbs were used: HPLC affinity-purified murine mAb IB4 (IgG_{2a}) [23] specific to human CD38; 5F49 (IgG_1) (made available by F. Bussolino, University of Turin, Italy) and Moon-1 (IgG_1) [15] mAb to CD31. An anti-CD2 (CBT11) mAb was used as control.

Cell lines

The human cell lines used [U937 (promonocytic lymphoma), THP-1 (monocytic), Raji (Burkitt lymphoma), Jurkat (T-lymphoblastoid) and K562 (erythroblastoid)] were maintained in suspension culture in RPMI-1640 supplemented with 10 % (v/v) FCS, L-glutamine and antibiotics.

Biochemical analysis of soluble CD38

Peritoneal ascites obtained by therapeutic paracentesis from patients with myeloma were used as the source of naturally occurring sCD38, which was purified as previously described [14]. The analysis of purified sCD38 was made after separation of the protein by SDS/PAGE (10 % polyacrylamide) according to the molecular mass [24]. After the run, one sample and the standard lanes were revealed by silver staining, while another sample lane was kept between glass slides at 4 °C. The stained and unstained sample lanes were aligned, the unstained lane was cut into slices, and the protein was electroeluted. To this end, the dialysis bag containing the gel slice was subjected to an electric field (100 mA, 40–60 V; 12 h at room temperature) in 0.1 % (w/v) SDS in Tris/glycine buffer, pH 7.8. The specific immunological activity of sCD38 was measured by its IB4 mAb-binding capacity and its enzymic activities were monitored as described previously [14–25].

A one-dimensional peptide mapping was performed by limited proteolysis using SDS/PAGE. Immunoprecipitated proteins were electrophoretically separated in a 7.5 % first slab gel. After staining briefly with Coomassie Blue, the bands of interest were excised from the gel and digested with 5 μg of *S. aureus* protease-V8 per lane, after electroelution into a stacking gel as described previously [26]. Following 30 min of digestion at room temperature, the peptides were resolved by SDS/PAGE into a 12.5 % gel and stained with silver.

Generation of soluble recombinant molecules

A soluble CD38 chimaera containing the extracellular domains of human CD38 and of murine CD8 (huCD38–muCD8α) was constructed as previously described [15]. Soluble recombinant muCD31 isoforms with three N-terminal Ig domains (muCD31-D_{1-3}, molecular mass $\approx$ 50 kDa) and with all six extracellular Ig domains (muCD31-D_{1-6}, molecular mass $\approx$ 95–100 kDa), associated with a murine Ck domain of Ig light-chain molecule, were used. They were produced in J558 myeloma cells and subsequently purified as reported previously [16]. A soluble recombinant huCD31 receptor globulin (huCD31-Rg), consisting of the entire extracellular domain of CD31 joined to the constant region of human IgG_1, was produced as described previously [21].

Preparation of radiolabelled soluble molecules

Purified sCD38 was radiolabelled by the chloramine-T method (0.2 mCi of $Na^{125}I$ for 1 min) while bound to a 200 μl-bed minicolumn of IB4 mAb–Sepharose. The ^{125}I-sCD38 was eluted with 4 M $MgCl_2$ in 5 mM Tris/HCl, pH 6.5, and dialysed versus PBS, pH 8.0, with a Centricon-10 in the presence of protease inhibitors. The specific activity of the labelled sCD38 ranged from 2 to 4×10^3 c.p.m./ng, and 90–95 % of radioactivity was precipitable by 10 % (v/v) trichloroacetic acid. To assess the bindability [27] of the labelled molecule, a trace concentration of ^{125}I-sCD38 was incubated with increasing concentrations of U937 cells (1×10^6–1×10^8 cells/ml). Specific binding was determined as described below, and the data were plotted in a double reciprocal plot. Bindability occurred at the intercept with the y axis, and > 65 % of the biological activity of binding was retained. Radiolabelling of soluble recombinant CD31 molecules and mAb was performed as described elsewhere [15,16].

Receptor binding assays

Cells (2×10^7/ml) were incubated in RPMI-1640 medium, containing 1 mg/ml BSA (RPMI/BSA), before use. The cells were then separated out into 1.5 ml microcentrifuge tubes (1.0×10^6 cells per assay, 0.15 ml per tube), to which were added increasing doses of ^{125}I-sCD38 also diluted in RPMI/BSA. After incubation (1 h at 4 °C), 100 μl of the cell suspension (5×10^5 cells) was layered on top of a 0.5 ml cushion of 20 % (w/v) sucrose in RPMI/BSA and centrifuged (10000 ***g*** for 1 min). Capped tubes were cut, and the tips containing the cell pellets were counted in a γ-counter (Cobra-Auto-Gamma; Packard Instrument Co., Meriden, CT, U.S.A.) to assess cell-bound radioactivity. Non-specific binding was determined by simultaneous incubations of cells in the presence of 100-fold excess unlabelled sCD38 and was always < 15 % of that totally bound to the cells. Cell viability

assessed by dye exclusion was always > 95 %. The mean number of ^{125}I-sCD38 molecules bound per cell was calculated from these corrected experimental data, the specific activity of ^{125}I-sCD38, and the cell number verified by microscope counting on several aliquots before separation. For sCD38 affinity analysis, the binding values obtained were analysed and plotted using a SAB program version [28].

To analyse the effects of mAbs specific for CD38 and CD31 on the binding of ^{125}I-sCD38, U937 cells (2.5×10^5/tube) were exposed (30 min at 4 °C) to unlabelled IB4, 5F49 and Moon-1 mAbs (10 μg/10^6 cells), before the addition of ^{125}I-sCD38 for 2 h. Cells were then assayed as described above. Control binding was measured on cells exposed to an irrelevant CBT11 mAb.

Ligand blot analysis

U937 cells (5×10^7) were lysed in 1 ml of buffer composed of 1 % (v/v) nonidet P-40, 140 mM NaCl, 1 mM PMSF, 15 μg/ml aprotinin and 10 μg/ml leupeptin, in 25 mM Tris/HCl, pH 8.0. The lysate was precleared by incubation with Gα mIg–agarose beads, and the precipitation was carried out by incubation with IB4 mAb- (or control CBT11 mAb-) coated Gα mIg–agarose beads. The material was eluted from the beads with sample buffer [2.3 % (w/v) SDS, 10 % (v/v) glycerol, 1 mM iodoacetamide, 5 % (v/v) 2-mercaptoethanol (2-ME) and 65 mM Tris/HCl, pH 6.8], heated at 90 °C for 5 min for analysis by SDS/PAGE (10 % polyacrylamide). Following SDS/PAGE, proteins were transblotted on to nitrocellulose in a continuous buffer system [25 mM Tris, 192 mM glycine and 20 % (v/v) methanol] for 1 h, at a constant voltage of 80 V. The membranes were stained with Ponceau S and blocked by incubation (3 h at 37 °C) in 10 mM Tris/HCl (pH 7.6)/150 mM NaCl/0.1 % (v/v) Tween-20/5 % (w/v) BSA (TBS-T). The nitrocellulose filters were extensively washed with TBS-T without BSA and then probed (12 h at 4 °C) with ^{125}I-muCD31-D_{1-3}, -D_{1-6} or ^{125}I-IB4 mAb, washed four times with TBS-T buffer, and exposed to Kodak X-OMAT X-ray films at −70 °C for autoradiography, using Cronex Dupont intensifying screens. Several blots were also stripped in 100 mM 2-ME/10 % (w/v) SDS/50 mM Tris/HCl, pH 7.4, and reprobed with muCD31-D_{1-3} or muCD31-D_{1-6} followed by rabbit anti-mouse Ig (L-chain) horseradish peroxidase conjugate. Immunodetection was performed with Gα mIg-HRP and an enhanced chemiluminescence procedure (ECL) according to the manufacturers' instructions.

Cross-linking of soluble molecules to a cell-surface target

Targeting of human sCD38

U937 cells (2×10^7/ml) were incubated (1 h at 4 °C) with saturating concentrations of ^{125}I-sCD38 in 0.5 ml of PBS (pH 8.0). Cells were separated from unbound ^{125}I-sCD38 as described above. The cell pellet was resuspended (4×10^7 cells/ml) in ice-cold PBS (pH 8.0), containing a 1–3 mM final concentration of the cross-linker reagent DSS freshly prepared in 1 mM DMSO. After incubation (30 min at 22 °C), the unreacted cross-linker was quenched in 20 mM Tris/HCl (pH 8.0). The cells were washed with cold PBS, and lysed (1.0×10^8 cells/ml) in 25 mM Tris/HCl (pH 8.0) containing 0.05 % (v/v) Nonidet P-40, 1 mM PMSF, 0.1 mM N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) and 20 mM iodoacetamide. After incubation (15 min at 4 °C) the cells were centrifuged (15 min at 4 °C and 12000 ***g***), and the cytoskeleton and nuclei removed. The receptor–ligand complex was processed by immunoprecipitation with IB4-coated GαmIg–agarose beads and analysed by SDS/PAGE (10 % polyacrylamide). After Coomassie Blue staining, the gel was dried and revealed by autoradiography.

Targeting of soluble recombinant murine CD31

^{125}I-muCD31-D_{1-6} was incubated with U937 cells and then chemically cross-linked as described above. The bifunctional cross-linker DSS was chosen because of its cell-impermeant property, which ensured that only those molecules on the surface of the cells would be cross-linked, and DSP, due to its cleavability. The cells were then lysed, and the covalent complexes were processed and analysed as described above.

Cross-linking among soluble molecules

Cross-linking of human sCD38 to soluble recombinant murine CD31

Human sCD38 or the soluble chimaeric molecule huCD38–muCD8α (500 μl of transfected or mock-transfected cell supernatant) were incubated (1 h at 22 °C) in PBS (pH 8.0) with ^{125}I-muCD31-D_{1-6}. The samples were cross-linked (30 min at 4 °C) with DSS, and the resulting covalent complexes were immunoprecipitated in a buffer containing 0.1 M Tris/HCl (pH 8.0), 0.3 M NaCl, 0.1 % (w/v) BSA, 0.1 % (w/v) Tween-20 and 20 mM Hepes, using IB4 mAb-coated GαmIg–agarose beads and analysed by SDS/PAGE (10 % polyacrylamide), under reducing conditions. The gels were dried and autoradiographed.

Cross-linking of human sCD38 to huCD31-Rg

^{125}I-sCD38 was incubated (1 h at 22 °C) in PBS (pH 8.0), in the presence or absence of 1 mM Ca^{2+}, with ^{125}I-huCD31-Rg. The samples were cross-linked by DSS (30 min at 4 °C), and the resulting adducts were immunoprecipitated in a buffer containing 0.1 M Tris/HCl (pH 8.0), 0.3 M NaCl, 0.1 % (w/v) BSA, 0.1 % (v/v) Tween-20 and 20 mM Hepes, using IB4 mAb-coated GαmIg–agarose beads. The samples were analysed by SDS/PAGE (7.5 % polyacrylamide) under reducing conditions and autoradiographed.

RESULTS

Biochemical characterization of sCD38

Initial studies were carried out to monitor the biochemical properties of the sCD38 used throughout this study. The immunopurified sCD38 was found to possess enzymic activity catalysing the synthesis of cGDP-ribose from NGD^+ and the hydrolysis of cADP-ribose, at a ratio of $\approx$ 1.0, similar to the value observed for the ectocellular domain constitutively expressed in normal and tumour cells [14]. A further structural analysis included a partial proteolytic peptide map of both sCD38 and mCD38 molecules. The digestion of 39 kDa (sCD38) and 45 kDa (mCD38) molecules with protease-V8 produced almost identical peptide profiles (Figure 1A, lanes a and b), clearly indicating a molecular identity of the proteins. SDS/PAGE analysis of the iodinated sCD38 molecule (^{125}I-sCD38) showed a 39 kDa single band (Figure 1B, lane b).

Binding of radio-iodinated sCD38 to cell lines for evaluation of the receptor activity

Radiobinding assays with the use of ^{125}I-sCD38 were performed on U937, THP-1, Jurkat, Raji and K562 cell lines as targets, to

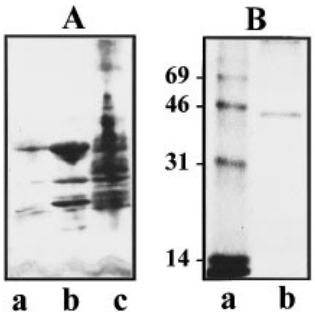

Figure 1 Biochemical characteristics of sCD38

(**A**) One-dimensional peptide-mapping analysis of unlabelled sCD38 and mCD38 performed by limited proteolysis with *S. aureus* protease-V8 using SDS/PAGE. Proteins electrophoretically separated in a 7.5% first slab gel were excised from the gel and digested with 5 μg of protease-V8 per lane, after electroelution into a stacking gel of a second 12.5% slab gel. The bands were revealed by silver staining. Lane a, sCD38; lane b, mCD38; lane c, control BSA. (**B**) Immunopurified sCD38 molecule was ^{125}I-labelled and analysed by autoradiography by SDS/PAGE (12.5% polyacrylamide) (lane b). Molecular masses of protein standards (lane a) are indicated (kDa).

Table 1 Binding of sCD38 to various human cell lines

Binding assays were performed in triplicate by incubation (2 h at 4 °C) of cells (5×10^5/tube) with saturating concentration of ^{125}I-sCD38 in PBS, in a final reaction volume of 200 μl with or without unlabelled sCD38. Values expressed in terms of maximum activity (B_{max}) represent means ± S.D.

Cell line	Radioactivity (B_{max}, c.p.m.)
U937	9850 ± 237
THP-1	7624 ± 189
Jurkat T	1584 ± 190
Raji B	1810 ± 154
K562	572 ± 75

ascertain the binding modes of sCD38. The different cell lines showed considerable variation among types in terms of maximum activity (Table 1). The receptor activity was detected on promonocytic (U937 and THP-1), Jurkat T- and Raji B-cell lines. Lower binding activity was indeed detected on the K562 cell line. Upon incubation of U937 cells with increasing amounts of ^{125}I-sCD38, saturation of the binding was observed at about 0.1 μg of sCD38 bound. The binding was completely displaced by excess unlabelled sCD38 (Figure 2A). The Scatchard analysis [29] of these experiments resulted in a linear plot, revealing that U937 cells expose $\approx 5 \times 10^4$ low-affinity homogeneous surface receptors per cell, with a K_d of 0.15×10^{-7} M (Figure 2B). Similar values were obtained for THP-1 cells (results not shown).

Characterization of the U937 cell-surface receptor targeted by the sCD38

The physical association of sCD38 with a cell-surface receptor was revealed by means of cross-linking experiments on intact cells. When U937 cells were equilibrated with ^{125}I-sCD38 and cross-linked with increasing concentrations of DSS, a product composed of the receptor and ^{125}I-sCD38 migrated at a molecular mass of 170 kDa (Figure 3A, lanes b and c). When only the solvent from the cross-linking reagent was added, no adduct was

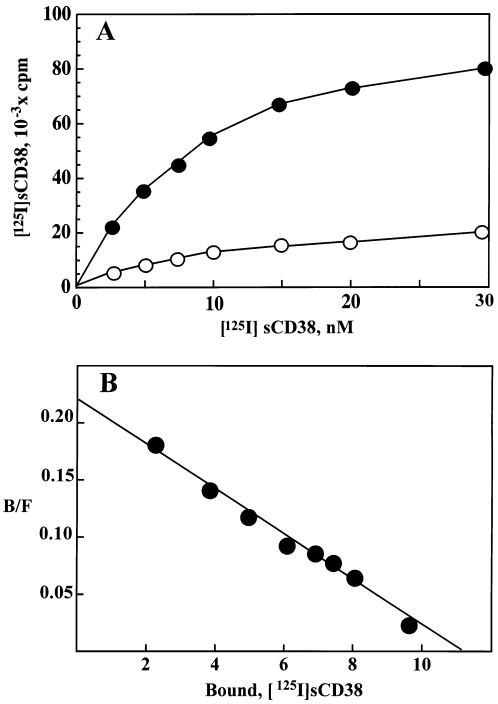

Figure 2 Binding activity of sCD38

To U937 cells were added increasing doses of ^{125}I-sCD38 in the presence (○—○) or absence (●—●) of unlabelled sCD38 diluted in RPMI/BSA. Cell-bound radioactivity was separated by centrifugation through a cushion of 20% (w/v) sucrose in RPMI/BSA, and the cell pellet was counted in a γ-counter. For sCD38 affinity analysis, the data from the filled circles (**A**) were analysed and plotted using a SAB program version for PC desk-top. The data shown are representative of two separate experiments. Abbreviations: B, bound; F, free.

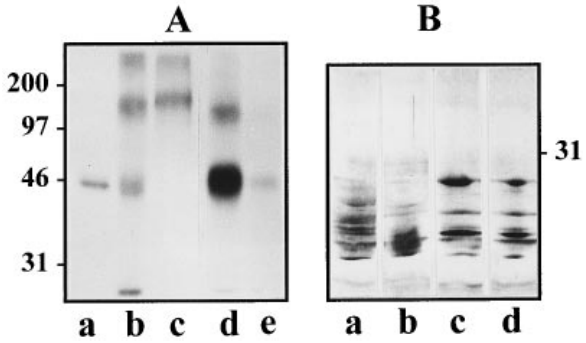

Figure 3 Cross-linking of ^{125}I-sCD38 to U937 cells

(**A**) Cells were incubated with ^{125}I-sCD38 for 1 h at 4 °C. After separation from unbound labelled ligand as described in the legend to Figure 2, the cells were cross-linked with DSS and solubilized with lysis buffer. The cross-linked complex was analysed by immunoprecipitation with IB4 mAb–Sepharose, SDS/PAGE and autoradiographed. Lane a, ^{125}I-sCD38 + U937 + solvent; lanes b and d, ^{125}I-sCD38 + U937 + 1 mM DSS; lane c, ^{125}I-sCD38 + U937 + 3 mM DSS; lane e, ^{125}I-sCD38 + U937 + unlabelled sCD38. (**B**) Peptide-mapping analysis: a one-dimensional peptide mapping of the radioactive cross-linked complex (lane c) and ^{125}I-sCD38 (lane d) cut and dried from the gel was performed as described in the legend to Figure 1(A). The peptides were detected by autoradiography. BSA (lane a) and IgG (lane b) were included as internal peptide mapping controls. Molecular masses of protein standards are indicated (kDa).

observed (lane a). As a test for specificity of the cross-linked product (lane d), the presence of > 100-fold molar excess unlabelled sCD38 included in the reaction mixture competed for

binding and cross-linking, resulting in the absence of the sCD38–receptor complex signal (lane e). The cross-linked band of 170 kDa represented the complex between the 39 kDa sCD38 and the cell-surface receptor. After subtracting the mass contribution of sCD38 and assuming the binding involves monomeric molecular forms, the receptor has a molecular mass of 130 kDa. Confirmation that the high-molecular-mass complex contained the ^{125}I-sCD38 molecule was obtained by peptide analysis following limited proteolysis. At least three identical peptides were observed when the cross-linked complex (Figure 3B, lane c) and ^{125}I-sCD38 (lane d) cut and dried from gels were subjected to V8-protease digestion.

These results fit a model where sCD38 binds a promonocytic-cell-associated receptor. In an attempt to identify the 130 kDa sCD38 receptor, we first tried to inhibit the binding of ^{125}I-sCD38 to the receptor expressed on U937 cells by pre-incubation of the cells with different mAb to human CD31. The 5F49 mAb produced a dose-dependent reduction in binding, whereas Moon-1 mAb had little effect on the binding of the ^{125}I-sCD38 to its receptor ($45 \pm 5\,\%$ *versus* $17 \pm 3\,\%$ binding inhibition respectively). Thus the sCD38 seems to identify a CD31 epitope that is far from the Moon-1 domain, but which partially overlaps the 5F49 epitope. The preincubation of ^{125}I-sCD38 with IB4 mAb resulted in an inhibition of binding ($65 \pm 5\,\%$), whereas the isotype-matched control CBT11 mAb did not affect the interaction between ^{125}I-sCD38 and the cell-surface receptor.

The binding activity of soluble CD38 is mediated by murine and human CD31

The receptor activity of sCD38 was also analysed through a direct association with soluble recombinant CD31 molecules of both murine and human origin.

Binding activity of murine CD31

The binding abilities of ^{125}I-muCD31-D_{1-3} and -D_{1-6} were used to prove the heterotypic interaction. First, a cell-protein adhesion screen was carried out to establish the bindability of the murine constructs. U937 and THP-1 cell lines bound both molecules, although ^{125}I-muCD31-D_{1-6} was much better able to sustain the binding (results not shown). The physical interaction between ^{125}I-muCD31-D_{1-6} and CD38 was characterized by using cross-linking experiments on intact cells. When U937 cells were exposed to ^{125}I-muCD31-D_{1-6}, the soluble construct was immunoprecipitated with antibodies against CD38 from cell lysate after stabilization of the binding with DSS (Figure 4A, lanes a and b) or DSP (lanes c and d), resulting in the detection of a $\approx$ 140 kDa protein complex. In the presence of 2-ME (lane d), the adduct was almost completely reduced. The interplay between the molecules was further confirmed in a ligand blot assay. Both ^{125}I-muCD31-D_{1-3} and -D_{1-6} reacted in a Western blot with a 45 kDa molecule immunoprecipitated with CD38 mAb IB4 from U937 cells (Figure 4B, lanes a and b). The molecule targeted by ^{125}I-muCD31-D_{1-3} and -D_{1-6} was recognized by ^{125}I-IB4 (lane c), indicating that the ligand on the blot is the CD38 antigen. The specific 45 kDa band on the blot was also highlighted by ECL when subjected to stripping and reprobing with muCD31-D_{1-3} or -D_{1-6} and revealed by a GαmIg (L-chain) antibody (lanes d and e). Thus the presence of CD31 molecules was confirmed as associated with the complex. No reaction was detected when U937 cell lysate was immunoprecipitated with a control CBT11 mAb-Sepharose (results not shown).

Having proven that the mCD38 is physically targeted by the soluble recombinant muCD31, the next step was the analysis of the interaction between both molecules in their soluble forms.

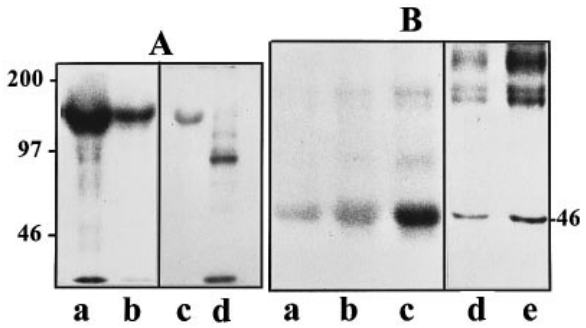

Figure 4 Binding of ^{125}I-muCD31-D_{1-6} to U937 cells

(**A**) Cross-linking analysis: ^{125}I-muCD31-D_{1-6} was incubated with U937 cells and cross-linked by means of DSS (lanes a and b) or DSP (lanes c and d). The cells were then lysed, and the resulting covalent complexes were immunoprecipitated with IB4 mAb-coated GαmIg–agarose. The samples were analysed by SDS/PAGE (10% polyacrylamide) under nonreducing (lanes a and c) and reducing conditions (lanes b and d). After Coomassie Blue staining, the gels were dried and autoradiographed. (**B**) Ligand blot analysis: U937 cell lysate was immunoprecipitated with IB4 mAb-coated GαmIg–agarose; the material eluted from the beads was analysed by SDS/PAGE (10% polyacrylamide). Gels blotted on to nitrocellulose membranes were probed with ^{125}I-muCD31-D_{1-3} (lane a), -D_{1-6} (lane b) or ^{125}I-IB4 mAb (lane c) and autoradiographed. Blots were also subjected to stripping and were reprobed with muCD31-D_{1-3} (lane d) or -D_{1-6} (lane e), and the product was revealed using ECL. Molecular masses of protein standards are indicated (kDa).

When sCD38 was cross-linked to ^{125}I-muCD31-D_{1-6} and processed by immunoprecipitation, a co-ligand complex of $\approx$ 140 kDa was revealed by SDS/PAGE and autoradiography (Figure 5A). After subtracting the mass contribution of sCD38, the receptor has a molecular mass of $\approx$ 100 kDa. Indeed, the muCD31-D_{1-6} molecule resolves under SDS/PAGE as a doublet of $\approx$ 95–100 kDa (Figure 5A, lane a), probably due to differential glycosylation. The degree of glycosylation seems to be important for co-ligand binding, the shift of molecular mass being prevalently observed for the $\approx$ 100 kDa band (results not shown). Activity was also detected at a molecular mass of $>$ 200 kDa, possibly due to aggregation or oligomerization of CD31 through a homotypic interaction. When ascites depleted of detectable sCD38 were used as a partner of cross-linking, only the high-

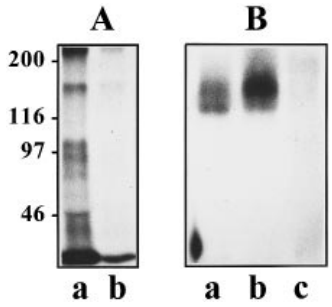

Figure 5 Cross-linking of murine sCD31 with sCD38 molecules

(**A**) ^{125}I-muCD31-D_{1-6} was incubated with human sCD38, and samples were cross-linked with DSS. The covalent complexes were immunoprecipitated, analysed by SDS/PAGE (10% polyacrylamide) under reducing conditions and autoradiographed. Lane a, ^{125}I-muCD31-D_{1-6} + sCD38 + DSS; lane b, ^{125}I-muCD31-D_{1-6} + ascites depleted of detectable sCD38 + DSS. (**B**) ^{125}I-muCD31-D_{1-6} was incubated with the chimaeric probe huCD38–muCD8α (500 μl of transfected or mock cell supernatant), and samples were analysed as in (**A**). Lanes a and b, ^{125}I-muCD31-D_{1-6} + huCD38–muCD8α + DSS; lane c, ^{125}I-muCD31-D_{1-6} + mock cell supernatant + DSS. Molecular masses of protein standards are indicated (kDa).

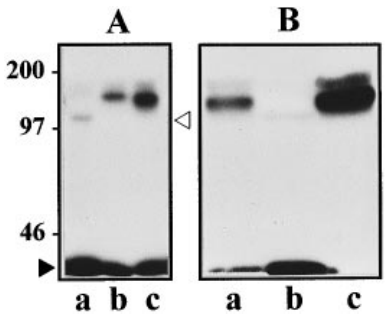

Figure 6 Cross-linking of human soluble molecules

(**A**) ^{125}I-sCD38 and ^{125}I-huCD31-Rg were cross-linked with DSS in the absence of Ca^{2+}, and the resulting immunoprecipitated covalent complexes were analysed by SDS/PAGE (7.5% polyacrylamide). The dried gel was Coomassie Blue stained and subjected to autoradiography. Lane a, ^{125}I-huCD31-Rg + ^{125}I-sCD38; lane b, ^{125}I-huCD31-Rg + ^{125}I-sCD38 + 1 mM DSS; lane c, ^{125}I-huCD31-Rg + ^{125}I-sCD38 + 3 mM DSS. (**B**) ^{125}I-sCD38 (lane b) was incubated with ^{125}I-huCD31-Rg in the absence (lane a) or in the presence (lane c) of 1 mM Ca^{2+}. Samples were cross-linked with 3 mM DSS, and the resulting covalent complexes were analysed as in (**A**). The positions of ^{125}I-sCD38 (▶) and ^{125}I-huCD31-Rg (▷) and molecular masses of protein standards (kDa) are indicated.

molecular-mass complex was visible (Figure 5A, lane b). Similar results were obtained using the huCD38–muCD8α molecule as a probe (Figure 5B). The binding and cross-linking with ^{125}I-muCD31-D_{1-6} converted almost all of huCD38–muCD8α into a 140–160 kDa complex (lanes a and b). This complex may result from formation of covalent bonds at two different points along the ligands yielding polypeptides with different migration. No evidence for complex formation was observed when the ^{125}I-muCD31-D_{1-6} molecule was cross-linked to a supernatant of mock transfectants (lane c).

Binding activity of human CD31

To explore the functional relationship between murine and human CD31 species, the human construct ^{125}I-huCD31-Rg was targeted to ^{125}I-sCD38 and then chemically cross-linked. The interaction of ^{125}I-sCD38 with ^{125}I-huCD31-Rg, followed by stabilization with increasing concentrations of DSS, resulted in the formation of a co-ligand complex, whose molecular mass corresponds to the addition of both molecules (Figure 6A, lanes b and c). Although this co-ligand complex was generated in the absence of Ca^{2+} (Figure 6B, lane a), the presence of 1 mM Ca^{2+} promotes migration on the CD31 ligand, producing a complete ^{125}I-sCD38 gel band shift (Figure 6B, lane c).

DISCUSSION

Leukocyte trafficking involves a finely modulated interaction between leukocytes and endothelial cells that depends on specific adhesion molecules and their soluble forms [1–3]. The recent identification of an active sCD38 [14], together with the availability of murine and human fusion protein isoforms of CD31 made it possible to show in the present study the CD38–CD31 co-ligation in human myeloid cells.

The receptor activity and the apparent avidity of the interaction between sCD38 and a cellular receptor were first examined. The rapid association and dissociation rate constants of adhesion mediated by selectins, required to permit rolling versus sticking during leukocyte trafficking [2], support our belief that the obtained K_d value (Figure 2) falls in the range expected for selectins. Indeed, it was recently found that (i) mCD38-mediated cell adhesion is effective in dynamic conditions, where they minimize the integrin functions, and (ii) upon occupancy intracellular Ca^{2+} levels of leukocytes were increased. These features are reminiscent of those exerted by selectins, although CD38 structure is not significantly homologous with selectins [13–15].

Next, a series of experiments, using a combination of receptor–ligand cross-linking and ligand blotting analysis, was conducted to identify the sCD38-receptor molecule on U937 cells. The cross-linking of ^{125}I-sCD38 on intact cells (Figure 3A) revealed a 130 kDa cell-surface receptor. This is consistent with the recent definition of the CD38-binding molecule on endothelial cells as CD31 [15,30]. Further proof of the CD38–CD31 interaction on promyelocytic cells was derived from the results of immunoprecipitation of the CD38 molecule from U937 cell lysate and probed in a ligand blot assay using soluble recombinant CD31 isoforms of murine origin (Figure 4B). The reactivity detected indicated that (i) the recognized epitope is determined by the tertiary structure of the molecule, and that (ii) the binding domain involved resides in the ectocellular portion of the CD31 molecule, more precisely in the first three N-terminal domains, as the interplay was also observed with the D_{1-3} truncated form of CD31. In fact, domain D_2 has been reported to be important for heterotypic binding to a cell-associated receptor [31,32]. Further, it is unlikely that (iii) the observed interaction is homotypic, since the lack of any of the D_{1-6} CD31 Ig domains leads to loss of this type of binding [33].

Cross-linking experiments also showed that soluble recombinant muCD31 was able to bind the human sCD38 antigen and the chimaeric huCD38–muCD8α probe (Figure 5), confirming the novel interplay in phylogeny. The observed interspecies ligand-binding activity of CD31 was not surprising, since cloning of the murine homologue of CD31 revealed an amino acid sequence with a similarity of 79% to human CD31 [34], suggesting the existence of highly conserved binding domains among species.

Recently, the integrin $\alpha_v\beta_3$ has been described as a heterotypic ligand for murine and human CD31 [16,20]. The binding to the integrin is supported by minimal truncated forms of CD31, whereas the Ig domains D_{1-6} did not further contribute to it [31]. In contrast, the CD38 binding reactivity was more apparent with CD31 displaying all six N-terminal Ig domains. Previous reports have shown that binding of CD31 to U937 cells was sensitive to cations, suggesting that the heterotypic counter-receptor on U937 cells might be the $\alpha_v\beta_3$ integrin [21]. However, a cation-independent binding also occurred when U937 cells or human T-cells bound to sCD31 molecules [22]. Data reported herein showed that the heterotypic binding following cross-linking of sCD38 to huCD31-Rg was also independent of cations, and yet, the addition of Ca^{2+} seems to enhance it (Figure 6). Although the actual reasons of increased sCD38 band shifting still await elucidation, some clues are derived from (i) general rules of sensitivity to divalent cations established for adhesion molecules [35], and (ii) the fact that the enzymic activity of integral type II ectoenzymes (e.g. CD38 and PC-1) increased in the presence of divalent cations [8,36]. Thus a Ca^{2+}-induced conformational change in individual receptors is the most likely explanation; however, no direct evidence on this proposal is available.

The biological functions of sCD38 are not yet clarified. Its immunological, enzymic and functional binding activities are relevant to confirmation of the fact that the soluble step of CD38 is part of the physiological life of the molecule. However, until evidence is obtained that sCD38 is endowed with signalling properties, like its membrane counterpart, it might be referred as a 'decoy molecule', which acts as a molecular trap for a

receptor ligand [37]. The activity of sCD38 as a decoy molecule is consistent with a model where the interplay of sCD38–CD31 modulates (i) the functional activity of mCD38, which serves as a biological drift directing leukocyte trafficking to their final destination; and (ii) the adhesion of cells that possess surface-exposed CD31. In this respect, the molecular mechanisms regulating the prevalence of homotypic or heterotypic adhesion of CD31-expressing cells remain unknown. However, the finding that sCD38 acts as a heterotypic ligand of CD31 offers some insight into its possible role as a regulatory mechanism.

Discernment of the physiological receptor/ligand role attributed to these molecules is important in view of their possible involvement in disease states: CD38 is expressed by myeloid precursor cells and their malignant counterparts. Despite marked improvement in survival rates, a significant number of leukaemic patients develop the Retinoic Acid Syndrome after treatment with all-*trans* retinoic acid, a potent CD38-inducing agent [38,39]. This is probably due to the increased expression of adhesion molecules on cells of the myeloid lineage [40], suggesting that the use of sCD38 may block this lethal side effect. The expression of CD31 is maintained during tumorous transformation, and the structure is important in the metastatic process [41]. Thus the sCD38 decoy molecule serves as a potentially exploitable pathway for anti-adhesive therapeutic intervention.

This work was supported in part by research grants from AIRC (Milan, Italy), Telethon (Milan, Italy), Special Project ACRO (CNR, Rome, Italy), AIDS and TB (Istituto Superiore di Sanità, Rome, Italy). A.L.H., on leave of absence from the National Research Council (Buenos Aires, Argentina), was supported by 'Fondazione CRT' (Torino, Italy). We are grateful to Dr. J. Banchereau (Schering-Plough, Dardilly, France) for providing the chimaeric huCD38–muCD8α probe. We thank A. Zanotto for technical assistance.

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Received 3 July 1997/6 November 1997; accepted 24 November 1997