Mapping the epitope of a functional P-selectin monoclonal antibody (LYP20) to a short complement-like repeat (SCR 4) domain: use of human–mouse chimaera and homologue-replacement mutagenesis

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P-selectin (CD62P), an adhesion molecule localized in platelet α -granules and endothelial cell Weibel–Palade bodies, is rapidly expressed on the surface of activated cells. This adhesion molecule, a member of the selectin family, mediates leucocyte interactions with activated platelets or endothelial cells. The aim of this study was to identify and characterize the epitope of a functional blocking P-selectin monoclonal antibody (mAb), LYP20. LYP20 recognizes human or rat, but not mouse, P-selectin. Human/mouse chimaeras and wild-type constructs, modified by homologue replacement mutagenesis, were expressed in COS cells. Blocking anti-(P-selectin) mAbs (G1, G3 or CLB-thromb/6) were observed, by flow cytometry, to bind to the lectin-like domain. In contrast, LYP20 was found to bind to one of the P-selectin short complement-like repeats (SCR domain 4). Homologue replacement mutagenesis of SCR domain 4 (region

delineated by amino acid residues 359–457) identified three amino acids (Cys⁴¹² → Ser, Cys⁴¹⁶ → Ser or Arg⁴¹⁵ → Lys) as being implicated in the LYP20 epitope. Deleting the region bearing the LYP20 epitope, from a wild-type CD62P construct, showed a decrease in polymorphonuclear leucocyte (PMNL) binding to transfected COS cells. In addition, mutation of one of the three amino acids, implicated in the LYP20 epitope, markedly affected PMNL binding to transfected COS cells but did not affect the binding of mAbs G1 and CLB-thromb/6. These results are the first to indicate (1) that a functional blocking anti-P-selectin mAb binds to SCR 4, a site other than the lectin-like/epidermal growth factor-like domain, and (2) that SCR domain 4 has a functional role in P-selectin–leucocyte interactions.

INTRODUCTION

P-selectin, a member of the selectin family [1,2], is rapidly expressed by activated endothelial cells or platelets [3–5]. Under resting conditions, P-selectin is present as an integral membrane component in Weibel–Palade bodies of human endothelial cells or in platelet α -granules [6–8]. Recent studies have shown the capacity of endothelial cells for P-selectin transcription *de novo* after stimulation with minimally oxidized low-density lipid, interleukin 1 or tumour necrosis factor [9–11]. This adhesion molecule is thought to have a crucial role in allowing leucocytes to roll on the activated vascular wall, at high shear stress, before adhesion and extravasation take place. In the presence of platelet-activating factor and activated $\alpha_v\beta_3$, P-selectin has a crucial role in allowing leucocyte–endothelial cell interactions [12,13]. P-selectin expression not only tethers leucocytes but also seems to be involved in their activation, with other cofactors, by inducing tissue factor expression, generation of O₂^{•−}, interleukin-8, monocyte chemotactic protein-1, phagocytosis via CD11b/CD18 activation, and synthesis of thrombospondin and heparan sulphate proteoglycan [14–16].

Members of the selectin family, which includes E-selectin, L-selectin and P-selectin, show structural similarities that include lectin-like, epidermal growth factor (EGF)-like, and short complement-like repeat (SCR) domains. The lectin-like and EGF-like domains are thought to play a critical role in functions of the selectin family [9,11,15]. Very little is known at present about the role of the SCR domains. Domain deletion studies,

performed on E-selectin, indicate that although the lectin-like and EGF-like domains are necessary to mediate cell adhesion, the additional six SCR domains contribute to enhancing the binding of E-selectin to its ligand [17].

Previous work from our laboratory has raised and characterized a monoclonal antibody (mAb) directed against human P-selectin [18]. This mAb, LYP20, was shown to inhibit the interaction of human or rat leucocytes with activated platelets or endothelial cells [13,19]. Moreover it was observed to decrease greatly the level of polymorphonuclear leucocytes (PMNLs) in a rat peritoneal inflammation model [19]. The aim of this study, with human/mouse P-selectin chimaeras and homologue replacement mutagenesis, was to identify the epitope of LYP20. Results indicate that the LYP20 epitope is located on P-selectin SCR domain 4. A significant decrease in PMNL binding to COS cells, expressing P-selectin, was observed when the domain bearing the LYP20 epitope was deleted or mutated. These results suggest a functional role for SCR domain 4 in leucocyte–P-selectin interactions.

MATERIALS AND METHODS

Materials

The M13 mutagenesis kit was obtained from Bio-Rad (Richmond, CA, U.S.A.). Oligonucleotides were from Genosys (The Woodlands, TX, U.S.A.). Restriction endonucleases were from

Abbreviations used: EGF, epidermal growth factor; hm, human–mouse chimaera; mAb, monoclonal antibody; mh, mouse–human chimaera; PMNL, polymorphonuclear leucocyte; SCR domain, short complement-like repeat domain; HBSS, Hank's balanced salt solution.

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New England Biolabs (Beverly, MA, U.S.A.). LipofectAMINE reagent was from Gibco-BRL. The COS-7 cell line was obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, Wilts., U.K.).

P-selectin constructs

Human and mouse P-selectin cDNA species were both subcloned into *Hind*III and *Xba*I sites of M13mp19. Restriction endonuclease sites were then introduced into each cDNA, exploiting codon degeneracy without altering the reading frame. The mutated cDNA species were then cloned into a pCDM8 expression vector and digested by the appropriate restriction endonuclease to allow the exchange of sequences between human and mouse cDNA species. The ligation products were then amplified in *Escherichia coli* MC1061p3 and purified on Qiagen columns. Amino acid substitutions were introduced into the human cDNA, again by oligonucleotide-directed mutagenesis. The three-letter code for amino acids is used for human P-selectin mutants. The number refers to the position in the P-selectin primary sequence of the first residue mutated. The corresponding mutation is given after the position number. All mutations were confirmed by DNA sequencing.

COS cell transfection

COS cell monolayers at 50% confluence in 10 mm Petri dishes were transfected with 6 µg of purified DNA by using 18 µl of LipofectAMINE in Dulbecco's modified Eagle's medium in accordance with the manufacturer's instructions. Cells were then grown for 48 h to allow the expression of CD62P.

mAbs

A panel of mAbs used (CLB-thromb/6, S12, G1 and G3) were gifts from the Fifth Leucocyte Workshop (Boston, MA, U.S.A.). Rb40-34 is an anti-(mouse P-selectin) rat IgG generously given by Dr. D. Vestweber (Institute of Cell Biology, Munster, Germany). LYP20 was produced and characterized in our laboratory [18].

Flow cytometry

Blood was drawn, in 0.38% trisodium citrate, pH 7.45 (0.9 ml/0.1 ml respectively), for measuring the expression of P-selectin on human, rat and mouse platelets. Human blood samples were used directly for flow cytometry assays, whereas mouse and rat blood samples were diluted (1 ml in 5 ml) in calcium-free Tyrode/BSA (3.5 mg/ml) solution (pH 7.45). Whole-blood platelet flow cytometry was performed as described by Shattil et al. [20] with a saturating concentration (40 µg/ml) of different antibodies. Platelet activation was performed by incubating blood samples with a 0.6 µM final solution of PMA. Platelet antibody binding was revealed by FITC-conjugated goat anti-(mouse IgG) antibody. Analysis was performed on a Becton-Dickinson Facscan® with the LYSISII software. COS cells 48 h after transfection were detached, for measurement of P-selectin expression, with 5 mM EDTA, washed in PBS and resuspended at a concentration of 10⁶ cells/ml in PBS/0.5% BSA. Anti-(P-selectin) mAbs were added at a concentration of 2.5 µg/ml and then incubated for 1 h at 4 °C. After two washes with PBS, cells were incubated for 1 h at 4 °C with the goat anti-(mouse IgG)-FITC conjugate. After washes with PBS, the level of P-selectin expression was analysed as for platelets. The ratio of the

binding of LYP20 and G1 to the wild-type construct, expressed in COS cells, was set equal to 100%. For the different mutated constructs the LYP20/G1 ratio was used, compared with the value obtained for the wild-type construct, to calculate the percentage of LYP20 binding.

PMNL-COS cell rosetting

PMNLs, isolated from blood by using a combination of dextran T500 sedimentation and Ficoll-Histopaque gradient centrifugation, were resuspended in Hanks balanced salt solution (HBSS)/0.5% BSA at a concentration of 1.2 × 10⁶ cells/ml. After 48 h of transfection, COS cells were detached with 5 mM EDTA, washed in HBSS and resuspended at a concentration of 4 × 10⁵ cells/ml in HBSS/0.5% BSA. For rosetting assays, 100 µl of the transfected COS cells was incubated with 100 µl of neutrophils (30 min, 37 °C). Rosettes (two or more bound neutrophils per COS cell) were evaluated in the cell suspension by using an Axioplan microscope (Zeiss, Oberkochen, Germany) by scoring a minimum of 200 COS cells. Triplicate assays for each experiment were scored by two different observers. An aliquot from each expressed construct was studied by flow cytometry to determine the percentage of transfection and the site densities. Appropriate mAbs were used to assay the expression of chimaera constructs (mAbs LYP20 and RB40-34) or homologue replacement constructs (mAbs CLB-thromb/6). A multiplicity factor, resulting from the ratio of percentage of transfection of wild-type/mutated construct, was used in calculating rosetting results. Rosetting results were multiplied by the above-mentioned factor to allow a comparison between different constructs and wild-type cell-cell interactions. Statistical analysis was performed with the unpaired Student's test.

RESULTS

Binding of anti-(P-selectin) mAbs to platelets

Flow cytometry performed on activated human, rat or mouse platelets showed LYP20 and a panel of mAbs (G1, G3 and CLB-thromb/6) binding to human but not to mouse P-selectin (Figure 1). LYP20 was the only mAb that recognized rat P-selectin (Figure 1).

P-selectin chimaeras

Human/mouse P-selectin chimaeras, expressed by COS cells, were used to identify the LYP20 epitope. mAbs LYP20, G1 and G3 recognized human wild-type P-selectin, but not its mouse equivalent, expressed by transfected COS cells. This recognition by LYP20 was cancelled when expressing a construct [human-mouse (hm)209] in which the human P-selectin SCR domains had been replaced by their mouse counterparts. In contrast, on expression of construct mh209, in which the human SCR domains had been kept and the lectin-like/EGF-like domains swapped for their mouse counterpart, LYP20 recognition was reinstated. mAbs G1 and G3, known to be directed against the human lectin-like domain of P-selectin [21], bound to expressed construct hm209 but not to mh209. Another anti-(P-selectin) mAb, CLB-thromb/6 [22], was observed in this study to have its epitope located in the same region as G1 and G3 (Figure 2 and Table 1). To confirm the position of the LYP20 epitope in the SCR region, a mutated form (construct h238-610) of wild-type human P-selectin, lacking SCR domains 2-7, was used. mAbs G1, G3 and CLB-thromb/6, but not LYP20 or S12, bound to mutant h238-610. To delineate further the LYP20 epitope, having established that the mAb bound to a site located between SCR domains 2

Figure 1 Flow cytometry of activated platelets with the use of P-selectin mAbs

Anti-(P-selectin) mAbs (40 μ g/ml final concentration) were used that bound to human, rat or mouse PMA-activated platelets and followed by an incubation with an FITC-conjugated goat anti-mouse immunoglobulin. PMA was used at 0.6 μ M final concentration.

and 7, a series of additional human–mouse or mouse–human chimaeras (hm359, mh359, hm507 and mh507) were tested. LYP20 bound to mh359 and hm507 but not to hm359 and mh507. Replacement of the lectin-like, EGF-like and three first human SCR domains (construct mh359) by their mouse counterparts did not affect LYP20 binding. In contrast, LYP20 binding was abolished when the first five human SCR domains (mh507), in addition to the lectin-like and EGF-like domains, were replaced by their mouse counterparts. These results suggest that the LYP20 epitope is located on SCR domains 4 or 5. To pinpoint the location of the LYP20 epitope, a chimaera (mh457), bearing the mouse lectin-like, EGF-like and SCR domains 1–4, was tested and found not to be recognized by LYP20. These results indicate that the LYP20 epitope is located on SCR 4 and delineated by amino acid residues 359–457. S12, a member of the panel of antibodies directed against P-selectin, bound to COS

Figure 2 Schematic representation of P-selectin human–mouse chimaeras

H and h designate human P-selectin (open squares); M and m designate mouse P-selectin (cross-hatched squares). SCR domains are indicated by numbers. Abbreviations: Lec, lectin-like domain; EGF, EGF-like domain; TM, transmembrane domain; Cyt, cytoplasmic domain; H w-t, human wild-type P-selectin; M w-t, mouse wild-type P-selectin.

cells expressing constructs mh359, mh457 or mh507, but not to hm359 or hm507. In view of these results, and the lack of recognition of construct h238-610, the S12 epitope is most probably located between SCR domain 6 and the start of SCR domain 7 (Figure 2 and Table 1).

Homologue replacement mutagenesis of the region spanning residues 359–457

A certain number of amino acids are conserved, in a region spanning residues 359–457, in human and rat, but not in mouse P-selectin (Figure 3). In that context it is of interest to note that mouse P-selectin is highly homologous, with the exception of SCR 2 [23], to its human counterpart. Homologue replacement mutagenesis of 10 conserved amino acids, spanning residues 359–457 was performed to locate those implicated in the LYP20 epitope. Within the above boundaries, starting from the end of the SCR3 to the beginning of the SCR5 domain, ten point mutants of P-selectin were constructed. LYP20 binding was decreased

Table 1 Anti-(P-selectin) antibodies binding to P-selectin chimaera-transfected COS cells

Binding percentages were determined by flow cytometry with the LYSISII analysis software and are results from five experiments, where minimal variations were observed (under 5%) arising from variations in transfection efficiency. LYP20, CLB-thromb/6, S12, G1 and G3 are anti-(human P-selectin) antibodies. Rb40-34 is an anti-(mouse P-selectin) antibody. Abbreviations: H w-t, human wild-type P-selectin; M w-t, mouse wild-type P-selectin; hm, human-mouse construct, the attached number indicating the homologous replacement position; mh, mouse-human construct.

P-selectin construct	Binding (%)					
	LYP20	RB40-34	CLB-thromb/6	S12	G1	G3
H w-t	35	< 3	30	28	33	17
M w-t	< 3	21	< 3	< 3	< 3	< 3
hm209	< 3	< 3	19	< 3	19	15
mh209	27	30	< 3	13	< 3	< 3
h238-610	< 3	< 3	30	< 3	35	23
hm359	< 3	< 3	25	< 3	27	18
mh359	33	30	< 3	19	< 3	< 3
hm457	20	< 3	21	< 3	31	21
mh457	< 3	30	< 3	21	< 3	< 3
hm507	30	< 3	25	< 3	30	19
mh507	< 3	32	< 3	14	< 3	< 3

Figure 3 Localization of LYP20 epitope on P-selectin

Alignment of human (H), mouse (M) and rat (R) P-selectin sequences in the region of residues 359–457. Bold letters indicate the amino acids conserved in human and rat but not in mouse.

(by 25%) with a construct bearing an Arg⁴¹⁵ → Leu mutation (Table 2). Moreover, constructs with mutations Cys⁴¹² → Ser or Cys⁴¹⁶ → Ser showed a marked decrease (by 80–85%) in the binding of LYP20 (Table 2). These findings suggest that amino acids Cys⁴¹², Cys⁴¹⁶ and Arg⁴¹⁵, located in SCR domain 4, are part of the LYP20 epitope. Homologue replacement mutagenesis constructs, performed in the region spanning residues 359–457, had no effect on the binding characteristics of mAb G1 (Table 2) or CLB-thromb/6 (results not shown).

Rosetting of PMNLs to COS cells expressing CD62P chimaeras

Binding of PMNLs to COS cells transfected with human P-selectin construct (h238-610), lacking SCR domains 2–7, was significantly decreased ($P < 0.01$) compared with that observed

Table 2 Binding of LYP20 to wild type and P-selectin mutants

Transfected COS cells (10^6 /ml) were incubated with LYP20 or G1 at 2.5 μ g/ml in PBS/0.5% BSA for 1 h at 4 °C. After two washes with PBS, cells were then incubated with FITC-conjugated anti-(mouse IgG) for 1 h at 4 °C. After washes with PBS, mAb binding was assessed by flow cytometry. The ratio of the binding of LYP20 and G1 to the wild-type construct expressed in COS cells was set equal to 100%. For the different mutated constructs, the LYP20-to-G1 ratio was used, compared with the value obtained for the wild-type construct, to calculate LYP20 binding percentage. Results are means $\pm$ S.D. for four different experiments.

P-selectin construct	LYP20 binding (%)
Wild type	100
D364S	96 $\pm$ 5
D370C	106 $\pm$ 15
D364N-D370G	94 $\pm$ 4
L389V	106 $\pm$ 4
S391A	100 $\pm$ 14
C412S	13 $\pm$ 6
R415K	93 $\pm$ 17
R415L	66 $\pm$ 7
C416S	29 $\pm$ 17
L433V	89 $\pm$ 11

with the wild-type construct (Table 3). These results suggest that the SCR domains are somehow implicated in the mechanism allowing the docking of P-selectin to its ligand on PMNLs. Moreover, when the first three SCR domains of human P-selectin and/or the lectin-like/EGF-like region of human P-selectin (constructs mh359 or mh209 respectively) were replaced by their mouse counterpart, a significantly lower binding of PMNLs was observed than with human wild-type P-selectin (Table 3). Interestingly, construct mh507 induced a significant ($P < 0.01$) decrease in rosetting compared with human wild-type P-selectin (Table 3). Moreover the binding of PMNLs to COS cells transfected with mh507 constructs was significantly decreased ($P < 0.05$) compared with results observed with the mh209 construct. These results suggest that the region from the end of SCR domain 3 to SCR domain 5 is implicated in PMNL rosetting. The above results are in line with the lower capacity of activated mouse platelets, compared with human platelets, to rosette to human PMNLs (E. Chignier, unpublished work). A control with mock-transfected or untransfected COS cells was performed to

Table 3 Human PMNL binding to COS cells expressing P-selectin human-mouse chimaeras

Freshly isolated PMNLs (1.2×10^6) were incubated with transfected or mock-transfected COS cells (4×10^4) for 30 min at 37 °C. Rosetting was assessed in triplicate by two different observers, by counting COS cells binding two or more PMNLs in a Neubauer chamber. The results are means $\pm$ S.D. for three experiments. Abbreviations: NT, non-transfected COS cells; H w.t., human wild-type P-selectin. * $P < 0.01$ compared with human wild-type P-selectin; ** $P < 0.05$ compared with human wild-type P-selectin; †† $P < 0.05$ compared with P-selectin human-mouse chimaera mh359.

P-selectin construct	PMNL binding
NT	26.16 $\pm$ 9.41
H w.t.	72.0 $\pm$ 2.5
h238-610	52.83 $\pm$ 6.93*
mh209	57.66 $\pm$ 7.09**
mh359	55.16 $\pm$ 4.54*
mh507	38.66 $\pm$ 7.09*††

Table 4 Human PMNL binding to COS cells expressing P-selectin mutants

Freshly isolated PMNLs (1.2×10^6) were incubated with transfected or mock-transfected COS cells (4×10^4) for 30 min at 37 °C. Rosetting was assessed in triplicate by two different observers, by counting COS cells binding two or more PMNLs, in a Neubauer chamber. The results are means $\pm$ S.D. for three experiments. Abbreviations: NT, non-transfected COS cells; H w.t., human wild-type P-selectin. * $P < 0.01$, ** $P < 0.05$ compared with human wild-type P-selectin.

P-selectin construct	PMNL rosetting
NT	26.47 $\pm$ 6.12
H w.t.	91.67 $\pm$ 10.01
Cys ⁴¹² $\rightarrow$ Ser	52.41 $\pm$ 7.47*
Arg ⁴¹⁵ $\rightarrow$ Leu	65.35 $\pm$ 7.97**
Cys ⁴¹⁶ $\rightarrow$ Ser	56.88 $\pm$ 10.32*

determine the binding of PMNLs independently of P-selectin expression. In addition, there was no significant difference between the wild type and each construct in the site densities (results not shown). The background level of PMNL binding to controls might conceivably be due to the presence on COS cells of constitutively expressed adhesion molecules (such as $\alpha v \beta 3$ integrins) implicated in cell–cell interactions. Background levels were also found to differ slightly, for PMNLs, from one donor to another.

Rosetting of PMNLs to COS cells expressing human CD62P mutants

The wild-type P-selectin construct, in comparison with mock transfection, resulted in a substantial number of PMNLs rosetting to COS cells (Table 4). There was no significant difference between the wild type and each mutant in respect of the site densities (results not shown). The mutations, Cys⁴¹² $\rightarrow$ Ser, Cys⁴¹⁶ $\rightarrow$ Ser or Arg⁴¹⁵ $\rightarrow$ Leu, on residues located in SCR domain 4 induced a significant decrease in PMNLs rosetting to transfected COS cells (Table 4). In contrast, a different mutation (Leu³⁸⁹ $\rightarrow$ Val) located in SCR domain 4, which did not decrease LYP20 binding, had no effect on PMNLs rosetting to transfected COS cells (results not shown). Taken together, these results show that the SCR domain 4 region, located around the LYP20 epitope, might conceivably be involved in P-selectin interactions with PMNL binding.

DISCUSSION

This study has identified, for the first time, the epitope of a functional mAb (LYP20) to SCR domain 4 of P-selectin. Moreover, the SCR domains seem to be implicated in the mechanism, as observed from human–mouse chimaeras and homologue replacement mutagenesis, allowing the docking of P-selectin with its ligand on PMNLs. The role of SCR domains in P-selectin functions is linked to the active participation of the lectin-like and EGF-like domains. Several lines of evidence support the above statements: (1) the deletion of SCR domains (residues 359–457) and (2) single mutations (residues Cys⁴¹², Cys⁴¹⁶ and Arg⁴¹⁵) inside SCR domain 4 prevent LYP20 binding to P-selectin; (3) the deletion of SCR domains, human–mouse chimaera or (4) single mutation homologue replacement mutagenesis of P-selectin greatly decrease the rosetting of PMNL to transfected COS cells.

Three mutations (Arg⁴¹⁵ $\rightarrow$ Leu, Cys⁴¹² $\rightarrow$ Ser and Cys⁴¹⁶ $\rightarrow$ Ser), of ten performed in a region spanning residues 359–457,

affect LYP20 binding. Arg⁴¹⁵ $\rightarrow$ Leu was the first identified mutation on SCR domain 4 that decreased LYP20 binding. Subsequently, the two cysteine residues (Cys⁴¹² and Cys⁴¹⁶), selected for their proximity to Arg⁴¹⁵, were mutated and observed to decrease LYP20 binding greatly. However, these mutations had no effect on the binding of other mAbs (G1 or CLB-thromb/6) directed to other regions of P-selectin. Cysteine residues are known to be implicated in maintaining three-dimensional loop-like structures, formed by each SCR domain, on P-selectin [24]. Our results suggest that cysteine residues at positions 412 and 416 are crucial, together with Arg⁴¹⁵, in maintaining the proper conformational structure recognized by LYP20.

Functional studies on P-selectin, performed by different workers, demonstrate the importance of the lectin-like and EGF-like domain in P-selectin functions [13,21,25–27]. Indeed, previous results showed that all other blocking mAbs, such as G1, G2 and G3, have their epitope on the lectin-like region of P-selectin [21,28]. In contrast, non-blocking antibodies directed against P-selectin, such as S12 [15], KC4 or AC1.2 [22], have their epitope located on SCR regions. To our surprise LYP20, a blocking mAb directed against P-selectin, was found to have its epitope located on SCR domain 4. Moreover we demonstrate, via human–mouse chimaeras and homologue replacement mutagenesis, that the region bearing the LYP20 epitope is very much involved in the mechanism allowing P-selectin to interact with PMNL. These results are supported by some very interesting recent observations that start to show the role of SCR in P-selectin functions. An anti-(P-selectin) mAb, directed against SCR domain 5, was observed to prevent venous thrombosis in dogs [29]. Increased adhesion of neutrophils was induced by a mAb directed against the ninth SCR domain of P-selectin. These last results are attributed to the cross-linking, by the mAb, of the specific SCR domain that facilitates P-selectin oligomerization [30].

Attention has been drawn to the importance of the complement-like repeats in other members of the selectin family. Lymphocyte and neutrophil adhesion, to post-capillary venules in lymph nodes and E-selectin-expressing cells respectively, were blocked by an anti L-selectin mAb (EL-246) directed against the SCR domains [31]. Both E- and L-selectin, but not P-selectin, are recognized by EL-246. The epitope of EL-246 was identified through the use of E-, L- or P-selectin chimaera techniques [31]. Interestingly, Watson et al. [32] suggest that the role of the SCR domains could differ from one selectin to another. Compared with other members of the selectin family, P-selectin has the largest number (nine) of SCR domains. This could perhaps explain the higher binding affinity of P-selectin glycoprotein ligand-1 (an important ligand expressed by leucocytes) for P-selectin than for E-selectin [33]. Moreover, SCR domains have recently been shown to be crucial for optimal neutrophil rolling under shear stress. In fact Patel et al. [34] demonstrate that at least four SCR domains are necessary to initiate PMNL rolling under shear flow conditions. In addition, another mAb, 10E9.6, recognizing the SCR1 and SCR2 domains of mouse E-selectin, blocked neutrophil recruitment into the inflamed peritoneal cavity of Balb/c mice, but without affecting neutrophil rolling *in vitro* or *in vivo* [35]. Taken together, these results indicate an important role of SCR domains in selectin functions.

Our results suggest a crucial role for the SCR domain (spanning residues 359–457) in P-selectin functions. It is probable that the EGF-like domain, as well as the SCR domains, maintain the lectin-like region in a functional conformational state. In fact, EGF-like and SCR domain-induced changes could result from a change in lectin-like domain affinity or from conformational

changes resulting in the acquisition of novel carbohydrate ligand recognition capabilities. Moreover it is also conceivable that the EGF-like and SCR domains might interact with different regions of the P-selectin ligand, help in cementing the initial contact made by the lectin-like region, and initiate the necessary transmission signals in leucocyte gene activation. Further work is under way to investigate these different possibilities implicated in cell-cell interactions.

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