Effects of Chemical Modification on the Binding Activities of P-Selectin Mutants

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ABSTRACT: P-Selectin (GMP140, CD62P, PADGEM), a 140-kDa glycoprotein found on activated platelets and endothelial cells, is involved in one of the early events in the inflammatory response due to its role in initiating the recruitment of circulating leukocytes. From a three-dimensional model of P-selectin and site-specific mutagenesis studies, a number of residues were previously identified as critical for the binding of P-selectin to HL-60 cells, a human myeloid cell line. Included among them were lysines 111 and 113 (K111 and K113). In this study, the roles of K111 and K113 were further characterized by the generation and specific chemical modification of two cysteine mutants, K111C and K113C, of a P-selectin−immunoglobulin fusion protein (P-selectin-Rg). Both K111C and K113C displayed significantly reduced binding activity compared to the wild-type P-selectin from which they were derived, further illustrating the importance of these particular lysines for ligand binding. Reaction of K111C with aziridine or nipsylcysteamine resulted in the formation of K111C-AZ and K111C-CY, both of which displayed significant increases in HL-60 binding activity. No such increase took place upon reaction of K111C with *N*-ethylmaleimide, indicating that a free amine at position 111 is important for binding. Residue length at position 111 is not critical, since the synthetic side chains are 0.5−2.0 Å longer than lysine yet still impart binding activity. Similar modification studies of K111A and K113C did not lead to any detectable increase in binding of these proteins to HL-60 cells. The loss of activity when K111 is mutated to cysteine, together with the increase in HL-60 binding that takes place upon chemical modification of K111C with aziridine or nipsylcysteamine, confirms the importance of this amino acid residue in P-selectin ligand binding.

P-Selectin (GMP140, CD62P, PADGEM) is a 140-kDa protein located in the α-granules of platelets and Weibel-Palade bodies of endothelial cells (Johnston et al., 1989; Larsen et al., 1989; McEver et al., 1989; Lasky, 1992). Stimulation of these cells by thrombin, histamine, or peroxides results in the rapid redistribution of P-selectin to the cell surface where it mediates vascular endothelial cell−leukocyte or platelet−leukocyte binding, one of the early events in the inflammatory response (Stenberg et al., 1985; Patel et al., 1991; Sugama et al., 1992; Larsen et al., 1989; Hattori et al., 1989; Mayadas et al., 1993). The selectins are a family of adhesion molecules that also includes L-selectin (CD62L, Leu8, LAM-1) and E-selectin (CD62E, ELAM-1), which are expressed on leukocytes and activated endothelial cells, respectively. These proteins have amino-terminal lectin and epidermal growth factor-like domains and a variable number of complement receptor-related repeats. The lectin domain is responsible for the binding of carbohydrates in a calcium-dependent manner. While sialyl-Lewis^x and sialyl-Lewis^a have been shown to be ligands of P-selectin, the exact carbohydrate ligand on the cell surfaces is not known and may consist of other sialyl lactosaminoglycans presented on specific glycoproteins (Lowe et al., 1991; Tyrrell et al., 1991; Tiemeyer et al., 1991; Sako et al., 1993; Varki, 1994).

The role of P-selectin in the binding of leukocytes to endothelial cells suggests that inhibition of its ligand interaction may provide control over inflammation. *In vitro* inhibition of the P-selectin−ligand interaction has been accomplished with agents such as sulfatide (Aruffo et al., 1991), glycyrrhizin (Rao et al., 1994), carbohydrates related to Lewis^x (Mulligan et al., 1993a), heparin oligosaccharides (Nelson et al., 1993), P-selectin peptides (Heavner et al., 1993), pertussis toxin peptides (Rozdzinski et al., 1993), and anti-P-selectin antibodies (Winn et al., 1993). Neutrophil-mediated damage of acute inflammation *in vivo* is inhibited by soluble P-selectin, an anti-P-selectin monoclonal antibody (mAb),[1] and by oligosaccharides known to bind P-selectin (Weyrich et al., 1993; Mulligan et al., 1992, 1993b; Winn et al., 1993)

An understanding of the precise nature of P-selectin−ligand interaction would assist in the development of therapeutically useful inhibitory compounds. Currently, there are no available crystal or NMR structures reported for P-selectin, although the X-ray crystal structure of E-selectin, a protein with a high degree of homology, has recently been reported (Graves et al., 1994). Prior to that, a structural model for P-selectin was proposed on the basis of its homology (approximately 25%) with rat mannose-binding protein (Hollenbaugh et al., 1993; Bajorath et al., 1994; Bajorath & Aruffo, 1994). This model provided the basis for site-specific mutagenesis of P-selectin-Rg, a fusion protein consisting of P-selectin and a modified human Fc domain. Several key residues were identified as being

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[1] Abbreviations: P-selectin-Rg, P-selectin−immunoglobulin fusion protein; K111C-AZ, K111C-CY, and K111C-NEM, aziridine, cysteamine, and *N*-ethylmaleimide adducts of the P-selectin mutant K111C, respectively; DTT, dithiothreitol; mAb, monoclonal antibody; NC, nipsylcysteamine; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell sorting.

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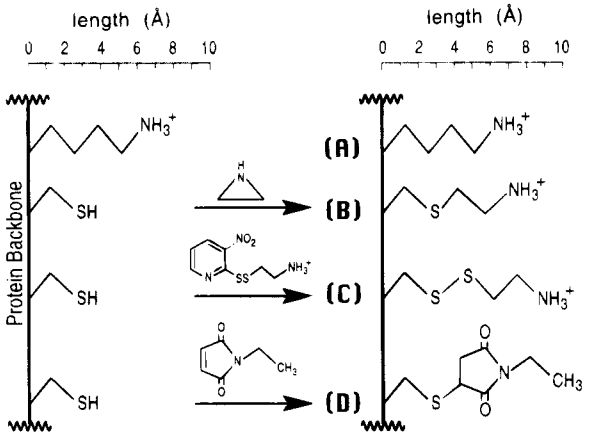

FIGURE 1: Structures of amino acid side chains. The residues shown in the right-hand portion of the figure correspond to (A) lysine, (B) reaction product of cysteine + aziridine (such as K111C-AZ), (C), reaction product of cysteine + nipsylcysteamine (such as K111C-CY), and (D) reaction product of cysteine + *N*-ethylmaleimide (such as K111C-NEM). The scale at the top of the figure gives the approximate length of the unfolded residues.

directly involved in ligand binding. In particular, mutations of the lysine residues at positions 111 and 113 to alanines (K111A, K113A) resulted in reduced binding to the cellular ligand (Hollenbaugh et al., 1993; Bajorath et al., 1994).

The interaction between P-selectin and its ligand may also be probed through chemical modification. Extending the original observations of Raftery and Cole (1966), a number of studies have shown that unnatural amino acid residues can be appended onto proteins by first introducing cysteines through site-specific mutagenesis and then using cysteine-specific alkylating reagents for further modification. Amino acid residues on proteins such as ribulose-bisphosphate carboxylase (Smith & Hartman, 1988), pseudomonas exotoxin (Lukac & Collier, 1988), thioredoxin (Wynn & Richards, 1993), and glutamine synthetase (Dhalla et al., 1994) have been mutated to cysteines, and the activities of the proteins after chemical modification have provided insight into enzyme-substrate interactions. We reasoned that similar studies of P-selectin would confirm the importance of residues K111 and K113 for ligand binding and allow a means to probe the binding site with residues of various lengths. One of the strengths of such an approach is that information regarding the importance of a particular amino acid residue is obtained by gaining binding activity. This is in contrast to site-specific mutagenesis studies, in which the interpretation of reduced binding activity that may result from amino acid substitution is complicated by potential changes in protein structure.

In this paper, we report the expression of two mutants of the P-selectin fusion protein, P-selectin-Rg, in which lysines at positions 111 and 113 were changed to cysteines. The resulting mutants, K111C and K113C, were chemically modified with aziridine, nipsylcysteamine (NC), and *N*-ethylmaleimide, forming selectin derivatives having the unnatural residues shown in Figure 1. The activities of the modified selectins are described.

MATERIALS AND METHODS

Materials. The L6 mAb (IgG_{2a}), which recognizes a 24-kDa tumor-associated antigen (Marken et al., 1992), was isolated as previously described (Hellström et al., 1986). NC was a generous gift from Dr. John Kadow. The compound was prepared according to a published procedure (Vyas et al., 1989). K111A was obtained as previously described (Hollenbaugh et al., 1993), and the anti-human P-selectin mAb 3D3 (Bajorath et al., 1994) was provided by Dr. Richard Darveau. The concentration of P-selectin-Rg and the P-selectin-Rg mutants were determined using the micro BCA assay (Pierce Chemical Co.) with bovine serum albumin as a standard. These results were within 10% of the values obtained spectrophotometrically at 280 nm using $E_{0.1\%}$ of 1.90 (from amino acid analysis of P-selectin Rg).

Construction and Isolation of Mutants. The gene for P-selectin-Rg was inserted into the pCDM8 plasmid, and protein was expressed in COS cells as previously described (Aruffo et al., 1991). This fusion protein contains three point mutations in the Fc region to reduce Fc receptor binding (Hollenbaugh et al., 1992). Mutants of P-selectin-Rg were constructed using overlap extension PCR (Ho et al., 1989) with *Pfu* polymerase as the catalyst. The oligonucleotide primers containing the desired mutations were as follows: K111C (forward), CTGCTTCTACAAAAAGCACGCGT-TGTGTTACACAGC; K111C (reverse), GGCTGTGTAAC-ACAACGCGTGCTTTTTGCACAAGCAGTG; K113C (forward), CTGCTTGAAGAAATGCCACGCGTTGTGTTAC-ACAGC; and K113C (reverse), GGCTGTGTAACACAA-CGCGTGGCATTTCTTCAAGCAGTG. An *Mlu*I restriction site was introduced in the primers in order to confirm that the resulting plasmid contained the mutated gene. The primers were used in separate PCR reactions with primers outside the P-selectin-Rg lectin domain. In the second PCR reaction, aliquots of the PCR products were mixed and further amplified using the external primers. The complete PCR products were digested with *Hin*dIII and *Kpn*I and ligated into the P-selectin-Rg gene in the pCDM8 plasmid. The presence of the K111C and K113C mutations and the absence of PCR-induced secondary mutations were confirmed by DNA sequencing. COS cells were transfected with the plasmids using DEAE-dextran and placed in serum-free Dulbecco's medium containing 4 mM L-glutamine the following day. Fresh medium was added after 3 days and the supernatant containing the fusion protein was harvested after 8−10 days. Purification of the fusion proteins was achieved by passing the supernatants through protein A−Sepharose columns and eluting the bound material with 0.1 M citrate (pH 3.0). The purified proteins were dialyzed against phosphate-buffered saline (PBS), concentrated using Centriprep-30 concentrators (Amicon), sterile-filtered, and stored at 4 °C.

Sulfhydryl Group Analysis. A modified procedure of Sueyoshi et al. (1985) was used to determine the number of free sulfhydryl groups present in the fusion proteins. Solutions of the proteins (14−40 μg) in PBS (0.32 mL) were treated with 8 M guanidine (0.16 mL), 0.5 M borate containing 1 mM EDTA at pH 9.5 (0.5 mL) and 0.4 mg/mL ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (0.05 mL) (Sigma). The solutions were heated at 60 °C for 45 min, allowed to cool to 23 °C, and precipitates (consisting of inorganic salts) were removed by centrifugation. Fluorescence was measured with excitation at 385 nm and emission at 515 nm, and the number of free sulfhydryl groups was determined by comparison to a standard curve obtained with reduced glutathione (0−1.6 nmol/sample) in place of protein. Protein concentrations were determined using the

micro BCA assay (Pierce Chemical Co.) with bovine serum albumin as a standard.

Chemical Modification of L6. Aziridine (Marshalton Research) was diluted in 0.5 M borate (pH 8.5). After the pH was readjusted to 8.5, the final aziridine concentration was 2.34 M. Similarly, solutions were prepared in 0.4 M phosphate at pH 4.0 and in PBS at pH 7.4. The solutions were kept on ice and were prepared just prior to use.

To a 2-mL solution of the L6 monoclonal antibody (7.6 mg/mL in PBS) (Hellström et al., 1986) on ice was added 0.2 mL of 0.5 M borate at pH 8.5, followed by 0.116 mL of 50 mM 2-iminothiolane (Pierce Chemical Co.). After 2.5 h, the solution was applied to a G-25 Sephadex column which was equilibrated in PBS containing 1 mM EDTA. The protein (12 mg) was collected in a total volume of 4 mL. The number of free sulfhydryl groups was determined using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Sigma) as previously described (Riddles et al., 1979). The thiol/L6 ratio was 7.5:1.

Thiolated L6 at 23 °C was treated with 2.34 M aziridine up to a final aziridine concentration of 1.56 M. After 30 min, the solution was applied to a G-25 Sephadex column which was equilibrated and eluted with PBS. Remaining sulfhydryl groups were measured using DTNB. The results were compared to those obtained for similarly processed thiolated L6 that was not treated with aziridine and for thiolated L6 (in PBS containing 1 mM EDTA) that was treated with 100 mM NC in H_2O (20 mM final concentration) instead of aziridine.

Chemical Modification of P-Selectin-Rg, K111C, and K113C. P-Selectin-Rg, K111C, and K113C (0.2 mg of each) in PBS were reduced with dithiothreitol (5 mM) for 30 min at 23 °C. Each protein solution was divided into three equal portions, which were then treated with either 50 mM NC in H_2O (20 mM final), 200 mM *N*-ethylmaleimide in dimethylformamide (20 mM final), or 2.34 M aziridine in 0.5 M borate at pH 8.5 (1.56 M final, prepared as described above). After 30 min at 23 °C, the samples were dialyzed against PBS at 4 °C. Protein concentrations were determined using the micro BCA assay (Pierce Chemical Co.) with bovine serum albumin as a standard. Samples were further analyzed by SDS−PAGE on 4−20% gels.

Binding Activity. HL-60 cells in RPMI medium containing 2% fetal bovine serum and 0.1% NaN_3 were incubated with excess murine IgG_{2a} (30−60 μg/mL, Sigma) to block Fc receptors before the addition of the selectin analogues (25 μg/mL) with or without the anti-P-selectin mAb 3D3 at 10 μg/mL. After 1 h at 4 °C, the cells were washed, FITC-conjugated goat anti-human Fc (TAGO, 1:500 dilution) was added, and incubation at 4 °C was continued for 45 min. The cells were washed, fixed with 1% paraformaldehyde in PBS, and analyzed with a FACScan flow cytometer (Becton-Dickinson). A previously reported assay (Hollenbaugh et al., 1993) involving the ability of the fusion proteins to mediate cell adhesion to plastic was used to determine the effects of aziridine on P-selectin-Rg binding. Briefly, fusion protein (2.5 μg/mL) is added to wells of a 96-well plate that has been coated with goat anti-human Fc antibody (5 μg/mL) and blocked with 1% BLOTTO. HL-60 cells are labeled with the fluorescent dye BCECF-AM (Molecular Probes) and allowed to adhere. The wells are washed, the adherent cells are lysed, and fluorescence is quantitated on a microplate fluorescence reader.

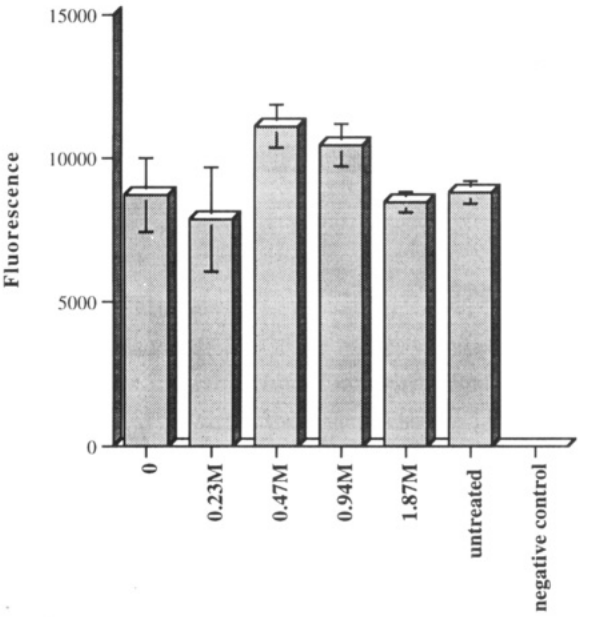

FIGURE 2: Binding of aziridine-treated P-selectin-Rg to HL-60 cells as assayed by adhesion to coated microtiter plates. The samples were treated with aziridine (pH 8.5) at the concentrations indicated. The negative control consists of wells treated with CD40-Rg, an unrelated fusion protein. The fluorescence values are the means of three points.

RESULTS AND DISCUSSION

Conditions for Chemical Modification. Model studies were undertaken with a readily available protein in order to establish conditions for efficient modification of the P-selectin mutants. Thiol groups were introduced on the L6 mAb (Hellström et al., 1986) through the reaction with 2-iminothiolane such that the ratio of SH groups to protein was 7.5:1. It was possible to block 82% of the thiols by reacting the antibody with 1.5 M aziridine at pH 8.5. The pH for the reaction appeared to be important, since very little reaction took place at pH 4.0 or 7.4. These results are consistent with previous reports indicating that high concentrations of aziridine are needed in order to effectively alkylate protein thiols (Raftery & Cole, 1966). In contrast, much lower concentrations of NC (20 mM) were required to effect a similar degree (80%) of thiol group modification. In addition, this reaction proceeded quite well at pH 7.4.

The conditions determined to be appropriate for derivatization, namely, high concentrations of aziridine at pH 8.5, could potentially affect the binding activity of the wild-type protein. To test this, wild-type protein was treated with varying concentrations of aziridine. The binding activity was then determined using a previously described cell adhesion assay (Hollenbaugh et al., 1993) in which the aziridine-treated P-selectin-Rg samples were captured with anti-human Fc antibody coated on plastic and then tested for their ability to bind to HL-60 cells, a human myeloid cell line. This adhesion is specific as demonstrated by the lack of binding to CD40-Rg. In addition, previous studies have shown that binding can be inhibited using antibodies against P-selectin (Bajorath et al., 1994). The results (Figure 2) indicated that aziridine concentrations as high as 1.9 M did not adversely affect P-selectin-Rg binding to HL-60 cells.

Expression and Characterization of P-Selectin Mutants. Overlap extension PCR (Ho et al., 1989) was used to mutate the lysines at positions 111 and 113 in P-selectin-Rg into

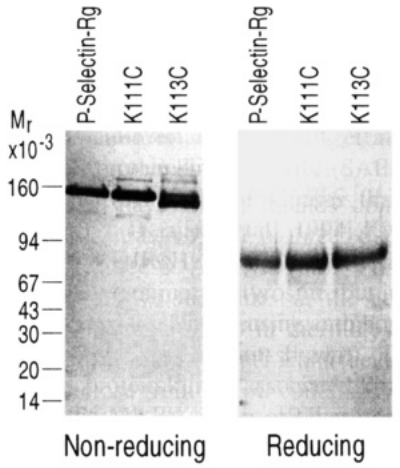

FIGURE 3: SDS−PAGE analysis (4−20% gradient gels) of P-selectin-Rg mutants under nonreducing and reducing conditions.

cysteines forming K111C and K113C, respectively. DNA sequence analysis verified that the correct mutations were inserted and that no additional changes had occurred elsewhere in the gene. The corresponding proteins were transiently expressed in COS cells as previously described (Hollenbaugh et al., 1993; Bajorath et al., 1994), and then purified by affinity chromatography on protein A−Sepharose. From 1-L culture supernatants, it was possible to obtain between 1 and 1.5 mg of P-selectin-Rg and K111C. The expression of K113C was consistently lower (0.4 mg/L). SDS−PAGE under nonreducing conditions indicated that small amounts (<10%) of higher molecular weight components were present in the K111C and K113C preparations (Figure 3). Under reducing conditions, the three proteins appeared to be identical. Thus, the vast majority of the K111C and K113C mutants did not form covalent adducts with other cysteine-containing proteins.

The number of free sulfhydryl groups present in the P-selectin analogues was assayed using ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (Sueyoshi et al., 1985) under denaturing conditions. This assay indicated the presence of approximately 2 thiol groups per P-selectin-Rg molecule. Surprisingly, only a single thiol group per protein molecule was detected in K111C and K113C. This might be due to the ability of the mutated cysteines of K111C and K113C to form internal disulfides, resulting in the net loss of one thiol group relative to P-selectin-Rg.

Effects of Chemical Modification on Selectin Binding. A FACS assay was used to assess the binding of the fusion proteins to HL-60 cells. Initial experiments failed to demonstrate improved binding activities for K111C and K113C after reaction with aziridine (data not shown). This is consistent with the reduced thiol content of K111C and K113C compared to P-selectin-Rg, suggesting that the critical sulfhydryl groups are not available for derivatization. As a result, the fusion proteins were reduced with 5 mM dithiothreitol (DTT) prior to further chemical modification. The reduction conditions are similar to those used in related studies with other cysteine-containing proteins (Stauffer & Karlin, 1994; Raftery & Cole, 1966) or proteins that were site-specifically mutated to contain new cysteine residues (Dhalla et al., 1994; Smith & Hartman, 1988). Figure 4

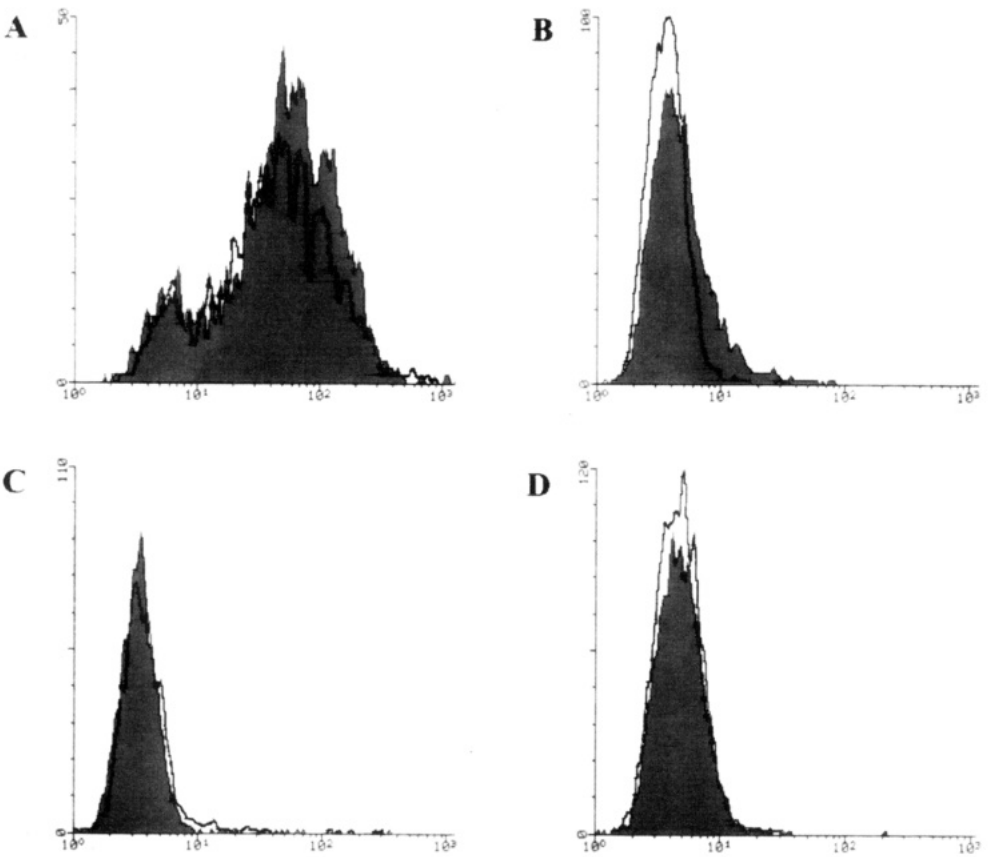

FIGURE 4: Effects of reduction on the binding activities of P-selectin-Rg proteins to HL-60 cells. (A) Wild-type P-selectin-Rg, (B) K113C, (C) K111C, and (D) K111A were treated with DTT (5 mM) for 30 min at 23 °C, and binding activities were determined by flow cytometry. Cells treated with unreduced proteins are represented by the filled areas, and solid lines represent cells that were treated with reduced proteins. Relative cell number is indicated on the *y*-axes, and fluorescence intensity is indicated on the *x*-axes.

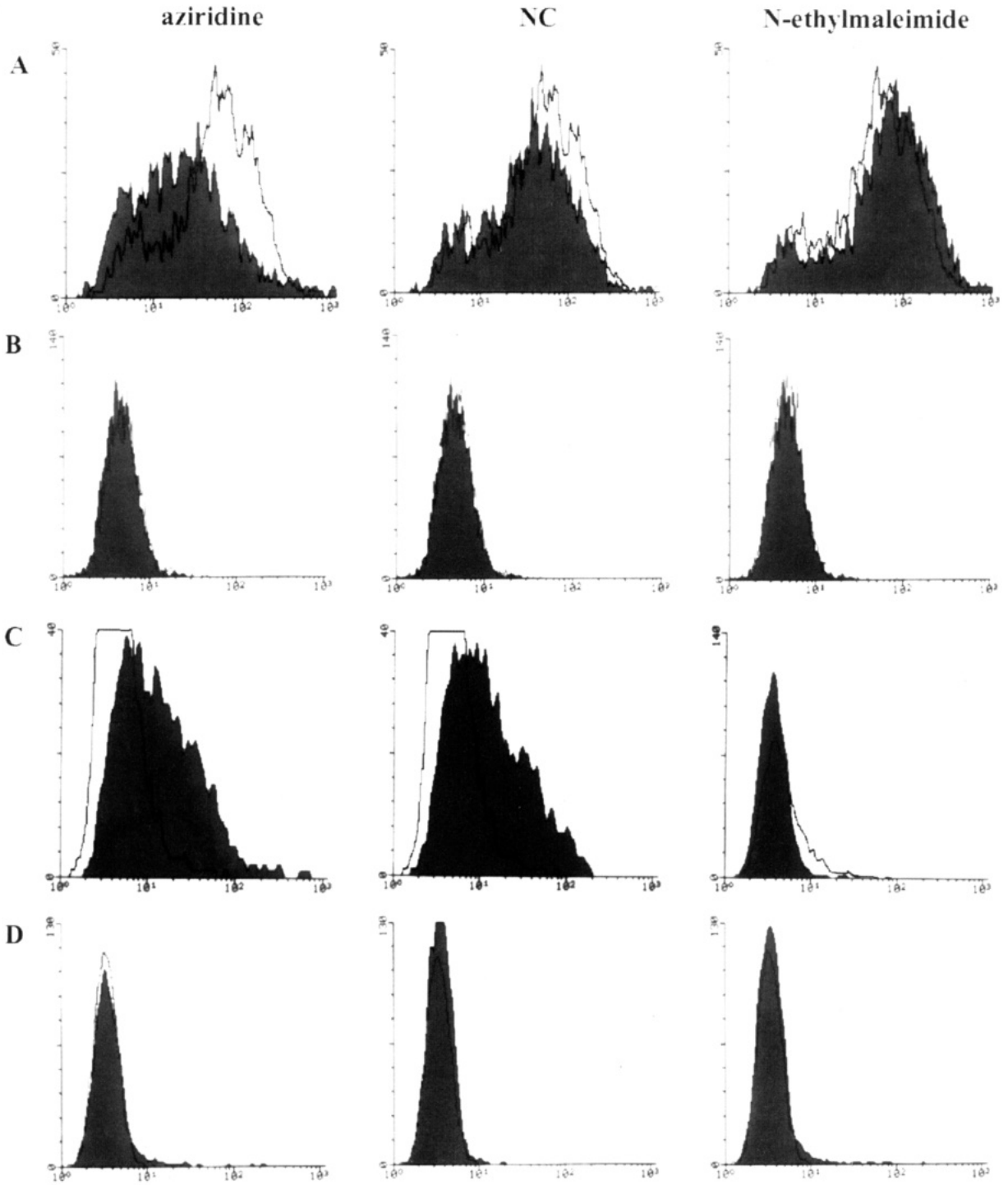

FIGURE 5: Binding of P-selectin-Rg proteins as assayed by flow cytometry. (A) Wild-type P-selectin-Rg, (B) K113C, (C) K111C, and (D) K111A proteins were reduced with dithiothreitol (5 mM) and treated with aziridine (1.56 M), NC (20 mM), or *N*-ethylmaleimide (20 mM). Cells treated with unreduced, untreated proteins are represented by solid lines, and the filled areas represent cells that were treated with the modified proteins. Relative cell number is indicated on the *y*-axes, and fluorescence intensity is indicated on the *x*-axes.

shows that the binding of the reduced proteins to HL-60 cells was approximately the same as the untreated proteins, indicating that DTT did not significantly affect ligand binding activity.

The binding of P-selectin-Rg to HL-60 cells was unaffected by treatment with DTT followed by NC or *N*-ethylmaleimide, while some loss in binding activity was observed with DTT treatment followed by aziridine (Figure 5A). K111C and K113C did not bind to the HL-60 cells in the FACS assay (Figure 5B,C), as expected on the basis of previous mutation studies of the lysine residues at these positions (Hollenbaugh et al., 1993). There was no increase in the ability of K113C to bind to HL-60 cells upon reaction with DTT followed by aziridine (K113C-AZ), NC (K113C-CY), or *N*-ethylmaleimide (K113C-NEM) (Figure 5B). In contrast, a significant increase in binding activity was observed by reacting K111C with DTT, followed by either aziridine (K111C-AZ) or NC (K111C-CY) (Figure 5C). The increase requires the formation of a lysine-like structure, since K111C-NEM had the same level of binding activity as K111C. K111A, which does not binding HL-60 cells in the FACS assay, was included as an additional control for

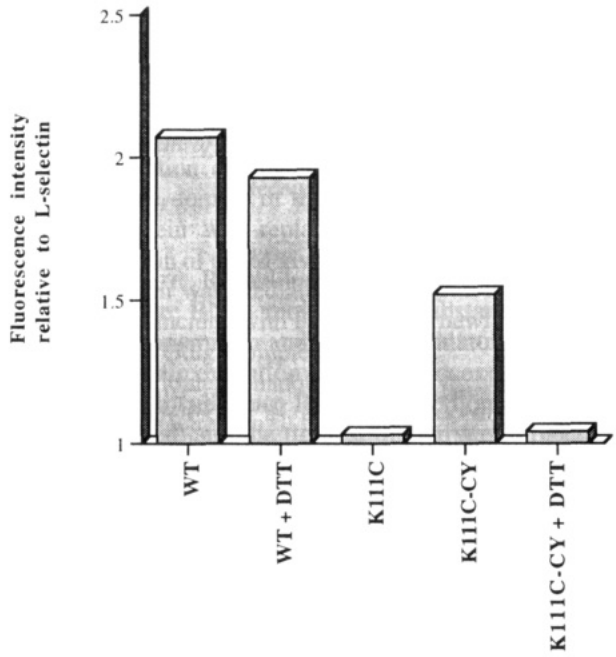

FIGURE 6: Flow cytometric analysis of the binding of K111C-CY before and after reduction with dithiothreitol (1 mM) for 1.5 h at 23 °C. The values shown are fluorescence obtained for the given sample divided by that obtained for L-selectin, the negative control.

these studies. The binding activity of K111A to HL-60 cells after treatment with DTT followed by aziridine, NC, or *N*-ethylmaleimide (Figure 5D) was the same as that of untreated K111A. These results indicate that by reacting K111C with an appropriate amine, the binding activity can be restored to approximate that of the wild type. The fact that K113C did not undergo similar activation may reflect inaccessibility of the introduced cysteine, perturbation of the protein fold, or a stricter structural requirement at this position. An indication of the sensitivity of this site is suggested by the loss of ligand binding activity by the conservative replacement of lysine 113 to arginine (Bajorath et al., 1994). It therefore appears that lysine 113 cannot be substituted without significant loss in ligand binding activity.

Additional confirmation that the amino group at position 111 is essential for binding to cells was obtained by subjecting K111C-CY to mild reduction with DTT. It was anticipated that these reduction conditions would effect disulfide bond reduction of the aminoethyl disulfide of K111C-CY, resulting in the formation of K111C (Figure 1). Figure 6 illustrates that the binding activity of reduced K111C-CY was equivalent to that of K111C. As expected, the conditions used for reduction did not significantly affect the binding of P-selectin-Rg. These results are consistent with the conversion of K111C-CY to K111C upon disulfide bond reduction. Similar studies were not performed with K111C-AZ since the thioether-containing residue is chemically inert toward DTT. To demonstrate that the mechanism of binding of K111C-AZ to HL-60 cells was related to that of P-selectin-Rg binding, the ability of a mAb to block binding was tested. The antibody 3D3 has previously been shown to inhibit binding of P-selectin-Rg to HL-60 cells (Bajorath et al., 1994). Figure 7 shows that 3D3 blocks the binding of P-selectin-Rg, as previously reported (Hollenbaugh et al., 1993), as well as K111C-AZ, providing further evidence for the specific nature of the binding of K111C-AZ to HL-60 cells.

Summary. The purpose of this work was to gain further insight into the molecular interactions between P-selectin and its cellular ligand and to develop a method for the specific labeling of P-selectin. The involvement of K111 and K113 of P-selectin-Rg was demonstrated by the loss in binding activity when either of the lysines was mutated to cysteine. Direct evidence for the importance of the amine at position 111 was obtained by showing that K111C-AZ and K111C-CY were capable of ligand binding, while similarly treated K111A was not. This is in spite of the fact that the unnatural side chains of K111C-AZ and K111C-CY are approximately 0.5 and 2.0 Å longer than lysine, respectively. Thus, it appears that, within the ranges tested, the length of the residue at the 111 position is not critical for binding. This may not be the case at the 113 position, since K113C-AZ and K113C-CY showed no detectable binding to HL-60 cells.

The methodology described here can be used to further characterize the interactions of P-selectin with natural ligands or inhibitors that block ligand binding. This can be accomplished by determining the effects that aziridine and NC have on ligand or inhibitor binding to K111C or by specific isotopic labeling of these reagents for incorporation into P-selectin as NMR probes. Studies such as these will be the subject of future investigations.

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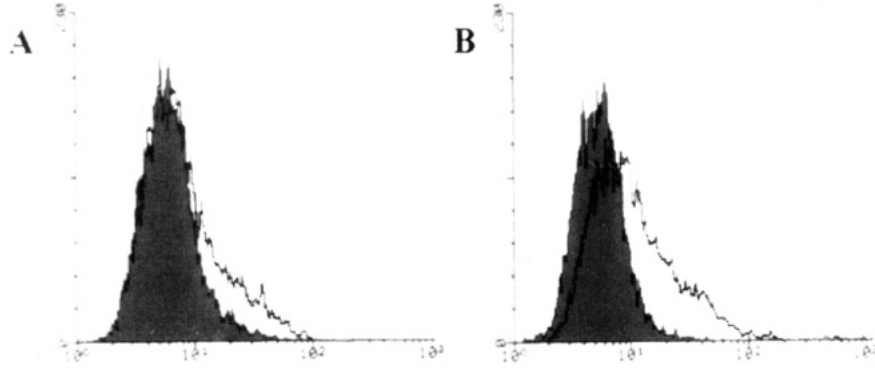

FIGURE 7: Binding inhibition by the anti-P-selectin mAb 3D3. (A) Wild-type P-selectin-Rg and (B) K111C-AZ were reduced with DTT (5 mM) and then treated with aziridine (1.2 M). The filled areas and solid lines represent staining of cells with and without preincubation of the fusion proteins with 3D3, respectively. Relative cell number is indicated on the *y*-axes, and fluorescence intensity is indicated on the *x*-axes.

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