Tyrosine Sulfation of P-selectin Glycoprotein Ligand-1 Is Required for High Affinity Binding to P-selectin*

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P-selectin glycoprotein ligand-1 (PSGL-1) is a mucin-like glycoprotein on leukocytes that is a high affinity ligand for P-selectin. Previous studies have shown that sialylation and fucosylation of PSGL-1 are required for its binding to P-selectin, but other post-translational modifications of PSGL-1 may also be important. We demonstrate that PSGL-1 synthesized in human HL-60 cells can be metabolically labeled with [35S]sulfate that is incorporated primarily into tyrosine sulfate. Treatment of PSGL-1 with a bacterial arylsulfatase releases sulfate from tyrosine, resulting in a concordant decrease in binding to P-selectin. These studies demonstrate that tyrosine sulfate on PSGL-1 functions in conjunction with sialylated and fucosylated glycans to mediate high affinity binding to P-selectin.

P-selectin is a calcium-dependent carbohydrate-binding protein that is expressed on the surfaces of activated platelets and endothelium in response to thrombin and other agonists (1–3). Through its binding to glycoconjugate-based counter-receptors on leukocytes, P-selectin mediates rolling adhesion of these cells on activated platelets and endothelium (4, 5). Both sialic acid and fucose are components of the PSGL-1 counter-receptors on leukocytes (6–8). Oligosaccharides containing sialyl Lewis x (sLe x) are a present on leukocyte surfaces, inhibit adhesion of leukocytes to P-selectin (9, 10). However, expression of sLe x on cell surfaces is not sufficient for high affinity binding of cells to P-selectin, since non-myeloid cells that express high levels of sLe x bind poorly to P-selectin compared to myeloid cells (11).

Leukocytes express a single high affinity ligand for P-selectin, termed P-selectin glycoprotein ligand-1 (PSGL-1) (5, 7, 8, 12, 13). PSGL-1 is a homodimeric glycoprotein with two disulfide-bonded 120-kDa subunits (7). The cDNA-derived sequence for PSGL-1 predicts a type 1 transmembrane protein of 402 amino acids (8). The extracellular domain has an N-terminal signal peptide from residues 1–18 and a putative propeptide from residues 19–41. Assuming deavage of the propeptide, the extracellular domain of the mature protein begins at residue 42 and extends to residue 308. The sequence concludes with a 25-residue transmembrane domain and a 69-residue cytoplasmic tail. The extracellular domain is rich in serines and threonines that are potential sites for O-glycosylation. There are also three potential N-glycosylation sites and three potential tyrosine sulfation sites at residues 46, 48, and 51 (8).

PSGL-1 must be sialylated and fucosylated to bind P-selectin (7, 8). Consistent with these observations, PSGL-1 is highly O-glycosylated (12) and contains sialylated and fucosylated O-linked poly-N-acetyllactosamine, including some glycans that terminate in sLe x (13). It is not clear, however, that sLe x or related glycans are sufficient for high affinity binding of PSGL-1 to P-selectin. For example, sulfated compounds lacking either sialic acid or fucose can inhibit adhesion of leukocytes to P-selectin (14–17). These data suggest that PSGL-1 may require sulfation to bind with high affinity to P-selectin. We demonstrate that PSGL-1 is sulfated, primarily on tyrosine residues. Furthermore, tyrosine sulfation of PSGL-1 is required for high affinity binding to P-selectin.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Carrier-free [35S]sulfate (1100–1600 mCi/mmol) was purchased from DuPont NEN. Emphaze™ affinity support resin was purchased from Pierce. Aerobacter aerogenes arylsulfatase, Arthrobacter ureafaciens neuraminidase, and sulfated monosaccharides were obtained from Sigma. Recombinant peptide-N-glycosidase F was purchased from Boehringer Mannheim. All cell culture reagents were obtained from Life Technologies, Inc. Authentic tyrosine sulfate was synthesized as described (18) using concentrated H2SO4 and tyrosine. Other chemicals were ACS grade or better and were obtained from Fisher Scientific.

Isolation of Radiolabeled PSGL-1—PSGL-1 was purified from human neutrophils and radiolabeled with Na125I as described (13). HL-60 cells (2–5 × 106 cells/ml) were labeled for 48 h with 100 μCi/ml [35S]sulfate at 37 °C in sulfate-deficient medium containing 10% dialyzed fetal bovine serum. [3H]PSGL-1 was prepared by metabolic labeling of HL-60 cells with [3H]glucosamine as described (7, 12). [3H]PSGL-1 and [35S]-PSGL-1 were purified using affinity chromatography with a column containing recombinant soluble P-selectin (19) coupled to Emphaze™ at a density of 5 mg/ml (13). The enriched EDTA-eluted samples were rechromatographed on the P-selectin column after dialysis into Ca2+ -containing buffer. Purified [3H]-PSGL-1 was treated with reducing or nonreducing SDS sample buffer and electrophoresed in a 7.5% polyacrylamide gel (20). Gels were dried and then exposed to Fuji RX x-ray film at −80 °C.

Detection of Tyrosine Sulfate—Tyrosine sulfation of PSGL-1 was determined by base hydrolysis of [3H]-PSGL-1. Following identification of [3H]-PSGL-1 by autoradiography, the dried gel was aligned with the exposed x-ray film, and the band from the nonreducing lane was subjected to strong base hydrolysis in 1.0 N NaOH at 110 °C, for 24 h as described (21). The hydrolysate was passed over a Dowex 50 (H⁺) column washed with water, lyophilized, and analyzed by anion exchange chromatography using a Varian AX-5 column (4 mm × 30 cm) that was eluted with a gradient of NaH2PO4, pH 3.0. Tyrosine sulfate was also detected using descending paper chromatography as described.
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To determine if PSGL-1 is post-translationally sulfated, human promyelocytic leukemia HL-60 cells were metabolically labeled with [35S]Sulfate, and PSGL-1 was purified from lysates of these cells by affinity chromatography on a P-selectin column. A [35S]Sulfate-labeled protein was detected that migrated in SDS gels with a relative molecular mass of 120,000 under reducing conditions and 240,000 under nonreducing conditions (Fig. 1). These mobilities are consistent with the disulfide-linked homodimeric structure of PSGL-1 (7). Furthermore, the [35S]Sulfate-labeled protein was immunoprecipitated with a specific rabbit antiserum generated against a synthetic peptide encoding residues 42–56 of the extracellular domain of PSGL-1 (13) (Fig. 1). Analysis of the supernatants showed that anti-42–56 precipitated all of the [35S]-PSGL-1, whereas NRS precipitated none of the [35S]-PSGL-1 (data not shown). These results demonstrate that PSGL-1 is post-translationally sulfated.

Sulfate can be incorporated into eukaryotic glycoproteins as tyrosine sulfate (27) or as sulfated carbohydrates (28, 29). In preliminary studies we failed to detect sulfated carbohydrates on PSGL-1, using techniques that detected such structures on other glycoproteins (25, 26). We then considered the possibility that PSGL-1 might contain tyrosine sulfate. The cDNA sequence of PSGL-1 predicts four extracytoplasmic tyrosine residues (8), three of which are clustered at positions 46, 48, and 51 within a predicted consensus sequence for tyrosine sulfation (27). To determine if PSGL-1 has tyrosine sulfate, the gel slice containing the 240-kDa [35S]-PSGL-1 was hydrolyzed with strong base and analyzed by both anion exchange chromatography and descending paper chromatography. In both systems, a single radioactive peak was recovered that comigrated with authentic tyrosine sulfate (Fig. 2, A and B). No radioactivity was recovered that comigrated with sulfated mono- or disaccharide standards.

We sought to test whether tyrosine sulfate is important for binding of PSGL-1 to P-selectin. Although the functions of tyrosine sulfation within proteins are not clear (27), some proteins require this modification for optimal activity (30, 31). The usual approach for assessing the importance of tyrosine sulfate is to prevent sulfation of newly synthesized proteins with chemical inhibitors or to replace tyrosine with phenylalanine by site-directed mutagenesis. We developed an alternative approach in which sulfate was enzymatically removed from tyrosine on intact PSGL-1. We first tested the ability of an arylsulfatase from A. aerogenes to release sulfate from [35S]tyrosine sulfate. Treatment of the hydrolysate of [35S]-PSGL-1 with 1000 milliunits of arylsulfatase quantitatively released [35S]sulfate from [35S]tyrosine sulfate (Fig. 3, A and B). In other experiments, as little as 50 milliunits of this arylsulfatase also quantitatively released [35S]sulfate from [35S]tyrosine sulfate derived from PSGL-1 (data not shown). Cleavage by arylsulfatase was specific, since 1000 milliunits of the enzyme did not release sulfate from Gal-6-sulfate, GlcNAc-6-sulfate, GalNAc-4-sulfate, and GalNAc-6-sulfate (data not shown).

We then examined the ability of the arylsulfatase to release sulfate from intact PSGL-1 and the effect of this release on rebinding of PSGL-1 to P-selectin. [35S]Sulfate released from [35S]-PSGL-1 by arylsulfatase was quantified by precipitation as insoluble BaSO4. Up to 50% of the [35S]sulfate on [35S]-PSGL-1 was released by 500 milliunits of arylsulfatase; increasing amounts of enzyme did not release more radioactivity (Fig. 4A).

The functional importance of the tyrosine sulfate on PSGL-1 was assessed by treating both [3H]-PSGL-1 from HL-60 cells and 125I-PSGL-1 from human neutrophils with arylsulfatase and measuring the rebinding of the treated ligands to a P-selectin column. Binding of both arylsulfatase-treated [3H]-PSGL-1 and 125I-PSGL-1 to P-selectin was reduced in a dose-dependent manner; the decreased binding was inversely related to the amount of sulfate released from [35S]-PSGL-1 (Fig. 4A). The reduced binding of PSGL-1 to P-selectin following arylsulfatase treatment was not due to general release of sialic acid and/or
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Arylsulfatase released ~50% of the [35S]sulfate from [35S]-PSGL-1 and decreased rebinding of 3H-PSGL-1 and 125I-PSGL-1 to P-selectin. 35S-PSGL-1 and 125I-PSGL-1 purified from HL-60 cells and 129I-PSGL-1 purified from human neutrophils were treated with increasing amounts of arylsulfatase. The [35S]sulfate released from 35S-PSGL-1 was quantified using BaSO4 precipitation (dashed line). Enzyme-treated 129I-PSGL-1 and 129I-PSGL-1 were analyzed for their ability to rebind to P-selectin (open symbols). B, [35S]tyrosine sulfate is present on PSGL-1 that binds P-selectin but is absent on PSGL-1 that does not bind P-selectin. Arylsulfatase-treated 35S-PSGL-1 and 129I-PSGL-1 were applied to a P-selectin column. The unbound (-) and bound (+) fractions were analyzed by SDS-PAGE under nonreducing conditions, followed by autoradiography.

PSGL-1 and decreased rebinding of 3H-PSGL-1 and 129I-PSGL-1 to P-selectin by the same degree. We considered the possibility that the fraction of 35S-PSGL-1 that rebound to P-selectin following treatment with arylsulfatase retained critical tyrosine sulfate residues, whereas the fraction that did not rebound to P-selectin had lost tyrosine sulfate. 35S-PSGL-1 was treated with 1000 milliunits of arylsulfatase and then applied to a P-selectin column. The bound and unbound fractions were analyzed by SDS-PAGE (Fig. 4B). A band corresponding to 35S-PSGL-1 was observed in the bound fractions, whereas no band was seen in the unbound fractions. The radioactivity in the unbound fractions represented free sulfate. When the band of 35S-PSGL-1 in the bound fractions was hydrolyzed, radioactivity was recovered in tyrosine sulfate (data not shown).

Tyrosine sulfate remaining in the P-selectin-bound subset of PSGL-1 may be resistant to arylsulfatase because of its inaccessibility or because of some other feature of PSGL-1 that blocks action of the enzyme. When 35S-PSGL was partly de-glycosylated by treatment with peptide N-glycosidase F and A. ureafaciens neuraminidase, subsequent treatment with arylsulfatase released up to 70% of the radioactivity as [35S]sulfate (data not shown). Since neuraminidase also eliminates binding of 129I-PSGL-1 to P-selectin, we could not determine whether the increased removal of [35S]sulfate further reduced binding of PSGL-1 to P-selectin. These results suggest that the extensive

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Tyrosine sulfate from PSGL-1 is sensitive to arylsulfatase. 35S-PSGL-1 was hydrolyzed with strong base as described under “Experimental Procedures.” The hydrolysates were either sham-treated with 1000 milliunits of boiled arylsulfatase (A) or treated with 1000 milliunits of active enzyme (B). The hydrolysates were then analyzed by anion exchange chromatography.
glycosylation of PSGL-1 may account for the inaccessibility of some tyrosine sulfate sites to arylsulfatase.

These results demonstrate that PSGL-1 contains tyrosine sulfate that is required for high affinity binding to P-selectin. It has been shown previously that PSGL-1 contains the sLex determinant on O-linked oligosaccharides and that both sialic acid and fucose are required for binding of PSGL-1 to P-selectin (7, 8, 12, 13). Tyrosine sulfate may be important because it promotes appropriate presentation of the glycans that bind directly to P-selectin. Alternatively, tyrosine sulfate may directly interact with P-selectin. This latter possibility seems more likely, since sulfatide and sulfated oligosaccharides are known to bind P-selectin (14–17). Sulfate is also a critical determinant on GlyCAM-1 to L-selectin, but it has been shown previously that PSGL-1 contains the sLex determinant for the binding of GlyCAM-1 to L-selectin, but sulfate is required for high affinity binding to P-selectin. It

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REFERENCES

1. McEver, R. P., Moore, K. L., and Cummings, R. D. (1995) J. Biol. Chem. 270, 11025–11028
2. Varki, A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7390–7397
3. Springer, T. A. (1991) Annu. Rev. Physiol. 53, 877–892
4. Lawrence, M. B., and Springer, T. A. (1991) Cell 65, 852–873
5. Moore, K. L., Patel, K. D., Bruehl, R. E., Li, F., J. Johnson, D. A., Lichenstein, H. S., Cummings, R. D., Bainton, D. F., and McEver, R. P. (1995) J. Cell Biol. 128, 661–673
6. Corral, L., Singer, M. S., Macher, B. A., and Rosen, S. D. (1990) Biochem. Biophys. Res. Commun. 172, 1349–1356
7. Moore, K. L., Stults, N. L., Diaz, S., Smith, D. F., Cummings, R. D., Varki, A., and McEver, R. P. (1992) J. Cell Biol. 118, 445–456
8. Sako, D., Chang, X.-J., Barone, K. M., Vachino, G., White, H. M., Shaw, G., Veldman, G. M., Bean, K. M., Ahern, T. J., Furie, B., Cummings, D., and Larsen, G. R. (1993) Cell 75, 1179–1186
9. Polley, M. J., Phillips, M. L., Wayner, E., Nudelman, E., Singhal, A. K., Hakomori, S.-I., and Paulson, J. C. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 6224–6228
10. Fovall, C., Watson, S. R., Dowbenko, D., Fennie, C., Lasky, L. A., Kiso, M., Nasegawa, A., Asa, D., and Brandley, B. K. (1991) J. Cell Biol. 117, 895–902
11. Zhou, Q., Moore, K. L., Smith, D. F., Varki, A., McEver, R. P., and Cummings, R. D. (1991) J. Cell Biol. 115, 557–564
12. Norgaard, K. E., Moore, K. L., Diaz, S., Stults, N. L., Ushiyama, S., McEver, R. P., Cummings, R. D., and Varki, A. (1993) J. Biol. Chem. 268, 12764–12774
13. Moore, K. L., Eaton, S. F., Lyons, D. E., Lichenstein, H. S., Cummings, R. D., and McEver, R. P. (1994) J. Biol. Chem. 269, 23318–23327
14. Aruffo, A., Kolanus, W., Wisn., F., Femand, P., and Bied, B. (1993) Cell 67, 35–44
15. Nelson, R. M., Cecconi, D., Roberts, W. G., Aruffo, A., Linhardt, R. J., and Bevilacqua, M. P. (1994) J. Biol. Chem. 269, 15060–15066
16. Cecconi, O., Nelsen, R. M., Roberts, W. G., Hanasaki, K. M., Mannioni, G., Schultz, C., Ulrich, T. R., Aruffo, A., and Bevilacqua, M. P. (1994) J. Biol. Chem. 269, 15060–15066
17. Skinner, M. P., Fournier, D. J., Andrews, R. K., Gorman, J. J., Chesterton, C. N., and Brier, M. C. (1989) Biochem. Biophys. Res. Commun. 164, 1373–1379
18. Huttner, W. B. (1984) Methods Enzymol. 107, 200–224
19. Ushiyama, S., Laue, T. M., Moore, K. L., Eriksson, H. P., and McEver, R. P. (1993) J. Biol. Chem. 268, 15229–15237
20. Malam, U. K. (1970) Nature 227, 680–685
21. Hartin, G., Tallefson, D. M., and Strauss, A. W. (1986) J. Biol. Chem. 261, 15827–15830
22. Dodson, K. S., Rose, F. A., and Tubbali, N. (1959) Bioche. J. 71, 10–15
23. Cummings, R. D., Kornfeld, S., Schneider, W. J., Hobgood, K. K., Tolleshaug, H., Brown, M. S., and Goldstein, J. L. (1989) J. Biol. Chem. 258, 15261–15273
24. Fowler, L. R., and Rammier, D. H. (1964) Biochemistry 3, 230–237
25. Shilatifard, A., and Cummings, R. D. (1995) Glycobiology 5, 291–297
26. Shilatifard, A., Merkle, R. M., Helland, D. E., Welles, J. L., Haseltine, W. A., and Cummings, R. D. (1993) J. Virol. 67, 943–952
27. Huttner, W. B., and Baeuerle, P. A. (1988) Mod. Cell Biol. 6, 97–140
28. Fiete, D., Srivastava, V., Hindsgaul, O., and Baenziger, J. (1991) Cell 65, 1103–1110
29. Kjell, L., and Lindahl, U. (1991) Ann. Rev. Biochem. 60, 443–475
30. Pittman, D. P., Wang, J. H., and Kawamura, R. (1992) Biochemistry 31, 3133–3135
31. Dong, J.-F., Li, C.-Q., and Lopez, J. A. (1994) Biochemistry 33, 13946–13953
32. Imai, Y., Lasky, L. A., and Rosen, S. D. (1993) Nature 361, 555–557
33. Henmerich, S., Bertuzzi, C. R., Leffler, H., and Rosen, S. D. (1994) Biochemistry 33, 4820–4829
34. Henmerich, S., Leffler, H., and Rosen, S. D. (1995) J. Biol. Chem. 270, 12035–12047
35. Lasky, L. A., Singer, M. S., Dowbenko, D., Imai, Y., Hanzel, W. J., Grimley, C., Fennie, C., Gillett, N., Watson, S. R., and Rosen, S. D. (1992) Cell 69, 927–938
36. Baumheuter, S., Singer, M. S., Hanzel, W., Henmerich, S., Rentz, M., Rosen, S. D., and Lasky, L. A. (1993) Science 262, 436–438
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