The Cytoplasmic Domain of P-Selectin Is Phosphorylated on Serine and Threonine Residues

By Tetsuro Fujimoto and Rodger P. McEver

P-selectin is an adhesion receptor for leukocytes that is redistributed from secretory granule membranes to the surfaces of activated platelets and endothelial cells. The cytoplasmic domain of P-selectin contains two serines, two threonines, and one tyrosine that could potentially be phosphorylated. We found that P-selectin was phosphorylated in both platelets and endothelial cells and that phosphorylation rapidly increased after cell activation. Approximately 0.02, 0.05, and 0.08 mol of phosphate/mol of P-selectin were incorporated, respectively, into resting, thrombin-activated, and phorbol ester-activated platelets. Phosphorylation was completely inhibited by the protein kinase C inhibitors, staurosporine, H-7, and chelerythrine, and was enhanced by the phosphatase inhibitors, okadaic acid and calyculin A. Phosphoamino acid analysis of 32P-labeled P-selectin showed that phosphorylation occurred predominantly on serine with lesser amounts on threonine. When expressed in transfected Chinese hamster ovary cells, P-selectin was also phosphorylated. Mutagenesis studies showed that Ser788 was the principal site of phosphorylation, with minor sites on the other serine and threonine residues of the cytoplasmic domain. Phosphorylation may regulate membrane trafficking or other functions of P-selectin.

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MATERIALS AND METHODS

**Materials.** 32Pi, carrier-free (8,500 to 9,120 Ci/mmol), was obtained from DuPont-New England Nuclear (Boston, MA). Phorbol myristate acetate (PMA), prostaglandin E1 (PGE1), staurosporine, okadaic acid, and calyculin A were purchased from Sigma (St. Louis, MO). H-7 [1-(5-isouquinolinesulfonyl)-2-methylpiperazine, di-HCL] was from Calbiochem (La Jolla, CA). Protein A-Sepharose CL-4B was from Pharmacia (Piscataway, NJ). Rabbit antimouse IgG was from Cappel-Organon Teknika Corp (Durham, NC). Phosphate-free RPMI medium 1640 was from GIBCO-BRL (Grand Island, NY). Chelerythrine chloride was a gift from Dr Anthony Ware (Beth Israel Hospital, Boston, MA). Human thrombin and HPC4, a monoclonal antibody (MoAb) to human protein C, were generously provided by Dr Charles Esmon (Oklahoma Medical Research Foundation). P-selectin was isolated from human platelets as previously described. The protein concentration of purified P-selectin was determined using a slightly revised extinction coefficient. GPIIb-IIIa was isolated from human platelets as previously described.

**P-labeling of platelets.** Platelets obtained from healthy donors were labeled with 32Pi as previously described, with slight modifications. Platelets were washed three times in 85 mmol/L sodium citrate, 111 mmol/L dextrose, 71 mmol/L citric acid, pH 7.0, containing 1 U/mL apyrase, and then resuspended at 2 × 10^10 cells/mL in incubation buffer (138 mmol/L NaCl, 12 mmol/L NaHCO3, 5.5 mmol/L glucose, 2.9 mmol/L KCl, 10 mmol/L HEPES, pH 7.4). The suspension was incubated with 1 mCi/mL of 32Pi at 37°C for 1 hour, then supplemented with 1 U/mL apyrase and 1 mmol/L EGTA, centrifuged at 800g for 15 minutes, and resuspended at 8 × 10^9 platelets/mL in incubation buffer containing 0.36 mmol/L NaH2PO4 and 1 mmol/L CaCl2. Aliquots (250 µL) of the labeled platelet suspension were incubated at 37°C with agonists (thrombin, PMA, or PGE1) as indicated in the text. As controls, platelets were incubated with the solvent in which each agonist was dissolved. After incubation for the specified time, 250 µL of ice-cold 2× lysis buffer (20 mmol/L Tris, 40 mmol/L K1PO4, 10 mmol/L sodium orthovanadate, 40 mmol/L molybdate acid, 80 mmol/L sodium pyrophosphate, 0.2 mmol/L trifluoroperazine, 2 mmol/L EGTA, 20 mmol/L benzamide, 2 mmol/L phenylmethylsulfonfluoride, 200 µg/mL leupeptin, and 2% Triton X-100, pH 7.2) were added. In samples treated with thrombin, 0.2 U/mL of hirudin was also added.
PHOSPHORYLATION OF P-SELECTIN

Endothelial Cells

Platelets

-P-Selectin

Fig 1. Phosphorylation of P-selectin in platelets and endothelial cells. Human platelets (left) or human umbilical vein endothelial cells (right) were labeled with $^{32}$P, lysed, and subjected to immunoprecipitation with an anti-P-selectin MoAb (S12) or a control antibody to human protein C (HPC4). The immunoprecipitates were resolved by SDS-PAGE on 5% to 20% gradient gels under reducing conditions, followed by autoradiography. Relative to unstimulated cells, the degree of phosphorylation was greater in platelets stimulated with thrombin (0.1 U/mL for 5 minutes) and in endothelial cells stimulated with PMA (10 pmol/L for 10 minutes). Molecular weight standards are shown on the left.

$^{32}$P-labeling of cultured human umbilical vein endothelial cells. Human umbilical vein endothelial cells were cultured in Medium-199 supplemented with 20% fetal bovine serum as previously described. Confluent first-passage cultures in 75-cm$^2$ flasks were washed three times with prewarmed phosphate-free RPMI 1640 and then incubated in phosphate-free medium containing 200 μCi/mL of $^{32}$P at 37°C for 3 hours. The cells were washed with Hanks' Balanced Salt Solution (HBSS) containing Ca$^{2+}$ and incubated an additional 10 minutes with or without 10 μmol/L PMA in the same buffer. The cells were then rapidly washed and lysed with 1 mL of ice-cold lysis buffer as described above.

Immunoprecipitation. The cell lysates from platelets or endothelial cells were centrifuged at 12,000g for 15 minutes and the supernatants were subjected to immunoprecipitation by a modification of a previously described method. Two MoAbs were used, S12 directed against human P-selectin and, as a negative control, HPC4 directed against human protein C. Both antibodies are of the IgG subclass. Cell lysates (500 μL) from $^{32}$P-labeled platelets or endothelial cells were incubated with 2 μg of MoAb for 1 hour at 37°C, and the mixture was then incubated for another 1 hour at room temperature with 100 μL of protein A-Sepharose CL-4B (3% wt/vol) that was preincubated with 10 μg of rabbit antimouse IgG. The beads were pelleted by centrifugation at 12,000g for 10 seconds and washed three times with lysis buffer. Proteins were eluted by boiling for 5 minutes in 100 μL of TBS (20 mmol/L Tris, 150 mmol/L NaCl, pH 7.4) containing 2% Nonidet P-40, 2% sodium deoxycholate, and 2% sodium dodecyl sulfate (SDS). Protein eluates were removed, diluted with an equal volume of TBS, and subjected to a second immunoprecipitation to reduce nonspecifically precipitated counts. The samples were incubated at 37°C for 1 hour with 4 μg of polyclonal rabbit IgG to P-selectin or 20 μL of goat antiserum to P-selectin. They were then incubated for an additional 1 hour at room temperature with 50 μL of protein A-Sepharose CL-4B or, for the samples to which goat antiserum was used, with 50 μL of protein G-Sepharose. The beads were washed three times as described above. The immunoprecipitates were eluted by boiling in SDS-sample buffer (62.5 mmol/L Tris-HCl, 2% SDS, 5% glycerol, 1% SDS, 0.1% bromphenol blue, 2% 2-mercaptoethanol) and subjected to electrophoresis on 10% polyacrylamide gels. The gels were stained with Coomassie Blue R-250 and destained in a solution of 10% acetic acid and 40% methanol. Relative to unstimulated cells, the degree of phosphorylation was greater in platelets stimulated with thrombin (0.1 U/mL for 5 minutes) and in endothelial cells stimulated with PMA (10 pmol/L for 10 minutes). Molecular weight standards are shown on the left.

Fig 2. Time course of phosphorylation of P-selectin in human platelets in response to agonists. (A) $^{32}$P-labeled platelets were incubated with 0.1 U/mL thrombin, 10 μmol/L PMA, or 5 μmol/L PGE$_1$ at 37°C. At various time points, the platelets were lysed, immunoprecipitated with S12, and analyzed by SDS-PAGE and autoradiography. (B) The relative amount of $^{32}$P incorporated into P-selectin was quantitated by densitometric analysis of the autoradiograms. The data represent the means ± SD of triplicate determinations and are representative of three (thrombin and PMA) or two (PGE$_1$) separate experiments.
Table 1. Stoichiometry of Phosphorylation of Platelet Proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Agonist</th>
<th>Mol P/Mol Protein</th>
<th>Reported Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-selectin</td>
<td>None</td>
<td>0.020 ± 0.006 (n = 9)</td>
<td>0.018</td>
</tr>
<tr>
<td></td>
<td>Thrombin</td>
<td>0.061 ± 0.018 (n = 6)</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>PMA</td>
<td>0.078 ± 0.014 (n = 5)</td>
<td>0.012^24</td>
</tr>
<tr>
<td>GP IIa</td>
<td>None</td>
<td>0.011 ± 0.003 (n = 11)</td>
<td>0.018</td>
</tr>
<tr>
<td>Myosin light chain</td>
<td>PMA</td>
<td>1.17 ± 0.11 (n = 4)</td>
<td>1.20^22</td>
</tr>
</tbody>
</table>

Platelets were labeled with ^32P at 37°C for 4 hours, separated into aliquots, and then stimulated with thrombin (0.1 U/mL for 5 minutes) or PMA (10 μmol/L for 10 minutes). The stoichiometry of phosphorylation (mol of phosphate/mol of protein) of the indicated proteins was determined as described under Materials and Methods.

Stoichiometry of P-selectin phosphorylation. The stoichiometry of protein phosphorylation in platelets was estimated according to the method of Hillery et al.24 Platelets were labeled at a concentration of 1 × 10^9/mL with 0.5 mCi/mL of ^32P for 4 hours. The specific activity of γ-[^32P]ATP was calculated indirectly by measuring the specific activity of newly synthesized phosphatidic acid. Phospholipids were extracted from unstimulated or thrombin (0.2 U/mL)-stimulated ^32P-labeled platelets (1 × 10^9) as previously described. The lipids were separated by thin-layer chromatography (TLC) on 20 × 20 cm silica G plates (Universal Scientific Inc, Atlanta, GA) in chloroform/methanol/NH_4OH. The positions of phosphatidic acids were detected by autoradiography and also by comparison with standard purified phosphatidic acid, which was visualized by iodine vapor. The phosphatidic acids were excised, extracted, and subjected to perchloric acid digestion. The released phosphates were then quantitated with malachite green as previously described, and the radioactivity was counted in Aquasol-2 in a liquid scintillation counter. The immunoprecipitated P-selectin from the same sample was quantitated by scanning of a Coomassie blue-stained gel with a densitometer and comparison with purified P-selectin. The radioactivity of P-selectin was determined by scintillation counting of P-selectin bands that were excised from the gels. The stoichiometry of P-selectin phosphorylation was calculated by dividing the specific activity of P-selectin by the specific activity of phosphatidic acid.

Effects of protein kinase C (PKC) inhibitors and protein phosphatase inhibitors. ^32P-labeled platelets were washed and incubated with the PKC inhibitors, staurosporine26 (1 to 10 μmol/L), H-727 (10 to 100 μmol/L), or chelerythrine29 (1 to 10 μmol/L) at 37°C for 10 minutes before stimulation. In other experiments, platelets were incubated with okadaic acid (10 μmol/L) or calyculin-A (1 μmol/L) at 37°C for 15 minutes. Okadaic acid and calyculin-A are inhibitors of protein phosphatase type 1 and type 2A. In control experiments, platelets were incubated with the same concentrations of dimethylsulfoxide (DMSO) in which the inhibitors were dissolved.
The final concentration of DMSO was 0.1% in all cases. Platelets were then lysed and immunoprecipitated as described above.

**Phosphoamino acid analysis.** P-selectin was immunoprecipitated as described above, except that the lysate from 1 x 10^9 32P-labeled platelets was used. After SDS-PAGE, phosphorylated P-selectin was visualized by Coomassie blue staining and autoradiography and excised from the dried gel. The excised gel pieces were washed three times with 0.1 mol/L ammonium acetate to remove the SDS and acetic acid, dried completely using a Speed-Vac (Savant Instruments, Farmingdale, NY), and incubated with 100 µL of TPCK-trypsin (0.1 mg/mL in 0.1 mol/L ammonium acetate) overnight at 37°C. The supernatant was removed and 200 µL of 0.1 mol/L ammonium acetate was added to the gel pieces. The supernatants were pooled and lyophilized. The tryptic digests were resuspended in 200 µL of 6 N HCl for 1.5 hours at 110°C under nitrogen gas. The hydrolysate was lyophilized and resuspended in 10 µL of H_2O containing 10 µg each of unlabeled phosphoserine, phosphothreonine, and phosphotyrosine as standards. The sample was spotted on a cellulose-coated TLC plate (20 x 20 cm) and subjected to electrophoresis in acetic acid/formic acid/H_2O (150:55:1795, vol/vol/vol), pH 1.9, at 500 V for 60 minutes. The plate was dried completely and phosphoamino acids were further separated by electrophoresis in the same direction in pyridine/acetic acid/H_2O (10:100:1890, vol/vol/vol), pH 3.5, at 500 V for 100 minutes. After electrophoresis, the positions of cold phosphoamino acid standards were visualized by staining with 0.25% ninhydrin in acetone, and 32P-labeled phosphoamino acids were identified by autoradiography.

**Expression vectors.** The cDNA clone encoding full-length human P-selectin was excised from the vector pBI20 and cloned into pcDNAI/Neo (Invitrogen, San Diego, CA) at the Xho I site. pcDNAI/Neo is a plasmid expression vector that contains a cytomegalovirus promotor. P-selectin mutants (see Fig 6) were constructed by using the polymerase chain reaction (PCR) according to the strategy previously described. In three mutants, the cytoplasmic domain was partially deleted. In the first construct (Tail-less), a stop codon was introduced at the junction between exon 14 and exon 15, resulting in deletion of the cytoplasmic tail after Asp762. In the second (C1 alone), a stop codon was created between exon 15 and exon 16, which resulted in deletion after Ser772. In the third (C2 alone), exon 15 was deleted. Four other constructs were made in which Ser772, Thr776, Thr778, or Ser788 was mutated to alanine. To mutate Ser772 (S772A), an 18-mer sense primer containing the Ser772→Ala mutation and its antisense oligonucleotide were prepared and two separate PCRs were performed: the first from the Xho I site at base 2240 to the antisense primer and the second from the sense primer to base

![Fig 5. Phosphoamino acid analysis of phosphorylated P-selectin.](image)

**Fig 5.** Phosphoamino acid analysis of phosphorylated P-selectin. 32P-labeled P-selectin was immunoprecipitated from resting, thrombin-, PMA-, or PGE-, treated platelets and separated by SDS-PAGE. P-selectin was localized by autoradiography and then isolated from excised gel slices. After partial hydrolysis, electrophoresis was performed on thin-layer cellulose plates with unlabeled phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr) as internal standards. The positions of the standards were determined by ninhydrin staining. 32P-labeled phosphoamino acids were identified by autoradiography. The data shown are representative of four experiments.

**Fig 4.** The effect of phosphatase inhibitors on P-selectin phosphorylation. (A) 32P-labeled platelets were incubated with two inhibitors of protein phosphatases, okadaic acid (10 µmol/L) or calyculin-A (1 µmol/L), at 37°C for 15 minutes and then stimulated with thrombin (1 U/mL for 5 minutes) or PMA (10 µmol/L for 10 minutes). Control platelets were incubated without inhibitors and then stimulated in the same manner. Cells were then lysed and immunoprecipitated by S12. Phosphorylated P-selectin was visualized by SDS-PAGE followed by autoradiography. (B) 32P incorporation into P-selectin was quantitated by densitometric analysis. The data are the means ± SD of three separate experiments.
2534. After gel purification the two PCR products were mixed and a second PCR was performed using the two outside primers. The mutation of Thr776 (T776A) or Thr781 (T781A) was made with sense and antisense primers containing each Thr → Ala mutation in the same manner. To mutate Ser772 (S772A), a single PCR was performed from the Xba I site at 2240 to an antisense primer that contained the Ser772 → Ala mutation and the stop codon after base 2534, followed by an additional Xba I site.

Three more constructs were made in which Ser772, Thr776, and Thr781 were mutated (S772:T776:T781A); Thr776, Thr772, and Ser772 were mutated (T776:T781:S788A); or all serines and threonines were mutated to alanine (S772A:T776A:T781A:S788A). In these constructs, PCR was repeated using single serine or threonine mutants as templates.

In all cases, PCR fragments were digested by Xba I, isolated by agarose gel electrophoresis, and then used to replace the Xba I site in the multicloning site of pcDNA/Neo. All mutations were verified by nucleotide sequencing by surface binding of 125I-labeled antibody.

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Fig 7. Phosphorylation of recombinant wild-type and mutant P-selectin in CHO cells. Stable CHO cell transfectants expressing various P-selectin cDNA constructs were established. After labeling with $^{32}$P, cell lysates were subjected to ELISA and to immunoprecipitation by S12 followed by SDS-PAGE and autoradiography. (A) An autoradiogram from a representative experiment is shown. (B) The degree of P-selectin phosphorylation was expressed as the relative densitometric unit per amount of protein in the cell lysates. The data from two independent experiments are shown.

Phosphorylation of P-selectin after platelet stimulation. $^{32}$P-labeled platelets were stimulated with thrombin or PMA for various times, lysed, and subjected to immunoprecipitation. The relative amount of $^{32}$P incorporation was measured by densitometry of autoradiograms. Incorporation of $^{32}$P into P-selectin increased 2.5-fold within 5 minutes after stimulation by 0.1 U/mL thrombin, and 4.5-fold within 10 minutes after stimulation by 10 μmol/L PMA (Fig 2). Phosphorylation levels decreased to basal levels after this time (data not shown). Unlike thrombin or PMA, PGE$_1$ did not affect the phosphorylation of P-selectin (Fig 2). P-selectin was not detected in the Triton X-100-insoluble cytoskeletal fraction of platelets before or after stimulation, suggesting that the increased labeling after stimulation reflected a true increase of $^{32}$P incorporation into the total pool of P-selectin (data not shown).

**Stoichiometry of P-selectin phosphorylation.** The stoichiometry of phosphorylation of other proteins has been evaluated by separating phosphorylated from unphosphorylated forms by isoelectric focusing or by double labeling with [$^{38}$S]methionine and $^{32}$P. Because these methods are not readily applicable to large phosphoproteins, we instead measured the stoichiometry of P-selectin phosphorylation in platelets by the method of Hillery et al. The specific activity of metabolic γ-[³²P]ATP was estimated from the specific activity of newly synthesized phosphatidic acid. In preliminary experiments, we found that $^{32}$P uptake into P-selectin approached steady state within 4 hours after incubation at 37°C. Therefore, all subsequent experiments used a 4-hour labeling period. In unstimulated platelets, approximately 0.02 mol of P/mol of P-selectin was phosphorylated (Table 1). After treatment with thrombin or PMA, the stoichiometry of P-selectin phosphorylation increased to 0.05 or 0.08 mol of P/mol of protein, respectively. The relative increases in phosphorylation were similar to those determined by densitometric analysis of autoradiograms (Fig 2).
The stoichiometry of phosphorylation of GPIIbα and myosin light chain, measured as controls (Table I), corresponded to published values.24,32

Effects of PKC inhibitors and protein phosphatase inhibitors on P-selectin phosphorylation. Because PMA directly activates PKC33 and also increases phosphorylation of P-selectin, the involvement of PKC in P-selectin phosphorylation was examined. Before thrombin stimulation, 32P-labeled platelets were preincubated with the potent PKC inhibitors, staurosporine, H-7, or chelerythrine. Of these three inhibitors, chelerythrine has been described as the most specific for PKC.29 All the inhibitors prevented the increased phosphorylation induced by thrombin (Fig 3A). At higher concentrations, staurosporine and chelerythrine reduced phosphorylation of P-selectin in thrombin-stimulated platelets to levels that were lower than those observed in control unstimulated platelets (Fig 3B). The inhibitors also reduced basal phosphorylation in unstimulated platelets and prevented the increased phosphorylation induced by PMA (data not shown). These results suggest that PKC regulates P-selectin phosphorylation in both unstimulated and stimulated platelets.

We next incubated platelets in the presence of okadaic acid or calyculin-A, which are inhibitors of protein phosphatase type 1 and type 2A. As shown in Fig 4A, the phosphorylation of P-selectin was significantly increased by both inhibitors. The effects of the phosphatase inhibitors were observed in both unstimulated and stimulated platelets, and the increases in phosphorylation by the phosphatase inhibitors were higher than those induced by thrombin or PMA (Fig 4B). The effects of okadaic acid were dose-dependent and were apparent at concentrations greater than 1 μmol/L (data not shown). We conclude that phosphorylation of P-selectin is regulated by okadaic acid-sensitive protein phosphatases (PP1 and/or PP2A).

Phosphoamino acid analysis. To determine which amino acids on P-selectin are phosphorylated, P-selectin was isolated from 32P-labeled platelets and then subjected to phosphoamino acid analysis. In both stimulated and unstimulated platelets, phosphoamino acid analysis showed that radioactive phosphate was incorporated mainly into serine residues (Fig 5). A relatively small amount of phosphothreonine was also observed. Phosphotyrosine was not seen under these conditions.

Phosphorylation of recombinant wild-type and mutant P-selectin in CHO cells. The cytoplasmic domain of P-selectin contains two serines at residues 772 and 788 and two threonines at residues 776 and 781.12 To determine which of these residues was phosphorylated, we first made P-selectin constructs containing various deletions in the cytoplasmic domain (Fig 6). CHO cells were transfected with these constructs and stable cell lines expressing the proteins at comparable levels were established. Immunoprecipitation of P-selectin from transfected 32P-labeled cells showed that wild-type P-selectin was phosphorylated (Fig 7). Phosphorylation was not detected in the tail-less molecule lacking all serines and threonines, confirming that P-selectin was phosphorylated within the cytoplasmic domain. The construct containing only the C2 segment that includes Ser788 and both threonines (encoded by exon 16) was phosphorylated, whereas the construct containing only the C1 segment that includes Ser772 (encoded by exon 15) was not phosphorylated.

We next examined phosphorylation of constructs in which each serine or threonine was mutated to alanine. By ELISA analysis of cell lysates and immunosupernatants, approximately 80% of P-selectin in the cell lysates was immunoprecipitated in all cases. We therefore compared the densitometric values of labeled P-selectin in the autoradiogram with the quantities of P-selectin in the cell lysates determined by ELISA. As shown in Fig 7, individual mutations of Ser772 (S772A), Thr776 (T776A), or Thr781 (T781A) did not significantly affect phosphorylation. In contrast, mutation of Ser788 (S788A) reduced phosphorylation by 70% to 80%. A construct in which Ser772, Thr776, and Thr781 were changed to alanine (S772:T776:T781A), preserving only Ser788, was phosphorylated at half the level of wild-type. A construct in which Thr776, Thr781, and Ser772 were mutated (T776:T781:S788A) was only minimally phosphorylated. No phosphorylation was detected when all serines and threonines were mutated to alanine (S772:T776:T781:S788A). We conclude that the principal phosphorylation site of P-selectin is Ser788, located between two prolines at the extreme C terminus of the cytoplasmic tail. Minor phosphorylation occurs at the other serine and threonine residues.

DISCUSSION

In this study, we showed that P-selectin was phosphorylated in platelets, in endothelial cells, and in CHO cells transfected with P-selectin cDNA, suggesting that phosphorylation is a general event in different cell types. Phosphorylation rapidly increased when platelets or endothelial cells were stimulated with thrombin or PMA. Although the stoichiometry of P-selectin phosphorylation was relatively low, it could be underestimated if the phosphatase inhibitors used did not block the activity of all phosphatases in the cell lysates. Furthermore, phosphatase inhibitors increased 32P incorporation into P-selectin in both resting and stimulated platelets, suggesting that there was rapid interconversion of phosphorylated and unphosphorylated forms of P-selectin. Therefore, phosphorylation of P-selectin may be a dynamic process in intact cells.

PKC may regulate the phosphorylation of P-selectin because PMA, which directly activates PKC, phosphorylated P-selectin and PKC inhibitors abolished phosphorylation. Whether PKC acts directly or indirectly through other cellular kinases remains to be determined. Because PGE1 did not affect phosphorylation, cyclic AMP-dependent protein kinase is probably not responsible for the phosphorylation of P-selectin. Phosphorylation does appear to be regulated by protein phosphatase type 1 and/or type 2A, which are inhibited by okadaic acid and calyculin-A.

Phosphoamino acid analysis showed that P-selectin in platelets was phosphorylated predominantly on serine with lesser amounts on threonine. No phosphotyrosine could be detected with this method. The mutagenesis studies indicated that Ser788 was the principal phosphorylation site for
P-selectin is phosphorylated in transfected CHO cells. Minor degrees of phosphorylation were observed on Ser^722 and on threonine, although we do not know whether Thr^776 and Thr^814 were both phosphorylated. P-selectin in both platelets and CHO cells was phosphorylated primarily on serine, with lesser amounts on threonine, and with no detectable phosphorylation on tyrosine, suggesting that the same phosphorylation sites are used in different cells. The phosphorylation of serine and threonine residues on P-selectin is consistent with a regulatory role for PKC, which is a serine/threonine-specific enzyme, and for phosphatases that are specific for serines and threonines. The two serines and two threonines in the consensus sequence for proline-directed protein kinase (-S/T-X-P-) resemble the consensus sequence for PKC phosphorylation site, S/T-X-P, except that the adjacent proline at residue 789 is at the extreme C-terminus of the molecule.

The functional significance of phosphorylation of P-selectin remains unclear. Although soluble forms of P-selectin lacking the cytoplasmic domain still bind leukocytes, phosphorylation of transmembrane P-selectin could modulate its adhesive function through conformational changes or clustering in the plane of the membrane. Phosphorylation does not promote binding of P-selectin to the cytoskeleton, as the protein is not detectably associated with the Triton X-100-insoluble cytoskeletal fraction of resting or activated platelets (our unpublished data).

Phosphorylation might affect the intracellular trafficking of P-selectin, thereby indirectly regulating its function. Phosphorylation of a specific serine in the cytoplasmic domain of the polymeric Ig receptor is required for efficient transcytosis. The mannose 6-phosphate/insulin-like growth factor II receptor is phosphorylated to a low level in the total cell population. However, the stoichiometry of phosphorylation increases dramatically, but transiently, in the cohort of proteins that are exiting the trans-Golgi network. Although phosphorylation of this receptor is not required for lysosomal enzyme sorting, treatment of cells with okadaic acid affects its recycling to the cell surface. P-selectin might also be phosphorylated quantitatively, but transiently, in specific subcellular locations. Further studies are necessary to determine whether phosphorylation plays a role in the trafficking or function of P-selectin.

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The cytoplasmic domain of P-selectin is phosphorylated on serine and threonine residues

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