Cell, Vol. 82, 279–286, July 28, 1995, Copyright © 1995 by Cell Press

Histidine Phosphorylation of P-Selectin upon Stimulation of Human Platelets: A Novel Pathway for Activation-Dependent Signal Transduction

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Summary

Transient phosphorylation of histidine characterizes the two-component systems in prokaryotes that control important physiological functions, but analogous events have not been implicated in signal transduction in mammalian cells. To explore histidine phosphorylation during activation of human cells, stimulated platelets were analyzed for the formation of protein phosphohistidine in a model system employing P-selectin. P-selectin, a leukocyte adhesion molecule, undergoes rapid phosphorylation and selective dephosphorylation of tyrosine, serine, and threonine. We now establish that phosphorylation following platelet activation with thrombin or collagen generates phosphohistidine at histidines on the cytoplasmic tail of P-selectin. With thrombin stimulation, the kinetics of phosphohistidine appearance and disappearance on P-selectin are very rapid. Platelets exhibit a novel ligand-induced signaling pathway to generate phosphohistidine. These results provide direct biochemical evidence for the induction of rapid and reversible histidine phosphorylation in mammalian cells upon cell activation and represent a novel paradigm for mammalian cell signaling.

Introduction

Phosphohistidine plays an important role as an intermediate in the formation of phosphoaspartic acid in prokaryotic signal transduction, but has not been directly implicated in cell signaling in eukaryotes, although phosphohistidine has been found in many eukaryotic proteins. These include nuclear proteins, such as histone H4, from rat and bovine liver (Walinder, 1968; Chen et al., 1974, 1977; Fujitaki et al., 1981) and from the slime mold Physarum polycephalum (Huebner and Matthews, 1985; Pesis et al., 1988) and G protein β subunits from human leukemic HL60 cells (Wieland et al., 1993). Protein histidine kinases responsible for phosphohistidine synthesis (Smith et al., 1974; Huang et al., 1991; Motojima and Goto, 1994) and phosphohistidine phosphatases capable of dephosphorylation of phosphohistidine on proteins (Kim et al., 1993; Wong et al., 1993; Motojima and Goto, 1994; Ohmori et al., 1993) have further suggested the potential for a critical physiological role for transient phosphorylation in eukaryotes. Homology between two-component bacterial signaling systems and parallel proteins in early eukaryotes (Swanson et al., 1994) has led us to explore possible formation of protein phosphohistidine as an immediate consequence of cell activation in mammalian cells in a model system. Such an observation would implicate transient histidine phosphorylation as a basis for molecular recognition and signaling.

Platelets have served as an important model for cell signaling. Platelets undergo rapid morphological changes, activation of quiescent biochemical pathways, degranulation, and receptor activation on a rapid time scale. Platelets constitutively express both a thrombin receptor and collagen receptors, although the functionally active collagen receptor is not known with certainty. The thrombin receptor is a member of the G protein–coupled receptor family (Vu et al., 1991; Rasmussen et al., 1991). Proteolytic cleavage of the thrombin receptor by thrombin leads to receptor activation by the new N-terminus, which functions as the ligand for cell activation (Vu et al., 1991). Thrombin receptor signaling activates phospholipase C, phosphoinositol 3-kinase, protein kinase C, and the tyrosine kinase Src (Hung et al., 1992; Zhang et al., 1993; Clark and Brugge, 1993). One platelet collagen receptor, glycoprotein Ia–IIa, is an $\alpha 2\beta 1$ member of the integrin family. Collagen stimulation of platelets results in tyrosine kinase activity (Haimovich et al., 1993), including the tyrosine phosphorylation of the focal adhesion molecule FAK and inositol triphosphate formation (Watson et al., 1985). Although serine, threonine, and tyrosine are constitutively phosphorylated on some platelet proteins, platelet activation leads to marked increase in protein phosphorylation. This increased phosphorylation appears to be closely linked to the occupancy of certain receptors inasmuch as binding of glycoprotein IIb–IIIa to fibrinogen requires prior tyrosine phosphorylation (Shattil, 1993). Phosphohistidine has not been observed in platelets.

P-selectin is a cell adhesion molecule that mediates the interaction of platelets (Larsen et al., 1989) and endothelial cells (Geng et al., 1990) with monocytes and neutrophils. An integral membrane protein with a molecular weight of 140,000 (Hsu-Lin et al., 1984), this protein is composed of a lectin domain, an epidermal growth factor domain, a series of concensus repeat domains, a transmembrane region, and a short cytoplasmic tail (Johnston et al., 1989). This protein resides in the membrane of the α granule (Stenberg et al., 1985; Berman et al., 1986). Upon platelet activation and degranulation, the protein is rapidly translocated to the plasma membrane. P-selectin binds to a counterreceptor, PSGL-1, expressed on leukocytes of the myeloid lineage (Sako et al., 1993; Moore et al., 1994). The interaction of P-selectin with leukocytes leads to capture of leukocytes on the vascular surface, as demonstrated by in vitro (Larsen et al., 1989; Geng et al., 1990; Lawrence and Springer, 1991) and in vivo experiments (Mayadas et al., 1993), and the induction of tissue factor activity on monocytes (Celi et al., 1994).

During cell activation, the cytoplasmic tail of platelet P-selectin undergoes rapid phosphorylation (Crovello et

al., 1993; Fujimoto and McEver, 1993); phosphoserine, phosphothreonine, and phosphotyrosine have been previously detected in P-selectin following platelet activation (Crovello et al., 1993). Although histidine phosphorylation has been implicated in prokaryotic signal transduction pathways that mediate certain processes, including chemotaxis (Hess et al., 1988) and porin expression (Stock et al., 1990), and phosphohistidine has been observed in mammalian cell proteins, activation of a histidine phosphorylation–dephosphorylation signaling pathway associated with mammalian ligand-induced cell activation has not been observed. We have demonstrated and characterized the reversible phosphorylation of histidine in human P-selectin following platelet activation and postulate the existence of a novel cell activation signaling pathway involving transient phosphohistidine formation in mammalian cells.

Results

We have previously demonstrated that platelet activation is associated with the rapid phosphorylation of serine, threonine, and tyrosine residues in the cytoplasmic tail of P-selectin, followed by dephosphorylation of phosphothreonine and phosphotyrosine (Crovello et al., 1993). However, because phosphoamino acid analyses were performed on P-selectin subjected to acid hydrolysis, we would have destroyed any phosphohistidine present on P-selectin under the conditions employed. Because of the functional importance of phosphohistidine in prokaryotes, the multiple sites of phosphorylation in the P-selectin cytoplasmic tail, and the presence of two histidine residues within this region, we were interested to learn whether phosphohistidine formation might accompany cell activation in a model mammalian system and, thus, represent a novel intracellular pathway for signal transduction. Platelets from normal human blood were isolated by gel filtration and were [^{32}P]orthophosphate loaded. After purification, platelets were activated by the addition of either thrombin or collagen and then disrupted at the indicated time using lysis buffer. P-selectin was isolated by immunoprecipitation, SDS gel electrophoresis, and electrophoretic transfer to Immobilon P. The phosphorylated P-selectin was detected using a phosphorimager (Figure 1). P-selectin from activated platelets was characterized by a band containing ^{32}P whose electrophoretic mobility was consistent with a molecular weight of 140,000. We have previously demonstrated that this band is a phosphorylated form of P-selectin (Crovello et al., 1993). In contrast, unstimulated platelets contain minimal phosphorylated P-selectin. The P-selectin band from thrombin-activated platelets or collagen-activated platelets was excised from Immobilon P, and P-selectin was subjected to alkaline hydrolysis. Phosphoamino acids were separated by thin-layer chromatography. As shown in Figure 2, chromatography of the base hydrolysate of P-selectin from activated platelets indicates the presence of both phosphohistidine and phosphotyrosine. A phosphohistidine standard, containing N1- and N3-phosphohistidine, comigrated with the [^{32}P]phosphohistidine. P-selectin isolated from resting platelets contains nearly undetectable amounts of phosphohistidine and phosphotyrosine. The small amount of phosphoamino acids observed in unstimulated platelets is likely due to the contamination of unstimulated platelets with platelets activated incidentally during the isolation process. P-selectin isolated from stimulated platelets demonstrates the presence of phosphohistidine and confirms the presence of phosphotyrosine. The amount of phosphohistidine in P-selectin from thrombin-stimulated platelets was about 20-fold higher than that of phosphohistidine in P-selectin derived from unstimulated platelets.

To eliminate the possibility that phosphohistidine was formed during alkaline hydrolysis of the phosphorylated P-selectin, possibly by β elimination of phosphoserine and nonenzymatic phosphorylation of an adjacent histidine, phosphohistidine was shown to be present in P-selectin not subjected to alkaline hydrolysis. The P-selectin band from thrombin-activated platelets was excised from Immobilon P, and P-selectin was subjected to Pronase digestion. Two-dimensional thin-layer chromatography of the enzymatic hydrolysate of P-selectin from activated platelets indicated the presence of phosphohistidine; the ^{32}P-labeled phosphohistidine derived from P-selectin cochromatographed with the phosphohistidine standard stained with ninhydrin (data not shown; see below).

The amino acid sequence of the cytoplasmic tail of P-selectin includes 35 residues (Figure 3). This sequence includes two serine residues, two threonine residues, one tyrosine residue, and two histidine residues. To prove that histidine residues on the cytoplasmic tail of P-selectin are phosphorylated following platelet activation, we isolated a tryptic peptide derived from the cytoplasmic domain of P-selectin and subjected it to Edman degradation and ^{32}P analysis. The phosphorylated form of P-selectin, labeled with ^{32}P, was isolated by immunoprecipitation, SDS gel electrophoresis, and electrophoretic transfer to Immobilon

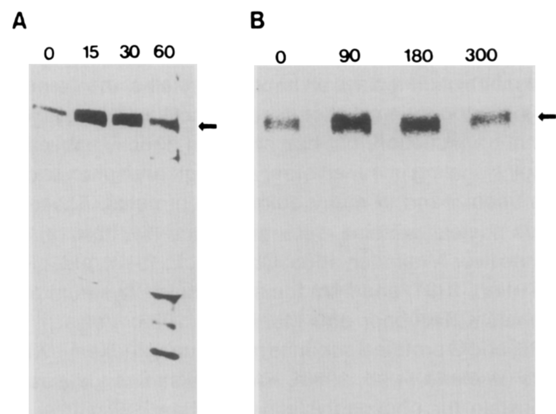

Figure 1. Phosphorylation of P-Selectin in Platelets during Thrombin or Collagen Stimulation

P-selectin was immunoprecipitated and subjected to SDS gel electrophoresis. ^{32}P in the gel was visualized with a phosphorimager; the position of P-selectin migration (MW 140,000) is indicated by the arrow. The time (seconds) between cell activation and termination of activation is indicated above each lane. (A) Thrombin-stimulated platelets. (B) Collagen-stimulated platelets.

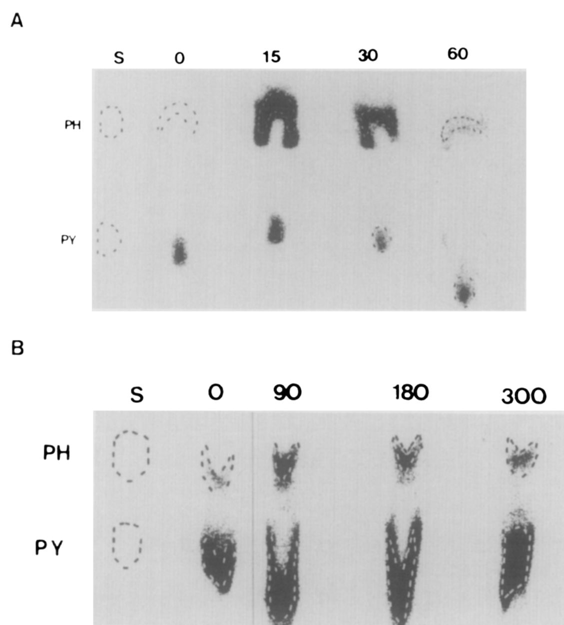

Figure 2. Phosphoamino Acid Analysis of P-Selectin from Activated Platelets

The P-selectin was excised from the transfer blot and phosphoamino acid analysis by thin-layer chromatography performed on the alkaline hydrolysate. The time (seconds) between cell activation and termination of activation is indicated above each lane. Lane S contains phosphohistidine and phosphotyrosine standards. (A) Thrombin-stimulated platelets. (B) Collagen-stimulated platelets. A phosphohistidine standard and phosphotyrosine standard were added to the alkaline hydrolysate of the radioactive phosphorylated P-selectin. ^{32}P was detected by autoradiography using a Molecular Dynamics phosphorimager. Phosphohistidine and phosphotyrosine standards (dashed line) were visualized with ninhydrin stain. The autoradiogram obtained is superimposed on a diagram of internal phosphohistidine and phosphotyrosine standards. The vertical line indicates a composite of two separate thin-layer chromatographs.

P. The purified protein was subjected to digestion with trypsin. The limit digest of P-selectin includes a C-terminal peptide derived from the cytoplasmic tail, from residues 766–789. This peptide, which contains all of the potential phosphorylation sites, was isolated by affinity chromatography using anhydrotrypsin as a ligand. Since anhydrotrypsin binds to all peptides that include at least one arginine or lysine residue and since trypsin cleaves C-terminal to lysine and arginine residues, all peptides in a tryptic hydrolysate of P-selectin except the C-terminal peptide will bind to anhydrotrypsin–Sepharose. When the tryptic hydrolysate of P-selectin was applied to an anhydrotrypsin–Sepharose column, ^{32}P was recovered quantitatively in the unbound fraction. Bound peptides were eluted with HCl, but no ^{32}P was associated with these peptides. Although HCl might cause some degradation of the acid-labile phosphohistidine on a bound peptide, acid-stable phosphoserine, phosphothreonine, and phosphotyrosine on the peptide would serve to identify this peptide following acid elution from the column. The phosphopeptide was further purified and characterized by reverse-phase high performance liquid chromatography (HPLC) (Figure 4). A single major protein peak was observed by monitoring absorption at 280 nm. This peak contained all of the peptide-bound ^{32}P and was reactive with an immunoaffinity-purified polyclonal rabbit antibody, anti-766–776, directed against the cytoplasmic tail of P-selectin. These results indicate the isolation of a single phosphopeptide from phosphorylated P-selectin.

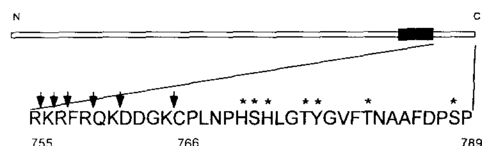

Figure 3. Cytoplasmic Tail of P-Selectin

The cytoplasmic tail is located on the C-terminus of P-selectin, from residue 755–789 (Johnston et al., 1989). The sequence of the cytoplasmic tail is shown, including potential sites of phosphorylation (asterisks). Potential sites of trypsin cleavage are indicated (arrows). The tryptic peptide 766–789 contains all of the potential phosphorylation sites.

The purified phosphopeptide derived from the tryptic digestion of P-selectin from thrombin-activated platelets was subjected to phosphoamino acid analysis following Pronase digestion. To confirm further the identity of the putative phosphohistidine species derived from P-selectin, we performed two-dimensional thin-layer chromatography on the phosphopeptide Pronase hydrolysate. The chromatogram of the ^{32}P-labeled hydrolysate, supplemented with phosphoserine, phosphotyrosine, phosphothreonine, and phosphohistidine standards, was developed with ethanol–ammonium hydroxide in the first dimension and methanol–ammonium hydroxide in the second dimension. As shown in Figure 5, the ^{32}P-labeled

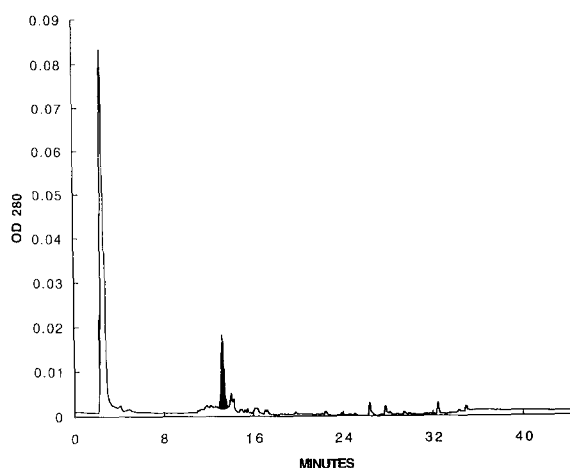

Figure 4. HPLC Purification of the Tryptic C-Terminal Phosphopeptide of P-Selectin

The phosphorylated P-selectin was subjected to trypsin digestion, and the hydrolysate was applied to an anhydrotrypsin–Sepharose column to isolate the C-terminal peptide lacking arginine or lysine. Bound peptides were eluted with HCl. All of the radioactivity was associated with the unbound fraction. This fraction, after concentration, was further purified by reverse-phase HPLC. The column was monitored at 280 nm (·), and fractions were assayed for ^{32}P (black).

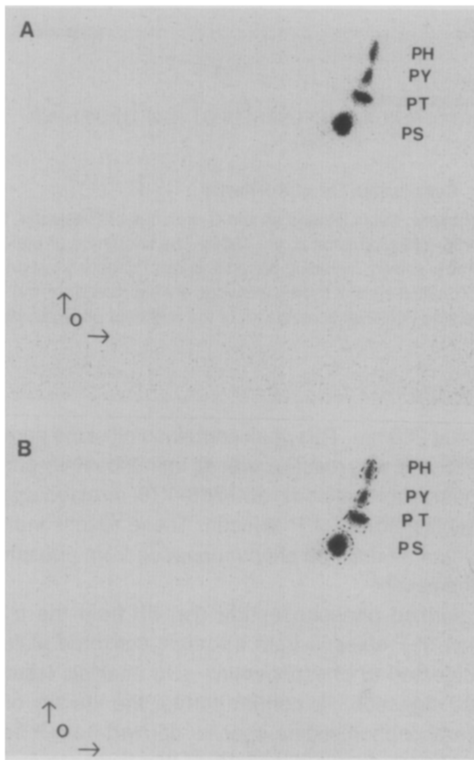

Figure 5. Phosphoamino Acid Analysis of the P-Selectin Tryptic Phosphopeptide from Activated Platelets Following Enzymatic Proteolysis

Two-dimensional thin-layer chromatography of the Pronase digest of the purified phosphopeptide was performed using an ethanol/ammonium hydroxide solvent system in the first dimension and a methanol/ammonium hydroxide solvent system in the second dimension. Radioactive phosphoamino acids (PH, PS, PY, and PT) were identified by autoradiography (top). The same image is shown (bottom), but the locations of the positions of the internal standards (phosphoserine, PS; phosphothreonine, PT; phosphotyrosine, PY; phosphohistidine, PH) identified by ninhydrin staining are overlayed (dashed line).

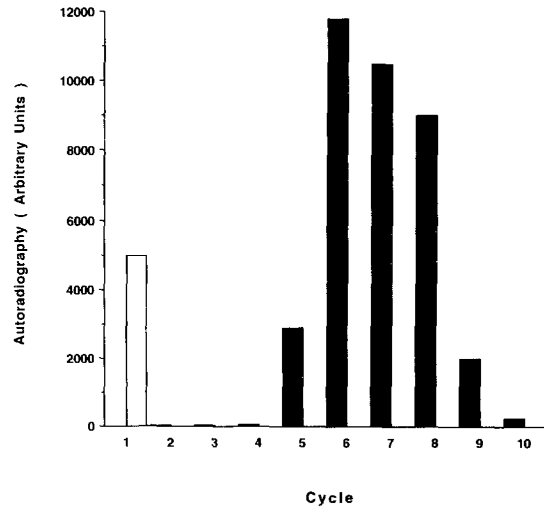

Figure 6. Identification of Phosphohistidine in the Phosphopeptide by Edman Degradation

The purified phosphopeptide (766–789) was subjected to automated Edman degradation under modified conditions to preserve and elute phosphoamino acids. Each residue was quantitatively analyzed for ^{32}P by autoradiography using a phosphorimager (closed bars). Residues 6, 7, and 8 contain ^{32}P. The presence of ^{32}P in residue 1 is due to free phosphate, demonstrated by thin-layer chromatography, derived from the phosphopeptide (open bar).

phosphoamino acids from the phosphopeptide included phosphohistidine, phosphotyrosine, phosphothreonine, and phosphoserine. These radioactive phosphoamino acids cochromatographed with the internal phosphoamino acid standards, including phosphohistidine, as identified with ninhydrin staining. These results prove the presence of phosphohistidine in the P-selectin phosphopeptide.

The purified phosphopeptide was subjected to Edman degradation and the ATZ derivatives of each residue were collected (Wettenhall et al., 1991). The amino acid derivative at each cycle was then quantitated for ^{32}P using a phosphorimager. As shown in Figure 6, cycles 6, 7, and 8 contained significant levels of ^{32}P; cycle 1 contained ^{32}P as free phosphate, as demonstrated by thin-layer chromatography. Positions 6, 7, and 8 correspond to His-771, Ser-772, and His-773, respectively. The small amount of radioactivity that appears in cycle 5 is due to preview during the Edman degradation. Owing to the repetitive yield, the diminished efficiency of degradation due to the presence of phosphoamino acids, and the quantity of initial material available, data in the experiment depicted were obtained only to cycle 10. However, in separate experiments, small amounts of ^{32}P were detected at positions 11 and 12, corresponding to Thr-776 and Tyr-777. These results, obtained in three independent Edman degradations with three independent preparations of phosphorylated P-selectin, confirm that phosphohistidine is a component of phosphorylated P-selectin and that both His-771 and His-773 in the cytoplasmic tail are phosphorylated upon platelet activation. Determination of the N-terminal sequence of the phosphopeptide was attempted, but was unsuccessful in multiple experiments. The low amount of phosphopeptide in the tryptic hydrolysate may be due to the palmitoylation of Cys-766 (Fujimoto et al., 1993).

Our previous studies have shown that the kinetics of phosphorylation of P-selectin following cell activation are very rapid. Furthermore, dephosphorylation of threonine and tyrosine is also rapid. To determine whether phosphohistidine formation paralleled the synthesis of phosphorylated serine, threonine, and tyrosine in P-selectin and whether the kinetics of phosphorylation and dephosphorylation of either serine, threonine, and tyrosine might most likely parallel that of phosphohistidine, the kinetics of phosphohistidine formation on P-selectin in thrombin-activated platelets was evaluated. As shown in Figure 7A, maximal phosphohistidine on P-selectin was observed at 15 s following thrombin stimulation. Dephosphorylation leads to diminished phosphohistidine at 30 s and barely detectable levels at 60 s. The half-disappearance time of phosphohistidine is estimated at about 10–15 s. By comparison, the half-disappearance times of phosphoserine, phosphothreonine, and phosphotyrosine on P-selectin from thrombin-activated platelets are estimated at about >300 s, 25 s, and 15 s, respectively (Crovello et al., 1993).

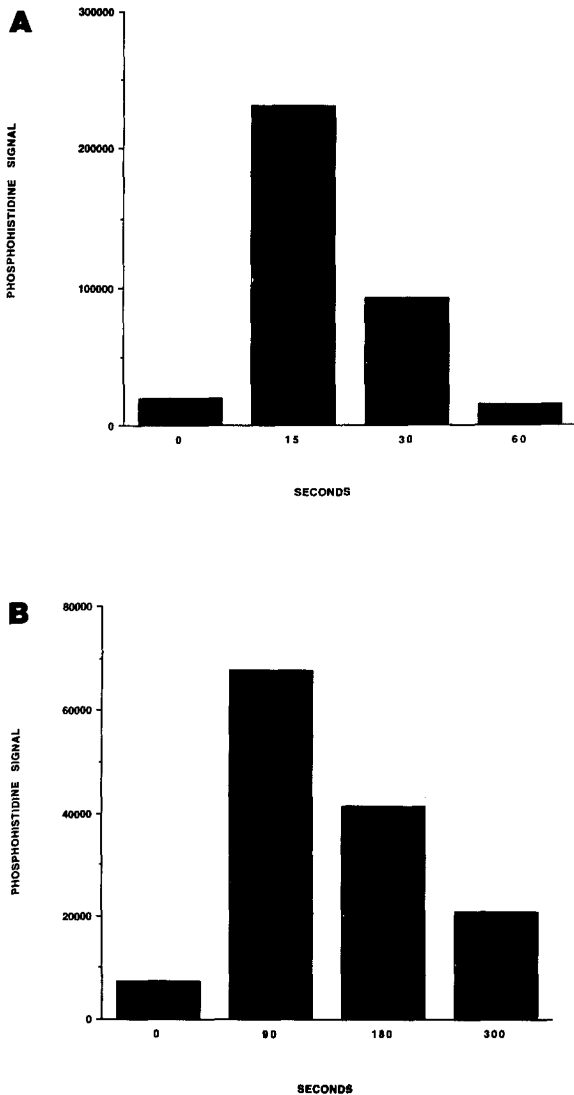

Figure 7. Kinetics of Histidine Phosphorylation and Dephosphorylation on P-Selectin Following Thrombin or Collagen Stimulation of Platelets

Platelets were activated for the indicated time before the reaction was stopped by the addition of lysis buffer. (A) Thrombin-stimulated platelets. (B) Collagen-stimulated platelets. Data were obtained by analyzing digitized images of autoradiograms of the amino acid analysis of alkaline hydrolysates.

The kinetics of phosphorylation and dephosphorylation of histidine differed in collagen-stimulated platelets. As shown in Figure 7B, collagen-induced histidine phosphorylation peaked at about 90 s. Dephosphorylation was also observed, but the half-disappearance time of phosphohistidine is about 60–90 s. Thus, the time scale of reversible histidine phosphorylation on P-selectin is about 4- to 6-fold longer with collagen-induced platelet activation than with thrombin-induced platelet activation. This is compatible with the observation that the peak of collagen-induced P-selectin phosphorylation in general occurs at about 90 s.

Discussion

In this work, we demonstrate that P-selectin phosphorylation during human platelet activation includes the transient generation of phosphohistidine on the C-terminal cytoplasmic tail. Histidine phosphorylation has not been previously implicated in mammalian signal transduction pathways. However, phosphohistidine is a known component of eukaryotic proteins (Zetterqvist, 1967; Walinder, 1968, 1969; Chen et al., 1974, 1977; Fujitaki et al., 1981; Huebner and Matthews, 1985; Pesis et al., 1988; Wieland et al., 1993; Motojima and Goto, 1993), and the enzymatic machinery for the synthesis of phosphohistidine and for its dephosphorylation is present in lower eukaryotic and mammalian cells. A protein histidine kinase has been isolated from yeast Saccharomyces cerevisiae (Huang et al., 1991), and protein histidine kinase activity has been identified in mammalian and slime mold cells (Smith et al., 1974; Huebner and Matthews, 1985; Motojima and Goto, 1993). Protein phosphatases that dephosphorylate phosphohistidine have also been identified, including protein phosphatases 1, 2A, and 2C (Kim et al., 1993; Motojima and Goto, 1994), all of which are known serine/threonine phosphatases, and phosphatases from rat brain (Ohmori et al., 1993; Wong et al., 1993).

Although the function of phosphohistidine residues on P-selectin remains to be elucidated, the importance of phosphohistidine as an intermediate in the two-component prokaryotic system strongly suggests a critical role for phosphohistidine in mammalian cell signaling (Swanson et al., 1994). Histidine phosphorylation is involved in prokaryotic signal transduction, including chemotaxis, porin expression, and nitrogen metabolism (Stock et al., 1990). In one process, a sensor histidine kinase is autophosphorylated on histidine in response to environmental stimuli and then rapidly transfers this phosphate to aspartyl residues on a response regulator protein in the next step of the signal transduction pathway (Bourret et al., 1991). In the family of prokaryotic two-component systems, the histidine kinase that autophosphorylates its own histidine and the regulator response domain that contains the phosphate receptor aspartic acid may be on independent proteins or domains of a single protein (Swanson et al., 1994). This two-component system may exist in lower eukaryotic signaling pathways as well, since genetic analysis of S. cerevisae has implicated a transmembrane histidine kinase in regulation of an osmosensing MAP kinase cascade (Maeda et al., 1994). Alternatively, mammalian cells may express independent histidine kinases and protein phosphatases that are highly regulated and responsible for transient phosphohistidine appearance. However, the triggering of events leading to the transient appearance of phosphohistidine upon cell activation, as we have now described in stimulated platelets, has no precedent in mammalian cells.

Thrombin is known to initiate a distinct signaling pathway through the thrombin receptor (Coughlin, 1994). Thrombin-induced platelet activation is rapid and is complete within 60 s. Collagen activates platelets through an

independent mechanism. Glycoprotein Ia–IIa, an α2β1 integrin, is one receptor that has been implicated in collagen binding (Kunicki et al., 1988; Staatz et al., 1989). Collagen-induced platelet activation is less potent, and the time scale for platelet activation is of the order of several minutes. The rate of thrombin-induced and collagen-induced platelet activation temporally parallels the rate of thrombin-induced and collagen-induced phosphohistidine formation and dephosphorylation observed in our experiments. Regardless of the rate of platelet activation with these two agonists, platelet activation is characterized in both cases by degranulation, surface receptor activation, activation of certain metabolic pathways, and morphological changes, including the extension of pseudopodia. These two agonists, working through two distinct and independent signal transduction pathways, both yield phosphohistidine on P-selectin and common platelet activation events.

We suspect that transient histidine phosphorylation occurs on many platelet proteins during platelet activation. Given the biochemical techniques used to investigate protein phosphorylation, phosphohistidine is too labile to facilitate direct observation. By revisiting this question, we and others will be able to ascertain the extent to which phosphohistidine appears on various classes of proteins during platelet activation and whether phosphorylation always occurs synchronously with the phosphorylation of serine, threonine, or tyrosine. Recently, two mammalian serine/threonine kinases, branched chain α-ketoacid dehydrogenase kinase (Popov et al., 1992) and pyruvate dehydrogenase kinase (Popov et al., 1993), have been cloned. These kinases regulate the activity of branched chain α-ketoacid dehydrogenase and pyruvate dehydrogenerase, respectively, in the mitochondria by phosphorylation–dephosphorylation. Although the residues phosphorylated on branched chain α-ketoacid dehydrogenase and pyruvate dehydrogenase are serines, branched chain α-ketoacid dehydrogenase kinase and pyruvate dehydrogenase kinase share considerable homology with prokaryotic protein histidine kinases. It has been hypothesized that the conserved histindine residues on branched chain α-keto acid dehydrogenase kinase may be autophosphorylated and, in turn, participate in phosphorylation of serine residues in branched chain α-ketoacid dehydrogenase. As P-selectin is unlikely to undergo autophosphorylation on histidine because of its short cytoplasmic tail, it is unclear whether the phosphorylation of histidine residues on P-selectin resembles in any way the two-component phosphohistidine–phosphoaspartic acid systems extensively studied in bacteria. Instead, phosphorylation of P-selectin histidines may be mediated by a true histidine kinase. The observation that G protein β subunits in HL60 cell membranes can be phosphorylated by GTP and that this high energy phosphate can be specifically transferred onto GDP via a phosphistidine intermediate (Wieland et al., 1993) raises further possibilities for mechanisms of histidine phosphorylation–dephosphorylation and potential physiological roles in cellular signaling pathways. To understand this system fully, it will be important to ascertain the immediate phosphate donor, establish the presence of a histidine kinase and phosphatases, and determine the recipient of phosphate transfer from phosphohistidine in platelets. Most importantly, the functional role of transient histidine phosphorylation in P-selectin specifically and other proteins more generally remains to be understood at the molecular level.

Experimental Procedures

Platelet Preparation

Fresh human platelets were isolated from the blood of normal subjects. Blood was anticoagulated with Wares buffer. Platelet-rich plasma was obtained by centrifugation of whole blood at 200 × g for 15 min and was adjusted to 1 mM EDTA. Platelets were isolated by gel filtration through Sepharose 2B (Crovello et al., 1993).

Isolation and Analysis of Phosphorylated P-Selectin

Platelets (5 ml, 1×10^9/ml) were incubated with ^{32}P-labeled orthophosphoric acid (1–2 mCi/ml) for 1 hr at 37°C. Platelets were separated into equivalent fractions. One fraction remained resting, and the other fractions were activated with agonist for the time period specified. After the indicated time interval, platelets were disrupted by the addition of 4× lysis buffer (40 mM Tris–HCl [pH 7.2], 4% Triton X-100, 632 mM NaCl, 10 mM EDTA, 4 mM PMSF, 4 mM sodium orthovanadate, 40 mM sodium fluoride, 20 mM tetrasodium pyrophosphate, 20 mM β-glycerophosphate, 20 μg/ml aprotinin) for 10 min at 0°C. Insoluble material was removed by centrifugation at 1400 × g for 5 min. The platelet lysate was first incubated with MOPC-21, an irrelevant isotype-matched murine monoclonal antibody, followed by AC1.2, a monoclonal anitibody directed against P-selectin (Larsen et al., 1989). MOPC-21 Sepharose (250 μl, 1 mg/ml) was incubated with the platelet lysate for 90 min at 4°C to remove proteins that nonspecifically bound to immunoglobulin IgG1. After the beads were removed by centrifugation, AC1.2 Sepharose (100 μl, 1 mg/ml) was added to the platelet lysate and incubated at 4°C for 90 min. After removal of the beads by centrifugation, the beads were washed extensively with 0.1% SDS, 0.5% Nonidet P-40, 0.5% bovine serum albumin, 0.5% deoxycholate, 2 mM EDTA, 100 mM Tris–HCl (pH 7.4), 2 mM PMSF, 20 mM tetrasodium pyrophosphate, 40 mM β-glycerophosphate, 2 mM sodium orthovanadate. Beads were then treated with Lamelli reducing sample buffer for 2 min at 100°C, and the supernatent was applied to a 6%–10% (linear) gradient SDS–polyacrylamide gel prior to electrophoresis. Following electrophoresis, proteins on the gel were electrophoretically transferred to Immobilon P (Millipore). ^{32}P contained within proteins on the membrane was detected using a Molecular Dynamics phosphorimager, model 425 B.

Isolation of the Phosphopeptide from P-Selectin

The ^{32}P-labeled P-selectin band was excised from Immobilon P, rewet in methanol, and rinsed with water. The membrane slice was incubated in 0.5% polyvinylpyrrolidene 360 (w/v) in 100 mM acetic acid for 15 min at 37°C. Under these conditions, we observed minimal dephosphorylation of the synthetic phosphohistidine standard. The membrane slice was then washed extensively with water, rinsed in 50 mM ammonium bicarbonate (pH 8.0), placed in 200 μl of 50 mM ammonium bicarbonate (pH 8.0), and 2 μg of TPCK–trypsin was added (Luo et al., 1991). The reaction was incubated at 37°C for 24 hr, and the supernatant was removed, adjusted to 1 mM diisopropyl phosphofluoridate, and lyophilized. After washing in water, the pellet was then resuspended in 50 mM sodium acetate, 20 mM $CaCl_2$ (pH 5.0), and applied to an anhydrotrypsin–Sepharose affinity column (Takara Biochemical) equilibrated in the same buffer. The unbound fraction from this column was collected, and the bound peptides were eluted with 5 mM HCl. Fractions were frozen in a dry ice–ethanol bath and lyophilized. The pellet was then resuspended in 0.1% TFA in water and immediately injected onto a C18 reverse-phase HPLC column (Vydac). The column was eluted with a gradient of acetonitrile and 0.1% TFA, and fractions were collected, frozen, and lyophilized. The fractions containing ^{32}P were further analyzed.

To positively identify the phosphopeptide, an anti-766–776 antibody was reacted with the HPLC-purified phosphopeptide. Immunoaffinity-purified anti-766–776 (Chong et al., 1994), a rabbit polyclonal antibody prepared against the synthetic peptide 766–776 of the P-selectin cyto-

plasmic tail sequence, was coupled to CNBr-activated Sepharose. The Sepharose–anti-766–776 beads were incubated with the HPLC-purified phosphopeptide for 8 hr, and the amount of ^{32}P bound to the beads was ascertained by assay using the phosphorimager.

Amino Acid Sequence Analysis

The purified phosphopeptide was covalently linked to an arylamine sequencing disk according to the instructions of the manufacturer (Milligen). The disk was subjected to automated Edman degradation using a Milligen protein sequencer, and the ATZ derivative of each residue was collected. Residues at each cycle were lyophilized and resuspended in water. Each residue was quantitatively spotted onto a grid, and the grid was analyzed using the phosphorimager to assay ^{32}P-labeled residues.

Phosphoamino Acid Analysis

The Immobilon P membrane was washed once in methanol and thrice in water. The ^{32}P-labeled P-selectin band was excised, rewet with methanol, and washed with water. The membrane slice was placed in a Pierce Reacti vial with 30 μl of 2 N KOH and incubated for 5 hr at 105°C. The hydrolysate was removed, and 7 N $HClO_4$ was added until the solution approached neutral pH. $KClO_4$ that precipitated was removed by centrifugation, and the supernatant was spotted onto a Whatman silica gel 60A thin-layer chromatography plate along with unlabeled phosphohistidine and phosphotyrosine standards. Phosphotyrosine was purchased from Sigma.

Alternatively, the ^{32}P-labeled P-selectin band was excised and subjected to total proteolytic digestion using Pronase. The ^{32}P-labeled P-selectin band was excised and rewet in methanol, rinsed in water, and then rinsed in 50 mM ammonium bicarbonate (pH 8.3). The band was immersed in 50 mM ammonium bicarbonate, and 10 μg of Pronase E (Sigma) was added from a stock of 1 mg/ml in the same buffer. The reaction was allowed to proceed overnight at 37°C. The reaction mixture was diluted to 1 ml with water and frozen in a dry ice–ethanol bath. The sample was twice lyophilized and then dissolved in 20 μl of water. The proteolytic hydrolysate was applied to a Whatman silica gel LK6 chromatography plate along with phosphohistidine, phosphotyrosine, phosphoserine, and phosphothreonine standards. For one-dimensional analysis, the plate was developed with two solvent cycles of ethanol:25% ammonia (3.5:1.6 [v/v]). For two-dimensional analysis, the plate was developed in the first dimension with the ethanol solvent system and was followed in the second dimension with two solvent cycles of methanol:25% ammonia (3.5:1.6 [v/v]). Phosphoamino acid standards were visualized by spraying the plates with 0.25% ninhydrin in butanol. ^{32}P-labeled phosphoamino acids were detected by autoradiography with the phosphorimager. The kinetics of phosphorylation and dephosphorylation of phosphohistidine was determined from the phosphorimager (Crovello et al., 1993).

Phosphohistidine Synthesis

An authentic phosphohistidine standard was prepared by the potassium phosphoramidate method (Wei and Matthews, 1993). Phosphoramidate was synthesized by the method of Sheridan et al. (1971). Potassium phosphoramidate (100 mg) was incubated with 15 mg of L-histidine in water for 60 min at room temperature. The composition of the synthetic phosphohistidine was confirmed by demonstration of its comigration on the thin-layer chromatograph with N1- and N3-phosphohistidine (gift of Dr. H. Matthews, University California at Davis). The chromatography plate was developed with two solvent cycles of ethanol:25% ammonium hydroxide (3.5:1.6 [v/v]), and the phosphoamino acid standards were visualized with ninhydrin spray (0.25% [w/v] in butanol).

Acknowledgments

We thank Dr. Harry Matthews for the phosphohistidine standard, Dr. Michael Berndt for the antibody to the P-selectin cytoplasmic tail, and Drs. Brian Schaffhausen, Andrew Wright, and Michael Mendelsohn for helpful discussions. We gratefully acknowledge the assistance of Margaret Jacobs in performing the Edman degradation. This work was supported by a grant (HL51926) from the National Institutes of Health. C. S. C. is a recipient of a Berlex Predoctoral Fellowship.

Received January 5, 1995; revised May 22, 1995.

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