Evidence for cAMP-dependent Platelet Ectoprotein Kinase Activity That Phosphorylates Platelet Glycoprotein IV (CD36)*

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The dephosphorylating enzyme alkaline phosphatase, by removing phosphate groups from the external platelet membrane proteins, modulates platelet activation (Hatmi, M., Haye, B., Gavaret, J. M., Vargaftig, B. B., and Jacquemin, C. (1991) Br. J. Pharmacol. 104, 554–558). This observation, together with findings reported by others (Ehrlich, Y. H., Davis, T. B., Bock, E., Kornecki, E., and Lenox, R. H. (1986) Nature 320, 67–70; Dusenbery, K. E., Mendiola, J. R., and Skubitz, K. M. (1988) Biochem. Biophys. Res. Commun. 153, 7–13), indicate the existence of ectoprotein kinase activity on the blood platelet surface.

In this study, we demonstrated that washed human platelets phosphorylate the synthetic heptapeptide kemptide in a cAMP-dependent mode. The intensity of the phosphorylation was concentration-dependent for kemptide. In addition, incubation of platelets with [γ-32P]ATP resulted in a rapid incorporation of [32P]phosphate into proteins at the outer membrane surface that was sensitive to alkaline phosphatase treatment. When cAMP was added to the medium, major phosphorylation of an 88-kDa ectoprotein occurred. Its isoelectric point determined by isoelectric focusing SDS-polyacrylamide gel electrophoresis was around pH 6.2. Phosphorylations of this 88-kDa polypeptide and of the exogenous kemptide substrate were both prevented by the specific protein kinase A inhibitor peptide. When platelets were preincubated with [32P]inorganic phosphate to label intracellular proteins, the protein phosphorylation pattern was different from that obtained with [γ-32P]ATP, indicating that the latter occurred at the outer surface of the cells. Prostacyclin, which induces the increase of intracellular cAMP levels and, consequently, its liberation into the extracellular medium, increased phosphorylation of both kemptide and platelet 88-kDa polypeptide. The major protein of 88-kDa, which was phosphorylated in the presence of cAMP and external [γ-32P]ATP, was identified by immunoprecipitation to GPIV (CD36), one of thrombospondin and collagen binding sites on platelets. The phosphorylation of CD36 also occurred in platelet-rich plasma, suggesting a physiological role for this ectoenzyme.

In the present study, we clearly demonstrated the presence of an ectoprotein kinase A activity at the surface of intact human platelets, and we revealed its principal endogenous substrate as being CD36.

The cell surface is directly involved in cell-cell interaction through receptors for extracellular signals. The phosphorylation and dephosphorylation of proteins are critical to the regulation of cellular functions, particularly in blood platelets (4–7). The dephosphorylating enzyme alkaline phosphatase, which removes phosphate groups from the external platelet membrane, prevents platelet aggregation and secretion by thromboxane mimetics (1). In addition, acid phosphatases that dephosphorylate the ectodomain of CD36, a collagen and thrombospondin receptor, decreased platelet aggregation to collagen and ADP (8). Also, ATP, the cAMP substrate for phosphorylation, is secreted by activated platelets from storage granules. Extracellular ATP is known to exert an inhibitory effect on platelet activation by competing with adenosine diphosphate, increasing intracellular cAMP levels and probably phosphorylating surface platelet proteins (9). The expression at the platelet surface of protein kinase and phosphatase activities may thus be an important mechanism for the regulation of platelet functions. Naik et al. (10) have reported protein kinase and phosphatase activities on the membrane surface of human platelets, which rapidly phosphorylated and dephosphorylated 39- and 42-kDa proteins, whose function was undetermined.

It is well known that intracellular protein kinase A (PKA)§ is also important in the regulation of various platelet functions (11–13), but ecto-PKA activity in the plasma membrane has not been demonstrated in blood platelets. The rate-limiting step for the ectophosphorylation activity depends on ATP and presumably on cAMP. ATP and other nucleotides, including cAMP, are intracellular constituents that may be released from platelets under well described conditions (14).

The objective of this study was first to investigate the existence of a PKA activity at the outer surface of platelets, using a specific synthetic substrate (kemptide) and the specific natural inhibitor (PKI) and second to characterize its putative endogenous platelet substrate. In this work, we provide direct evidence for the existence of an ecto-PKA activity that mainly phosphorylates a protein substrate identified as GPIV (CD36). The ectoprotein phosphorylation/dephosphorylation states of CD36 were shown to modulate the interaction of platelets with adhesive proteins, thrombospondin and collagen (8). Our results suggest that rephosphorylation of CD36 by ecto-PKA could restore the binding properties of the resting state.

EXPERIMENTAL PROCEDURES

Materials—Reagents and materials were from the following sources. Prostacyclin (PGI2), cAMP, cGMP, alkaline phosphatase, bovine serum albumin (Fraction V), ATP, kemptide (Leu-Arg-Ala-Ser-Leu-Gly), natural protein kinase inhibitor peptide of Cheng et al. (15), and anti-

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1 The abbreviations used are: PKA, protein kinase A; PAGE, polyacrylamide gel electrophoresis; PG12, prostaglandin I2 (prostacyclin); PKI, protein kinase A inhibitor; PRP, platelet-rich plasma.
bodies mouse IgG1 and rabbit polyclonal anti-mouse IgG were purchased from Sigma. The cAMP-acetylcholinesterase tracer, Ellman's reagent, anti-cAMP, and mouse monoclonal antibodies were obtained from SPI-Bio (Saclay, France). The stable endoperoxide/TxA2 analogue U46619 was from Upjohn Co. (Kalamazoo, MI). Monoclonal antibody anti-GPIV, FA6–152 (16) was kindly provided by Dr. J. L. McGregor (INSERM U 331, Lyon) or purchased from Immunotech SA (Marseille, France). Nitrocellulose membranes (BA83) were from Schleicher and Schuell (Dassel, Germany). [32P]Inorganic phosphate ([32P]Pi), [γ-32P]ATP and ECL Western blotting detection system were purchased from Amersham (Buckinghamshire, United Kingdom). The luciferin-luciferase solution was from Lumac (Schaeberg, The Netherlands).

Preparation of Human Platelets—Blood obtained from healthy human volunteers who had not received any medication for at least 10 days was anticoagulated with 1/6 of its final volume of citric acid-citrate-dextrose (7, 93, and 139 mM, respectively, pH 6.4) containing heparin (20 IU/ml). Washed platelets were prepared according to Mustard et al. (17), in which apyrase was omitted and PGI2 (1 nm) was added for the first two steps of washing. The platelets were resuspended in the Tyrode's buffer, and their number was adjusted to a final concentration of 5 × 10^8 cells/ml. Platelet-rich plasma (PRP) was obtained from citrated (0.38% final concentration) blood by standard differential centrifugation.

Measurement of ATP Release—ATP secretion was determined at 3 min, after the addition of the stimulating agent U46619, by the bioluminescence assay (luciferin-luciferase) using a Pico-ATP device from Jobin Yvon (Paris, France).

Measurement of cAMP—0.4-ml suspensions of washed platelets (5 × 10^8 cells/ml) were exposed to PGI2 for 10 min before the addition of 1 ml of glacial ethanol. cAMP contained in each sample was determined by enzyme immunoassay method, according to Pradelles et al. (18).

Radioactive Labeling of Platelet Proteins—Intact human platelets (5 × 10^7 cells/100 µl of Tyrode's buffer) were incubated at 37°C with [γ-32P]ATP (10 µCi, 50–70 nm) for 10 min or with [32P]Pi (0.25 µCi/ml) for 45 min. The cells were washed and used following appropriate experimental procedures.

Analysis of Platelet Protein Phosphorylation—For monodimensional separation (SDS-PAGE), phosphorylation reactions in washed labeled platelets were stopped by adding of the buffer solution (10% glycerol, 5% β-mercaptoethanol 1 mM, 3% SDS, and 0.0625 × Tris-HCl, pH 6.8). Then, samples were boiled for 5 min, and platelet proteins were separated according to Laemmli (19). The gels were dried and exposed to XAR films (Kodak) for 3–4 days.

For two-dimensional separation, gel electrophoresis isoelectric focusing/SDS-PAGE was performed as described by O'Farrell (20). Platelets were treated with a lysis buffer containing 9.5 M urea, 2% Nonidet P-40, 1% ampholines (pH 5–7) and 0.4% ampholines (pH 3–10) and 5% β-mercaptoethanol 1 mM.

Analysis of Kemptide Phosphorylation—Protein kinase activity on kemptide was assayed in Tyrode's buffer containing platelet suspension (5 × 10^6 cells), [γ-32P]ATP (7–10 µCi/sample), unlabeled ATP (5 µM), cAMP (5 µM), and paranitrophenyl phosphate (10 µM). Reactions were initiated by the addition of kemptide at appropriate concentration and were continued for 10–15 min, except for the kinetic studies, at 37°C. In order to stop the reactions, 20 µl of each sample were placed in plastic tubes containing 10 µl of glacial acetic acid. Aliquots of 20 µl of reaction mixture were deposited on Whatman P-81 phosphocellulose filter papers according to Glass et al. (21). After 30 s, the filter papers were introduced in 30% acetic acid containing 2 mM ATP for at least 60 min. The filter papers were washed 3 times for 15 min in acetic acid 15% and then introduced in either/ethanol (∼1/1) and finally in ether and dried. Phosphorylation was evaluated by the determination of the radioactivity in each aliquot (filter paper), using a β-counter (Tri-carb, Packard).

Immunoblotting—Briefly, after gel separation platelet proteins were transferred onto nitrocellulose membranes BA83 by semidry transfer (40 mM aminoacproic acid, 300 mM Tris, 0.1% SDS, and 20% methanol) at 2.5 mA/cm² for 1 h. Membranes were blocked overnight with 3% bovine serum albumin in the following buffer: Tris 50 mM, pH 7.5, NaCl 150 mM and 0.1% Tween 20. Blots were probed 2 h with anti-CD36 (diluted to 1:1000). The primary antibody was removed, and immunoreactive bands were visualized using a peroxidase mouse immunoglobulins (diluted to 1:1000) followed by ECL reaction and autoradiography.

Immunoprecipitation—Immunoprecipitation was performed according to a slight modification of the method of McGregor et al. (22). Phosphorylated platelets with [γ-32P]ATP were treated for 1 h at 4°C in the lysis buffer (20 mM HEPES, 1% Triton X-100, 0.1% SDS, 10% glycerol, 1 mM phenylmethanesulfonyl fluoride, 20 mM NaF, 1 mM Na3VO4, 1 mM paranitrophenyl phosphate). The lysate was clarified by protein A-Sepharose CL 4B. The soluble material was incubated with anti-CD36 monoclonal antibody (1:500) or mouse IgG1 (1:500, control) at 4°C for 2 h and then with rabbit polyclonal anti-mouse IgG for 1 h. Immunoprecipitate complexes were incubated with protein A for 1 h and washed 4 times with lysis buffer. Finally, samples were boiled for 10 min in Laemmli buffer (4% SDS) and then subjected to SDS-PAGE.

RESULTS

Phosphorylation of Exogenous Kemptide by Intact Cells—To test the existence of ecto-PKA activity on intact platelets, kemptide, a specific protein kinase substrate that requires cAMP for its phosphorylation (23), was added to reaction mixtures. The incubation of various concentrations of kemptide with intact washed human platelets in the presence of [γ-32P]ATP (10 µCi) and cAMP (5 µM) resulted in a Michaelian incorporation of radioactivity in the kemptide (Km, evaluated from reciprocal plot of the data of Fig. 1A is of 20 µM). As expected, no significant radioactivity incorporation was observed in the absence of cAMP (Fig. 1A). Furthermore, the radioactivity incorporation was stimulated by the lowest used concentrations of cAMP and increased in a concentration-dependent manner. The reaction reached a plateau at cAMP levels above 30 µM (Fig. 1B).

Kinetic experiments illustrated in Fig. 2, showed that extracellular kemptide was rapidly and highly phosphorylated by intact human platelets in the presence of exogenous cAMP. By...
contrast, only a moderate stimulation of phosphorylation occurred in the presence of prostacyclin, an adenylate cyclase activator that enhances intracellular cAMP levels. No significant increase in the phosphorylation of kemptide was observed when exogenous cAMP was omitted or when the specific PKA inhibitor peptide (PKI) was added (Fig. 2).

The substitution of cAMP by cGMP at the same concentration resulted only in a weak response, slightly above the control (cAMP-untreated platelets) (data not shown).

To investigate whether PKA activity was released during cell preparation, freshly washed platelet suspensions were incubated at 37 °C for 30 min and then centrifuged. Platelet pellets resuspended in their own supernatants and platelet-free supernatants were tested for their PKA activity. No significant release of PKA activity by resting washed platelets was observed. Indeed, kemptide phosphorylation intensity after 15 min was 5952 ± 6647 cpm for platelet suspensions versus 557 ± 262 cpm for platelet-free supernatants (n = 3).

Phosphorylation of Cell Surface Proteins—To examine the physiological role of this ectoprotein kinase activity it was necessary to investigate its major endogenous substrate. As shown in Fig. 3, when washed platelets were incubated in the presence of cAMP and [γ-32P]ATP for 10 min a few proteins were phosphorylated including a major protein of 88 kDa. Addition of PKI to the reaction medium selectively inhibited the phosphorylation of the protein substrate of 88 kDa. Phosphorylation of the 88-kDa protein in the presence of 0.03 μM PGI2 was low and increased significantly with 0.1 μM PGI2. This phosphorylation was completely abolished when PKI was present in the extracellular medium (Fig. 3). The cAMP amounts released into the supernatant were 84 and 167 pmol/ml when platelets were exposed to 0.03 and 0.1 μM of PGI2, respectively (n = 2). Finally, the addition of alkaline phosphatase (1 unit/ml) to the medium suppressed phosphorylation of the 88-kDa protein both in the presence of exogenous cAMP (5 μM) or of PGI2 (0.1 μM) (Fig. 3).

ATP, the cosubstrate for phosphorylation, is released by activated platelets. Indeed, when platelet suspensions were exposed to 0.3, 1, and 3 μM of U46619, the amounts of released ATP were 4.5 ± 0.4, 5.9 ± 0.2, and 6.7 ± 0.1 nmol/ml, respectively (n = 3).

Furthermore, we verified, using monodimensional analysis, that the ecto-PKA functions also in PRP. PRP was phosphorylated with [γ-32P]ATP alone (lane 1) and in the presence of cAMP (5 μM) without (lane 2) or with (lane 3) PKI (5 μg/ml). Then samples were treated according to the legend of Fig. 3.
was lacking in the intracellular phosphoprotein pattern (Fig. 5A). As shown in Fig. 5C, PKI abolished the phosphorylation of this ectoprotein.

Characterization of an 88-kDa Phosphoprotein—The protein that became strongly phosphorylated in the presence of $\gamma^{32}$P]ATP and cAMP has an apparent molecular weight similar to that of GPIV (CD36). Moreover, the ectophosphoprotein, as CD36, migrates similarly on SDS gel when either unreduced or reduced by $\beta$-mercaptoethanol (data not shown).

In order to characterize better the 88-kDa platelet ectophosphoprotein, we used an antibody against CD36. By multidimensional gel electrophoresis separation, we showed that the 88-kDa phosphoprotein (Fig. 6A, lane 1) comigrated with CD36 detected by immunoblotting (Fig. 6A, lanes 2 and 3). In addition, many entities (pH 5.7–6.8) of CD36 were also detected by bidimensional gel electrophoresis (Fig. 6B). Only 4 or 5 of these spots of isoelectric point around 6.2 were phosphorylated as shown earlier in Fig. 5B. Finally, the exact nature of the 88-kDa protein corresponding to platelet CD36 was confirmed by an immunoprecipitation experiment (Fig. 6A, lanes 4–7).

DISCUSSION

Protein phosphorylation is an important regulatory mechanism in many cells. Whereas most studies of protein phosphorylation have been centered on intracellular protein kinases (24–26), recent studies indicate the existence of ectoprotein kinase activities on the surface of various cells, including platelets (1–3, 10). The detection of platelet surface-located PKA activity might be particularly interesting in the modulation of some platelet functions.

Using kemptide, a heptapeptide currently employed in assays for cell surface-located ecto-PKA activity (23, 27), we clearly demonstrated the existence of an ecto-PKA activity on the outer surface of platelet membranes. Indeed, the addition of labeled ATP ($\gamma^{32}$P]ATP) to intact platelets in the presence of cAMP resulted in a rapid incorporation of radioactive phosphate into kemptide. When cAMP was replaced by cGMP, no significant phosphorylation of kemptide occurred, confirming the specificity of this kinase for cAMP.

When the specific inhibitory peptide for cAMP protein kinase (PKI) (15) was used, kemptide phosphorylation observed in the presence of cAMP was abolished. Since it is known that PKI does not cross the membrane barrier of intact cells (15), this result not only confirms the cAMP-dependent type of kinase reaction but also points out its outer surface location. The sensitivity to alkaline phosphatase of the $^{32}$P]kemptide also argues that this PKA activity is located on the platelet surface. A similar model was reported by Vilgrain and Baird (28), demonstrating a PKA activity located on the outer surface of human hepatoma cells, which can phosphorylate the basic fibroblast growth factor.

In order to understand the physiological role of this ectoprotein kinase activity in platelet functions, it was important to identify and characterize its endogenous major substrate. Thus, when intact platelets were used both as substrate and enzyme sources, we observed the major phosphorylation of an 88-kDa protein substrate. Its labeling was sensitive to the specificity of this kinase for cAMP.
into the extracellular medium.

Similar characteristics were observed when the phosphorylation took place in PRP, except that other membrane proteins were phosphorylated as well in a CaMP-dependent mode. Likely, these proteins either belong to contaminating cells, mainly red cells, or represent platelet peripheral species, lost during platelet washing steps.

In washed platelets, the protein intensively phosphorylated in the presence of $[^{32}P]ATP$ and CaMP had electrophoretic properties similar to those of CD36: an apparent molecular mass of approximately 88 kDa, a mean isoelectric point of 6.2, and an unchanged electrophoretic migration under reducing and nonreducing conditions. No phosphoprotein with these analytical characteristics was detected in the $[^{32}P]$inorganic phosphate-prelabeled platelet preparation (Fig. 5A). The nature of 88-kDa phosphoprotein as CaMP was confirmed by immuno blotting and immunoprecipitation experiments.

CD36 is a transmembrane glycoprotein expressed in various cells including platelets, monocytes, and endothelial cells. It is a receptor for the adhesive proteins, thrombospondin (30–31) and collagen (32). It was recently demonstrated by Asch et al. (8) that CD36 possesses an ectodomain constitutively phosphorylated by PKC at the level of a threonine residue, present in the common binding sequence. Under this phosphorylated state, i.e. in resting platelets, CD36 binds collagen but not thrombospondin. In contrast, when platelets were degranul ated, acid phosphatase was released and thought to dephosphorylate thrombospondin. In addition, when platelets were degranulated, acid phosphatase was released and thought to dephosphorylate CD36, promoting thrombospondin binding and suppressing collagen binding. Just as it is, this sequence of events seems irreversible. Indeed, after its synthesis in the megacaryocyte, CD36 must be phosphorylated by PKC inside the cell, before being translocated to the cell surface. The temporal link between synthesis and phosphorylation of CD36 was confirmed by the inability of resting platelets to phosphorylate intracellulary the ectodomain from $[^{32}P]$inorganic phosphate (Fig. 4A).

Finally, intracellular PKA activity released in the incubation medium by activated platelets (33, 34) could contribute to ectophosphorylation of CD36. Nevertheless, under our experimental conditions, using resting washed platelets, no release of PKA activity was detected.

Different questions can be raised concerning the ectophosphorylation of CD36. Are there physiological conditions where ATP and CaMP are simultaneously present outside the platelet? Experimentally, this situation may be obtained by the stimulation of platelets by the thromboxane mimetic U-46619, followed by their treatment with PG12, the first phase extruding ATP from granules and the second allowing CaMP release. These conditions mimic the interaction between endothelial cells (synthesis and liberation of PG12) and activated platelets (ATP release) in the blood vessel. It is tempting to speculate that these conditions lead to the phosphorylation of CD36 by ecto-PKA. In the lack of direct identification of the phosphorylated site, we may present two, not exclusive, hypotheses. The first one results from the search of canonical substrate sequences recognized by PKA and allows us to propose Ser$^{99}$, which is included in the motif RXXS$^9$ (35). The other, very attractive, would be that the sequence RGPTYTVRFQL, including Thr$^{99}$, the substrate of PKC, is also phosphorylated by PKA. Results by Asch et al. (8), reporting that PKA and other kinases were less efficient than PKC on phosphorylation of the peptide 87–99, do not favor this hypothesis but do not exclude it. In any event, this ectophosphorylation may restore the binding properties of CD36 described in resting platelets (8).

In conclusion, our findings provide direct evidence for CaMP-dependent platelet ecto-PKA activity that phosphorylates platelet CD36 and constitute the basis for investigating the role of surface protein phosphorylation on some platelet functions. This phosphorylation of CD36 by CaMP-dependent ectoprotein kinase introduces a new concept supporting the possibility that CaMP, which is known for its intracellular function, might act also as an extracellular messenger. Further studies are required to identify the phosphorylation site on the extracellular extension of CD36 and to determine whether this phosphorylation state by ecto-PKA may modulate extracellular events such as the interaction of platelets with adhesive proteins.

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