Dephosphorylation of cofilin in stimulated platelets: roles for a GTP-binding protein and Ca^{2+}

Monica M. L. DAVIDSON* and Richard J. HASLAM*†‡

Departments of *Pathology and †Biochemistry, McMaster University, Hamilton, Ontario, Canada L8N 3Z5

In human platelets, thrombin not only stimulates the phosphorylation of pleckstrin (P47) and of myosin P-light chains, but also induces the dephosphorylation of an 18–19 kDa phosphoprotein (P18) [Imaoka, Lynham and Haslam (1983) *J. Biol. Chem.* **258**, 11404–11414]. We have now studied this protein in detail. The thrombin-induced dephosphorylation reaction did not begin until the phosphorylation of myosin P-light chains and the secretion of dense-granule 5-hydroxytryptamine were nearly complete, but did parallel the later stages of platelet aggregation. Experiments with ionophore A23187 and phorbol 12-myristate 13-acetate indicated that dephosphorylation of P18 was stimulated by Ca^{2+} , but not by protein kinase C. Two-dimensional analysis of platelet proteins, using non-equilibrium pH gradient electrophoresis followed by SDS/PAGE, showed that thrombin decreased the amount of phosphorylated P18 in platelets by up to 70% and slightly increased the amount of a more basic unlabelled protein that was present in 3-fold excess of P18 in

unstimulated platelets. These two proteins were identified as the phosphorylated and non-phosphorylated forms of the pH-sensitive actin-depolymerizing protein, cofilin, by sequencing of peptide fragments and immunoblotting with a monoclonal antibody specific for cofilin. The molar concentration of cofilin in platelets was approx. 10% that of actin. Platelet cofilin was phosphorylated exclusively on serine. Experiments with electroporated platelets showed that dephosphorylation of cofilin could be stimulated by guanosine 5'-[γ -thio]triphosphate (GTP[S]) in the absence of Ca^{2+} or by a free Ca^{2+} concentration of 10 μM . This GTP[S]-induced dephosphorylation reaction was inhibited by 1-naphthyl phosphate, but not by okadaic acid. Our results add cofilin to the actin-binding proteins that may regulate the platelet cytoskeleton, and suggest that platelet cofilin can be activated by dephosphorylation reactions initiated either by a GTP-binding protein or Ca^{2+} .

INTRODUCTION

Protein phosphorylation plays a major role in signal transduction following the activation of blood platelets by physiological stimuli [1–16]. Early studies with platelets that had been pre-incubated with [^{32}P]P_i demonstrated that aggregating agents induce major increases in the ^{32}P -labelling of proteins that migrate on SDS/PAGE at 40–47 kDa and 20 kDa [2–4]. The 40–47 kDa protein is the major platelet substrate of protein kinase C [5] and many studies have shown that its phosphorylation is associated with secretion of platelet granule constituents (e.g. [3,6,7]). Although this protein, now named pleckstrin, has been purified [8] and cloned [9], its precise function is still unclear. The 20 kDa group of phosphorylated proteins contains the P-light chains of platelet myosin [10], phosphorylation of which may play a key role in the contractile events associated with platelet shape change [11] and possibly secretion [10,12]. Three isoforms of the P-light chain exist in platelets and can often be resolved on SDS/PAGE as a 20 kDa doublet and a minor 19 kDa species [1,13,14]. These proteins are phosphorylated by both the Ca^{2+} /calmodulin-dependent myosin light-chain kinase [15] and protein kinase C [14,16]. In addition to these major increases in protein phosphorylation associated with platelet activation (and minor increases in the phosphorylation of multiple unidentified proteins [1,8]), one conspicuous phosphoprotein of 18–19 kDa (P18) present in resting platelets has been observed to undergo dephosphorylation after stimulation of platelets by collagen [1,4] and particularly by thrombin [8]. To our knowledge, this protein has not been studied since these initial reports were published. We now show that P18 is

cofilin, a pH-sensitive actin-depolymerizing protein that binds to both G- and F-actin [17,18], and that about 25% of platelet cofilin is phosphorylated on serine in resting platelets. Experiments with permeabilized platelets showed that dephosphorylation of this protein could be initiated by independent guanine-nucleotide- and Ca^{2+} -dependent mechanisms. A preliminary report describing some of these findings has been published [19].

EXPERIMENTAL

Materials

Carrier-free [^{32}P]P_i, [γ - ^{32}P]ATP (3000 Ci/mmol) and 5-hydroxy-[side chain-1,2- ^3H (n)]tryptamine ([^3H]5-HT) (28 Ci/mmol) were obtained from Du Pont Canada Inc. (Mississauga, ON, Canada), and 5-hydroxy[side chain-2- ^{14}C]tryptamine ([^{14}C]5-HT) was from Amersham Canada Ltd. (Oakville, ON, Canada). Ionophore A23187 was supplied by Calbiochem (San Diego, CA, U.S.A.) and okadaic acid by Upstate Biotechnology Inc. (Lake Placid, NY, U.S.A.). Guanosine 5'-[γ -thio]triphosphate (GTP[S]) and *Staphylococcus aureus* V8 endoproteinase Glu-C were from Boehringer Mannheim Canada (Laval, PQ, Canada). Phorbol 12-myristate 13-acetate (PMA), 1-naphthyl phosphate, 3-(cyclohexylamino)-1-propanesulphonic acid (CAPS) and protein molecular-mass markers for SDS/PAGE were obtained from Sigma (St. Louis, MO, U.S.A.). Ampholytes (Bio-Lyte 5/8 and Bio-Lyte 3/10) were obtained from Bio-Rad Laboratories Ltd. (Mississauga, ON, Canada), and poly(vinylidene fluoride) membranes (Immobilon-P and Immobilon-P⁸⁰) from Millipore (Canada) Ltd. (Mississauga, ON, Canada). Human α -thrombin was a gift from Dr. J. W. Fenton II of New York State

Abbreviations used: P18, 18–19 kDa phosphoprotein; 5-HT, 5-hydroxytryptamine; GTP[S], guanosine 5'-[γ -thio]triphosphate; PMA, phorbol 12-myristate 13-acetate; CAPS, 3-(cyclohexylamino)-1-propanesulphonic acid; NEPHGE, non-equilibrium pH-gradient electrophoresis.

‡ To whom correspondence should be addressed.

Department of Health, Albany, NY, U.S.A., and a monoclonal anti-cofilin antibody (MAB-22) was generously provided by Dr. T. Obinata of the Department of Biology, Chiba University, Chiba, Japan. Other materials were from sources listed previously [8,13].

Isolation and labelling of platelets for studies on protein phosphorylation in relation to aggregation and secretion

Human platelets were isolated, washed and labelled with [32 P]P_i and [3 H]5-HT, essentially as described previously [3]. However, in most experiments, Ca²⁺- and phosphate-free Tyrode's solution containing 5 mM Pipes buffer, pH 6.5, 50 units of heparin/ml, 60 μ g of apyrase/ml and 0.35 % BSA was used in the first wash. These platelets were resuspended at 3×10^9 platelets/ml, and then incubated for 1 h at 37 °C with 0.2–0.3 mCi of [32 P]P_i/ml and 28 μ Ci of [3 H]5-HT/ml (1 μ M). After a further wash, the platelets were finally resuspended in Tyrode's solution containing 6 μ g of apyrase/ml. For measurement of platelet aggregation and secretion, platelet suspension (3×10^8 platelets) was stirred with additions (final vol. 1 ml) in an aggregometer (Payton Associates, Scarborough, ON, Canada). Samples for measurement of [3 H]5-HT secretion were mixed with 0.2 vol. of solution containing 9 % (w/v) paraformaldehyde, 0.03 M EDTA and 0.15 M NaCl and, after centrifugation, the supernatants were counted for 3 H radioactivity [3]. Alternatively, for measurements of protein phosphorylation, platelet suspension was mixed with 0.2 vol. of 30 % (w/v) trichloroacetic acid and the precipitated protein was dissolved in electrophoresis sample buffer and analysed by SDS/PAGE, in 13 %-acrylamide gels [8]. Proteins were stained with Coomassie Brilliant Blue R [4]; in some experiments, the relative amounts of different proteins were determined by elution of the dye from the dried gels [20]. The 32 P-labelled proteins were detected by autoradiography [8] and, in some experiments, counted for 32 P radioactivity [4].

Two-dimensional electrophoresis of platelet proteins

Acid-precipitated protein was dissolved by sonication in a solution containing 9.5 M urea, 2 % (w/v) Nonidet P-40 and 2 % (v/v) mercaptoethanol and mixed with ampholytes (final concns. 1.5 % Bio-Lyte 5/8 and 0.5 % Bio-Lyte 3/10). Samples containing 0.5–1 mg of protein were subjected to non-equilibrium pH-gradient electrophoresis (NEpHGE) in 3 mm $\times$ 130 mm tube gels run at 1000 V for 1.6–2 h, as described in [21]. SDS/PAGE in the second dimension [8,21] was carried out in 13 %-acrylamide gels. Proteins were stained by Coomassie Brilliant Blue R and quantified as described above.

Peptide mapping and sequencing

Proteins from 32 P-labelled platelets were resolved by two-dimensional electrophoresis, located by lightly staining with Coomassie Brilliant Blue R, excised and subjected to proteolysis by 0.2 μ g of endoproteinase Glu-C at the interface of stacking and separating polyacrylamide gels, as described by Cleveland et al. [22]. The fragments were resolved by SDS/PAGE, using a separating gel containing 16 % acrylamide, and either stained with silver [23] and subjected to autoradiography, or electroblotted on to Immobilon-P[®] by using 10 mM CAPS buffer, pH 11, containing 20 % (v/v) methanol [24]. Peptides on Immobilon-P[®] were stained with Coomassie Brilliant Blue R and sequenced with an Applied Biosystems pulsed liquid sequencer model 473A equipped with micro-gradient phenylthiohydantoin analysis.

Immunoblotting and phosphoamino acid analysis

Proteins from 32 P-labelled platelets were resolved by two-dimensional electrophoresis as above, and electroblotted [25] on to Immobilon-P using buffer (pH 8.3) containing 25 mM Tris, 192 mM glycine, 20 % methanol and 0.05 % SDS. Proteins of potential interest were located by staining with Ponceau S [24]. Blots were blocked with 1.5 % gelatin and probed with monoclonal antibody to cofilin (MAB-22) [26]; immunoreactive proteins were detected by using goat anti-(mouse IgG)-alkaline phosphatase conjugate. 32 P-labelled phosphoamino acids present in proteins bound to Immobilon-P were identified as described by Kamps and Sefton [27].

Studies with electroporomeabilized platelets

Platelets that had been labelled with [14 C]5-HT were electroporomeabilized and isolated by gel filtration in a medium (pH 7.4) containing 12.5 mM MgCl₂ and the potassium salts of glutamic acid (160 mM), Hepes (20 mM), EGTA (2.5 mM) and EDTA (2.5 mM) (buffer A) [13]. After dilution to $(3\text{--}5) \times 10^8$ platelets/ml and addition of 5 mM ATP (final concn.), this suspension was stored at 0 °C for up to 2 h without loss of the capacity to secrete [14 C]5-HT in response to Ca²⁺ or GTP[S] [13,28,29]. Secretion was determined in mixtures comprising 80 μ l of suspension of permeabilized platelets with 10 μ l of CaCl₂ and KOH in buffer A (to give defined pCa values at pH 7.4 [13]) and a total of 10 μ l of other additions in buffer A. Some incubation mixtures contained an additional 10 mM EGTA in place of CaCl₂ to give an essentially Ca²⁺-free condition (pCa > 9) [29]. These mixtures were equilibrated for 15 min at 0 °C and then incubated at 25 °C, usually for 10 min, before measurement of the [14 C]5-HT released into the medium [13]. Protein phosphorylation was determined in identical (100 μ l) incubation mixtures, but permeabilized platelet suspension containing 16 μ Ci of [γ - 32 P]ATP/sample [13] was used. Incubations were terminated by addition of 0.5 ml of 10 % (w/v) trichloroacetic acid, and the precipitated protein was analysed by SDS/PAGE in 13 %-acrylamide gels, followed by autoradiography of the dried gels.

RESULTS

Relationships between dephosphorylation of P18, secretion of dense-granule 5-HT and platelet aggregation

When protein from platelets labelled with [32 P]P_i was analysed by SDS/PAGE in 13 %-acrylamide gels, P18 was resolved as a well-defined 32 P-labelled species that co-migrated with a distinct stained protein band [1]. By dye elution, we have now found that the mass amount of this protein present in platelets is equivalent to 6 ± 1 % of platelet actin (mean $\pm$ S.E.M., $n = 5$). In experiments in which 32 P-labelled platelets were stirred with a high concentration of thrombin (1 unit/ml), a small initial increase in the labelling of P18 (at 5 s) was followed by a loss of 32 P that was detected after 15 s and reached a maximum after 1 min (e.g. Figure 1). As determined by counting the 32 P radioactivity in gel slices containing P18 (which could include small amounts of other labelled proteins of similar molecular mass), this maximum decrease in labelling averaged 42 ± 4 % (mean $\pm$ S.E.M., $n = 4$). The time course of P18 dephosphorylation differed markedly from that observed for the phosphorylation of the P-light chains of platelet myosin and the secretion of 5-HT, which were well under way only 5 s after addition of thrombin. However, the loss of 32 P from P18 closely paralleled the later stages of platelet aggregation, measured as the decrease in attenuation of the platelet suspension (Figure 1). The effects of a range of thrombin concentrations were studied in 30 s incubations. Loss of 32 P from

Figure 1 Relationships between thrombin-induced secretion of platelet 5-HT, platelet aggregation and the phosphorylation of platelet proteins

Samples of washed platelets that had been labelled with both [32 P]P_i and [3 H]5-HT were added to aggregometer tubes containing thrombin (1 unit/ml; final vol. 1 ml) and incubated at 37 °C with stirring for periods from 0 to 2 min, during which platelet aggregation was recorded. Some incubations were terminated by addition of trichloroacetic acid, and the precipitated protein was analysed by SDS/PAGE. The 22–16 kDa section of an autoradiograph is shown; lane 1, unincubated control; lane 2, 5 s; lane 3, 15 s; lane 4, 30 s; lane 5, 1 min; lane 6, 2 min. Other incubations were terminated by addition of formaldehyde/EDTA for measurement of the secretion of [3 H]5-HT. Values for platelet aggregation ($\Delta D \times 100$) and for the percentage secretion of [3 H]5-HT are shown below each lane of the autoradiograph. Abbreviations: Myosin P-LCs, P-light chains of platelet myosin; P18, 18 kDa phosphoprotein.

P18 was detected with 0.1 unit of thrombin/ml and was optimal with 1 unit/ml, and at this time interval correlated with both platelet aggregation and the secretion of 5-HT (results not shown). Addition of neither 5 mM EGTA nor 5 mM EDTA affected the dephosphorylation of P18 measured 1 min after addition of thrombin at 1 unit/ml. Since EGTA decreased and EDTA abolished platelet aggregation (without affecting secretion of 5-HT), it follows that the dephosphorylation reaction is not an effect of platelet aggregation.

Since signal transduction by thrombin involves synergistic effects mediated by the mobilization of Ca²⁺ and activation of protein kinase C [6,7], we examined the actions of ionophore A23187 and PMA, added alone or in combination, on the dephosphorylation of P18 (Figure 2). These experiments were carried out in the presence of indomethacin and an ADP-scavenging enzyme system (creatine kinase and phosphocreatine) to prevent platelet activation by secondary mediators. Under these conditions, 0.2 μ M A23187 caused a marked loss of 32 P from P18 by 1 min, at which time only small increases in the phosphorylation of the myosin P-light chains and pleckstrin were detected. These changes in protein phosphorylation were associated with platelet aggregation, but not with secretion of 5-HT (Figure 2, lane 2). In contrast, 100 nM PMA did not affect the 32 P labelling of P18, but caused a marked increase in the phosphorylation of pleckstrin and a smaller increase in that of the myosin P-light chains (Figure 2, lane 3). PMA alone did not induce significant platelet aggregation or secretion by 1 min, but in longer incubations (4 min, and especially 10 min) platelet aggregation was observed without dephosphorylation of P18. When added together, ionophore A23187 and PMA exerted potent synergistic effects on platelet aggregation and secretion, as initially reported by Yamanishi et al. [30]. This was associated with enhanced myosin phosphorylation, presumably reflecting the combined effects of myosin light-chain kinase [15] and protein kinase C [16], whereas the observed changes in the phosphorylation of P18 and pleckstrin reflected the individual effects of A23187 and PMA respectively (Figure 2, lane 4). These results

Figure 2 Effects of ionophore A23187 and PMA on the phosphorylation of platelet proteins; relationship to secretion of 5-HT and aggregation

Samples of washed platelets that had been labelled with [32 P]P_i and [3 H]5-HT were preincubated for 5 min at 37 °C with phosphocreatine (2 mM), creatine kinase (100 units/ml) and indomethacin (10 μ M) and then stirred in an aggregometer for 1 min with the following additions (final vol. 1 ml); lane 1, none; lane 2, 0.2 μ M A23187; lane 3, 0.1 μ M PMA; lane 4, 0.2 μ M A23187 and 0.1 μ M PMA. Samples (0.05 ml) of each incubation mixture were then removed for measurement of the secretion of [3 H]5-HT. Protein was precipitated from the remainder of each sample with trichloroacetic acid and analysed by SDS/PAGE. An autoradiograph is shown, with the values for platelet aggregation and secretion of [3 H]5-HT below each lane. Abbreviations as in Figure 1.

show that loss of 32 P from P18 can be mediated by Ca²⁺, but not by protein kinase C.

To establish that the loss of 32 P reflects a decrease in the phosphorylation rather than just in the labelling of P18, platelet proteins were analysed by two-dimensional electrophoresis. Preliminary experiments established that P18 could only be resolved by NEpHGE/SDS/PAGE [20], indicating that it is a much more basic protein than the myosin P-light chains [10,14]. By this technique, three stained proteins with molecular masses close to that of P18 were detected (Figure 3, proteins 1, 2 and 3). Two of these (proteins 1 and 3) were unlabelled, whereas one (protein 2, P18) was a 32 P-labelled species that showed marked decreases in both protein and radioactivity after treatment of platelets with thrombin (Figure 3). In these experiments, in which minor phosphorylated proteins could be excluded, the decrease in the 32 P present in P18 amounted to $70 \pm 2\%$ after incubation of labelled platelets for 3 min with 2 units of thrombin/ml (mean $\pm$ S.E.M., $n = 3$). This loss of P18 was not accompanied by the appearance of a new more basic or a lower-molecular-

Figure 3 Analysis of P18 dephosphorylation by electrophoresis in two dimensions

Samples of washed platelets that had been labelled with [^{32}P] were stirred for 3 min at 37 °C in an aggregometer in the absence or presence of thrombin (2 units/ml). Incubations were terminated by addition of trichloroacetic acid, and the precipitated protein was analysed by NEpHGE (horizontal dimension) and SDS/PAGE (vertical dimension) and stained with Coomassie Brilliant Blue R. The regions of the gels containing proteins 1, 2 and 3, which migrate with the same apparent molecular mass as P18, are shown for platelets incubated (a) without thrombin and (b) with thrombin; the corresponding autoradiographs, (c) and (d), are below. The position of a 20 kDa protein marker added during SDS/PAGE is shown on the left (arrowhead).

mass protein species. However, small increases were usually detected in the staining of protein 1, which was more basic than protein 2 and present in much larger amounts (Figure 3). These results suggested that the latter protein is either the dephosphorylated form of P18 (protein 2) or co-migrates with dephosphorylated P18. Elution of the dye from proteins 1 and 2 on three gels from each of two experiments indicated that, in each case, these proteins were present in a ratio of 3:1 under resting conditions. Analyses of protein 2 (P18) for ^{32}P -labelled phosphoamino acids [27] showed that all the label was in phosphoserine.

Identification of P18

Proteins 1 and 2 (see Figure 3) were compared by peptide mapping in one dimension (SDS/PAGE) after partial proteolysis with endoproteinase Glu-C, as described by Cleveland et al. [22]. Both proteins reproducibly yielded four major peptides (Figure 4a). For the non-phosphorylated species (protein 1), the apparent molecular masses of these peptides were 12.8 kDa (A), 10.7 kDa (B), 9.2 kDa (C) and 7.2 kDa (D). Protein 2 (P18), on the other hand, yielded two peptides similar in size to A and C (A' and C'), and two that migrated more slowly than B and D and contained ^{32}P (B' and D') (Figure 4b). This result supports the idea that protein 1 is the dephosphorylated form of protein 2 (P18). Peptides A, B, C and D from protein 1 were electroblotted on to a poly(vinylidene fluoride) membrane and sequenced. Although both peptides B and D proved to be blocked at their N-termini,

Figure 4 Peptide mapping of proteins related to P18

Protein 1 (putative non-phosphorylated P18) and ^{32}P -labelled protein 2 (P18) were separated by two-dimensional electrophoresis as in Figure 3, proteolysed by endoproteinase Glu-C [22] and analysed by SDS/PAGE in 16%-acrylamide gels. (a) A silver-stained gel showing major peptides A, B, C and D derived from protein 1 (lane 1) and peptides A', B', C' and D' derived from P18 (lane 2); (b), an autoradiograph of (a). The positions of protein molecular-mass markers are shown on the right; in descending order these are: 17.0, 14.4, 10.6, 8.2, 6.2 and 3.5 kDa.

Figure 5 Reaction of phosphorylated and non-phosphorylated P18 with anti-cofilin antibody

Samples of protein from ^{32}P -labelled control platelets were analysed by two-dimensional electrophoresis as in Figure 3 and transferred to Immobilon-P by electroblotting. (a) Blot stained with Coomassie Brilliant Blue R; (b) blot of a corresponding area probed with a monoclonal antibody to cofilin (MAB-22); (c) and (d), autoradiographs of (a) and (b) respectively. The positions of proteins 1, 2 and 3 are indicated.

reliable sequence was obtained from peptide A (GKEILVGD-VGQTVDDPYATF) and peptide C (TKESKKEDLVFI). A computer search [31] of NCBI databases using the BLAST network service showed that these sequences are found only in pig [32] and human [33] cofilin, in which they follow glutamic

Figure 6 Dephosphorylation of P18 (cofilin) in electroporabilized platelets

Samples of permeabilized platelets containing [^{14}C]5-HT were equilibrated with additions for 15 min at 0 °C and then incubated for 10 min at 25 °C (see the Experimental section). Secretion of [^{14}C]5-HT and protein phosphorylation by [$\gamma\text{-}^{32}\text{P}$]ATP were determined in separate otherwise identical incubations. Autoradiographs of the 22–16 kDa parts of SDS/PAGE gels from three separate experiments are shown, with the corresponding secretion of [^{14}C]5-HT below each lane. The latter are mean values from three determinations. Abbreviation: Myosin P-LCs, P-light chains of platelet myosin. (a) Incubations were carried out under the following conditions: lane 1, pCa 7; lane 2, pCa 7 with 100 μM GTP[S]; lane 3, pCa 6; lane 4, pCa 6 with 100 μM GTP[S]; lane 5, pCa 5; lane 6, pCa 5 with 100 μM GTP[S]. (b) All incubations were at a pCa > 9. Other additions were: lane 1, none; lane 2, 1 μM okadaic acid; lane 3, 10 mM 1-naphthyl phosphate; lane 4, 100 μM GTP[S]; lane 5, 100 μM GTP[S] and 1 μM okadaic acid; lane 6, 100 μM GTP[S] and 10 mM 1-naphthyl phosphate. (c) In this experiment, permeabilized platelet suspension was preincubated for 10 min at 25 °C with [$\gamma\text{-}^{32}\text{P}$]ATP (or unlabelled ATP) before equilibration of samples with additions at 0 °C and their further incubation at 25 °C. Lane 1, protein from permeabilized platelets that had been preincubated with [$\gamma\text{-}^{32}\text{P}$]ATP and then returned to 0 °C. Lanes 2–5, protein from the same permeabilized and preincubated platelets after their equilibration and incubation under the following conditions: lane 2, pCa > 9; lane 3, pCa > 9 with 100 μM GTP[S]; lane 4, pCa 7; lane 5, pCa 7 with 100 μM GTP[S].

acid residues. The only closely related mammalian protein for which the sequence is available, pig destrin [34], differs from cofilin in ten of the 32 residues sequenced (at the positions shown in bold) and can therefore be excluded. The sizes of the above four peptides and the N-terminal sequences of peptides A and D lead us to conclude that endoproteinase Glu-C cleaved each cofilin molecule at only one of two possible sites, so generating two pairs of peptides, either B (residues 1–90) and C (residues 91–166), or, in somewhat larger amounts, D (residues 1–51) and A (residues 52–166). Although less protein 2 (P18) was available, useful sequence (XKXEDLVFI) was obtained from peptide C', indicating that this too is derived from cofilin and not destrin, though, for unknown reasons, cleavage appeared to occur at a slightly different site, giving a peptide consisting of residues 93–166. Since only the N-terminal peptides B' and D' were ^{32}P -labelled, we conclude that one or more of the five serine residues

in the first 51 residues of cofilin is phosphorylated in protein 2 (P18).

To confirm our identification of proteins 1 and 2 as the non-phosphorylated and phosphorylated forms of cofilin, we used a monoclonal antibody to this protein (MAB-22) that does not react with destrin [26]. As shown by Western blotting (Figure 5), both proteins 1 and 2 were immunoreactive, whereas protein 3 was not. In some blots of NEpHGE/SDS/PAGE gels, a minor immunoreactive species that was not labelled with ^{32}P was detected between proteins 1 and 2; this could be a second isoform of cofilin.

Factors affecting the dephosphorylation of P18 (cofilin) in permeabilized platelets

Previous work has shown that guanine nucleotides decrease the free Ca^{2+} concentration required to initiate secretion of granule constituents from electroporabilized platelets [28], and that 100 μM GTP[S] can induce some secretion in the effective absence of Ca^{2+} (pCa > 9) [29]. Ca^{2+} and GTP[S] also caused enhanced phosphorylation of the P-light chains of myosin and of pleckstrin in this system [13,29], as observed with thrombin in intact platelets. In the present study, we utilized permeabilized platelets to study the possible roles of guanine-nucleotide-dependent and Ca^{2+} -dependent mechanisms in the dephosphorylation of P18. Little or no labelling of any platelet protein with ^{32}P occurred during equilibration of permeabilized platelets with [$\gamma\text{-}^{32}\text{P}$]ATP at 0 °C, but labelling of P18 was observed during incubation of the platelets at 25 °C, either in the presence of low free Ca^{2+} concentrations (pCa 7 and 6 in Figure 6a, lanes 1 and 3) or in the absence of Ca^{2+} (pCa > 9 in Figures 6b and 6c). In contrast, no labelling of P18 was observed during incubation of the platelets at pCa 5 (Figure 6a, lane 5). These results differ from those obtained for the myosin P-light chains, labelling of which was strikingly enhanced by Ca^{2+} (Figure 6a). The labelling of the latter, which was marked at pCa 6, was more sensitive to Ca^{2+} than was the loss of label from P18, which required a pCa of 5.

Incubation of permeabilized platelets with GTP[S] not only increased ^{32}P incorporation into pleckstrin and the P-light chains of myosin, but also markedly inhibited the labelling of P18, even in the effective absence of Ca^{2+} (Figures 6b and 6c). To help to establish that GTP[S] caused dephosphorylation of P18, rather than an inhibition of its phosphorylation, P18 was labelled with ^{32}P by preincubation of permeabilized platelets with [$\gamma\text{-}^{32}\text{P}$]ATP at 25 °C before addition of GTP[S]. This led to a decrease in the extent of 5-HT secretion, probably as a result of resealing of the permeabilized platelets, but, as shown in Figure 6(c), GTP[S] was still able to decrease the ^{32}P labelling of P18 well below control values. In all the experiments with permeabilized platelets, the secretion of 5-HT in response to GTP[S], Ca^{2+} or both, correlated with the phosphorylation of the myosin P-light chains and with the loss of ^{32}P from P18.

Addition of okadaic acid, an inhibitor of protein phosphatases 1 and 2A [35], had no effect on the labelling of P18 in control incubations at pCa > 9 and failed to prevent the loss of ^{32}P from this protein caused by either GTP[S] at pCa > 9 (Figure 6b) or by Ca^{2+} at pCa 5 (results not shown). Okadaic acid did, however, enhance both the phosphorylation of the P-light chains of myosin and the secretion of 5-HT caused by GTP[S] at pCa > 9. In contrast, a high (10 mM) concentration of a non-specific phosphatase inhibitor, 1-naphthyl phosphate, which alone had no consistent effect on the labelling of P18 in permeabilized platelets incubated at pCa > 9, partly prevented the decrease in the labelling of P18 caused by GTP[S] (Figure 6b). This was observed in each of three separate experiments, in which GTP[S] decreased

^{32}P incorporation into P18 by an average of $44 \pm 6\%$ in the absence of 1-naphthyl phosphate and by $19 \pm 10\%$ in the presence of this phosphatase inhibitor (means $\pm$ S.E.M.). Although 1-naphthyl phosphate enhanced the phosphorylation of the P-light chains of myosin, it differed from okadaic acid in that it inhibited the GTP[S]-induced secretion of 5-HT (Figure 6b). 1-Naphthyl phosphate did not prevent the loss of ^{32}P from P18 observed when permeabilized platelets were incubated at pCa 5 (results not shown). To exclude the possibility that this Ca^{2+} -dependent decrease in the labelling of P18 might result from the proteolytic action of calpain, we studied the effect of 0.2 mM leupeptin. Neither the labelling of P18 in the absence of Ca^{2+} nor the loss of ^{32}P from P18 in platelets incubated at pCa 5 were influenced by this proteolytic inhibitor.

DISCUSSION

This investigation derives from early observations in this laboratory which showed that stimulation of platelets by aggregating agents, such as collagen and thrombin, led not only to major increases in the phosphorylation of some proteins, but also to dephosphorylation of an 18–19 kDa species that we termed P18 [1,4,8]. We have now shown that P18 is a phosphorylated form of the cytoskeletal protein cofilin, and that resting platelets contain both phosphorylated and dephosphorylated cofilin in a ratio of 1:3. The presence of these two forms of cofilin in platelets was established both by sequencing peptide fragments from these proteins after their isolation by two-dimensional electrophoresis and by immunoblotting with a specific monoclonal antibody to cofilin. Both methods clearly distinguished platelet cofilin from destrin, a closely related actin-depolymerizing factor that is present in many of the same mammalian tissues but migrates on SDS/PAGE with a slightly lower apparent molecular mass [34]. To our knowledge, cofilin has not previously been reported in platelets.

Cofilin is a pH-sensitive actin-depolymerizing protein that can bind to both G- and F-actin [17,18]. Although initial studies estimated the molecular mass of pig brain cofilin as 21 kDa [17], molecular cloning gives a value of 18.5 kDa for the unprocessed protein [32]. Human cofilin differs from the pig protein only in the replacement of Cys-108 by Ser [33]. Three possible mechanisms have been suggested by which the F-actin-depolymerizing activity of cofilin might be regulated. The known pH-sensitivity of cofilin [18] implies that an increase in cellular pH above 7.3 could enhance actin depolymerization. However, the available evidence indicates that the intracellular pH does not increase above this level in stimulated platelets [36]. Second, the actin-depolymerizing activity of cofilin is inhibited by polyphosphoinositides and phosphatidylinositol, which bind the protein and prevent its interaction with F-actin [37]. In this case, the lack of specificity of the interactions of cofilin with these phosphoinositides makes it unlikely that a change in their relative concentrations could regulate the action of cofilin. Third, there is increasing evidence that the phosphorylation status of cofilin can affect its biological activity. Thus, Ohta et al. [38] have reported that about 50% of the cofilin in 3Y1 fibroblasts was phosphorylated on a serine residue and that exposure of these cells to heat shock or dimethyl sulphoxide led to dephosphorylation of the protein and its accumulation in the cell nucleus as actin/cofilin rods. In preliminary experiments, these workers have shown that, *in vitro*, cofilin can be phosphorylated by Ca^{2+} /calmodulin-dependent protein kinase II and suggest that Ser-24 is the most likely phosphorylation site. This possibility is consistent with our conclusion that platelet cofilin is phosphorylated on serine in the N-terminal region of the protein. Recent studies on chick actin-

depolymerizing factor, which is the avian homologue of destrin, have demonstrated the presence of the phosphorylated protein in myocytes and have shown that this form is unable to depolymerize actin [39]. If, as seems likely [39], this finding can be extrapolated to mammalian cofilin, dephosphorylation of the platelet protein could generate additional cofilin with actin-depolymerizing activity. This possibility is strongly supported by our preliminary experiments, in which gel filtration of platelet proteins on AcA 54 partly separated ^{32}P -labelled cofilin from the dephosphorylated protein, which behaved as though it was bound to a larger protein, probably G-actin (M. M. L. Davidson and R. J. Haslam, unpublished work).

The relatively slow time course of cofilin dephosphorylation in intact platelets indicates that this process is not required for dense-granule secretion. Presumably, then, the quantitative correlation between secretion and dephosphorylation of cofilin in permeabilized platelets is coincidental. However, the time course of cofilin dephosphorylation did parallel the later stages of platelet aggregation and, as shown by our experiments with EDTA, is not an effect of aggregation itself. A role for the dephosphorylation of cofilin in platelet aggregation is thus conceivable, but is not easily reconciled with the ability of PMA to induce substantial aggregation without measurable dephosphorylation of the protein. In any case, it remains unclear whether newly formed dephosphorylated cofilin could have an important role in the cytoskeletal remodelling that occurs during platelet aggregation, since the net change is an increase in the F-actin content of the platelets [40]. Calculation suggests that the amount of dephosphorylated cofilin that becomes available during platelet aggregation is relatively small. As measured by the elution of dye from proteins separated by SDS/PAGE, cofilin was equivalent in mass to about 6% of the platelet actin. Allowing for contaminating protein and the difference between the molecular masses of cofilin and actin, it follows that the molar concentration of cofilin in platelets is about 10% of that of actin. Since only 25% of this was phosphorylated in resting platelets and dephosphorylation by thrombin did not exceed 70%, the newly available dephosphorylated cofilin is probably equivalent to no more than 1.5% of the total platelet actin. For this to have a significant effect, dephosphorylation of cofilin would probably need to coincide with the release of other actin-binding proteins from F-actin. In this context, it is significant that cofilin has been shown to inhibit the interactions between F-actin and tropomyosin, myosin or caldesmon [17,41].

The enzymes involved in the phosphorylation and dephosphorylation of platelet cofilin have not yet been characterized. Although it has been suggested that Ca^{2+} /calmodulin-dependent protein kinase II may phosphorylate cofilin [38], chick actin-depolymerizing factor is a poor substrate for this enzyme [39]. Moreover, our results indicate that the phosphorylation of cofilin was not Ca^{2+} -dependent in permeabilized platelets. Activation of protein kinase C by PMA did not increase the ^{32}P labelling of cofilin (P18) or prevent dephosphorylation of this protein in intact platelets. Finally, increases in platelet cyclic AMP or cyclic GMP did not enhance the ^{32}P labelling of P18 [1]. Our results with permeabilized platelets show that dephosphorylation of cofilin can proceed by independent GTP[S]-stimulated and Ca^{2+} -dependent mechanisms. If, as seems probable, this action of GTP[S] is mediated by a GTP-binding protein that can be activated directly or indirectly by membrane receptors, thrombin may stimulate the dephosphorylation of cofilin through both of the mechanisms that we have identified. At least three protein phosphatases have been identified in platelets, namely the Ca^{2+} /calmodulin-dependent protein phosphatase 2B [42] and protein phosphatases 1 and 2A [43,44], both of which are inhibited by

okadaic acid [35]. An additional okadaic acid-sensitive enzyme has also been described in platelets [44]. The Ca^{2+} -dependent dephosphorylation of cofilin observed in both intact and permeabilized platelets in this study is consistent with a role for protein phosphatase 2B. However, the failure of okadaic acid to influence the GTP[S]-induced dephosphorylation reaction in permeabilized platelets, despite partial inhibition by the non-specific inhibitor 1-naphthyl phosphate, excludes protein phosphatases 1 and 2A from consideration. This suggests that GTP[S] must act either through the Mg^{2+} -activated protein phosphatase 2C, of which there is little in platelets [45], or on an as yet unidentified platelet protein phosphatase. There is a precedent for G-protein-mediated regulation of protein phosphatase activity, in that GTP[S] has been reported to inhibit myosin light-chain phosphatase in vascular smooth muscle [46]. In platelets, activation of a protein phosphatase by a GTP-binding protein could be direct or indirect. Thus, studies in this laboratory have shown that, in permeabilized platelets incubated in the absence of Ca^{2+} , GTP[S] stimulates the formation of phosphatidic acid by phospholipase D, and have suggested that this phosphatidic acid may act as a second messenger that potentiates secretion [47]. Although GTP[S] slightly enhanced the phosphorylation of the P-light chains of myosin in permeabilized platelets incubated in the absence of Ca^{2+} , this could reflect the activation of protein kinase C, possibly by phosphatidic acid [29,47], rather than an inhibition of a protein phosphatase [46]. 1-Naphthyl phosphate and okadaic acid both enhanced the control and GTP[S]-induced phosphorylation of myosin P-light chains in permeabilized platelets, but had opposite effects on the secretion of 5-HT. 1-Naphthyl phosphate inhibited the secretion of 5-HT from permeabilized platelets, although this compound has been reported to enhance secretion of noradrenaline from PC12 cells [48]. Okadaic acid, on the other hand, potentiated the GTP[S]-induced secretion of 5-HT from permeabilized platelets, although it inhibits aggregation and secretion in intact platelets [45,49], probably because of inhibitory effects on Ca^{2+} mobilization [49] and phospholipase C activation [45], which are not required for GTP[S]-induced secretion from permeabilized platelets [29,47].

Note added in proof (received 9 May 1994)

The presence of cofilin in platelets has also recently been demonstrated by Professor T. Obinata and colleagues, of Chiba University, Chiba, Japan (personal communication).

This work was supported by a grant (MT-5626) from the Medical Research Council of Canada. We thank Christina Lyons of the Sequencing and Amino Acid Analysis Laboratory at Queen's University, Kingston, Ontario, for sequencing the peptides described in this paper.

REFERENCES

- Haslam, R. J., Salama, S. E., Fox, J. E. B., Lynham, J. A. and Davidson, M. M. L. (1980) in *Platelets: Cellular Response Mechanisms and their Biological Significance* (Rotman, A., Meyer, F. A., Gitler, C. and Silberberg, A., eds.), pp. 213–231, Wiley, Chichester
- Lyons, R. M., Stanford, N. and Majerus, P. W. (1975) *J. Clin. Invest.* **56**, 924–936
- Haslam, R. J. and Lynham, J. A. (1977) *Biochem. Biophys. Res. Commun.* **77**, 714–722
- Haslam, R. J., Lynham, J. A. and Fox, J. E. B. (1979) *Biochem. J.* **178**, 397–406
- Sano, K., Takai, Y., Yamanishi, J. and Nishizuka, Y. (1983) *J. Biol. Chem.* **258**, 2010–2013
- Kaibuchi, K., Takai, Y., Sawamura, M., Hoshijima, M., Fujikura, T. and Nishizuka, Y. (1983) *J. Biol. Chem.* **258**, 6701–6704
- Walker, T. R. and Watson, S. P. (1993) *Biochem. J.* **289**, 277–282
- Imaoka, T., Lynham, J. A. and Haslam, R. J. (1983) *J. Biol. Chem.* **258**, 11404–11414
- Tyers, M., Rachubinski, R. A., Stewart, M. I., Varrichio, A. M., Shorr, R. G. L., Haslam, R. J. and Harley, C. B. (1988) *Nature (London)* **333**, 470–473
- Daniel, J. L., Molish, I. R., Holmsen, H. and Salganicoff, L. (1981) *Cold Spring Harbor Conf. Cell Proliferation* **8**, 913–928
- Daniel, J. L., Molish, I. R., Rigmaiden, M. and Stewart, G. (1984) *J. Biol. Chem.* **259**, 9826–9831
- Painter, R. G. and Ginsberg, M. H. (1984) *Exp. Cell. Res.* **155**, 198–212
- Haslam, R. J. and Davidson, M. M. L. (1984) *Biochem. J.* **222**, 351–361
- Kawamoto, S., Bengur, A. R., Sellers, J. R. and Adelstein, R. S. (1989) *J. Biol. Chem.* **264**, 2258–2265
- Hathaway, D. R. and Adelstein, R. S. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1653–1657
- Naka, M., Nishikawa, M., Adelstein, R. S. and Hidaka, H. (1983) *Nature (London)* **306**, 490–492
- Nishida, E., Maekawa, S. and Sakai, H. (1984) *Biochemistry* **23**, 5307–5313
- Yonezawa, N., Nishida, E. and Sakai, H. (1985) *J. Biol. Chem.* **260**, 14410–14412
- Davidson, M. M. L. and Haslam, R. J. (1993) *Mol. Biol. Cell* **4** (suppl.), 260a
- Fenner, C., Traut, R. R., Mason, D. T. and Wikman-Coffelt, J. (1975) *Anal. Biochem.* **63**, 595–602
- O'Farrell, P. Z., Goodman, H. M. and O'Farrell, P. H. (1977) *Cell* **12**, 1133–1142
- Cleveland, D. W., Fischer, S. G., Kirschner, M. W. and Laemmli, U. K. (1977) *J. Biol. Chem.* **252**, 1102–1106
- Morrissey, J. H. (1981) *Anal. Biochem.* **117**, 307–310
- LeGendre, N. and Matsudaira, P. (1989) in *A Practical Guide to Protein and Peptide Purification for Microsequencing* (Matsudaira, P. T., ed.), pp. 49–69, Academic Press, New York
- Towbin, D. W., Staehelin, T. and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- Ono, S., Abe, H., Nagaoka, R. and Obinata, T. (1993) *J. Muscle Res. Cell Motil.* **14**, 195–204
- Kamps, M. P. and Sefton, B. M. (1989) *Anal. Biochem.* **176**, 22–27
- Haslam, R. J. and Davidson, M. M. L. (1984) *FEBS Lett.* **174**, 90–95
- Coorssen, J. R., Davidson, M. M. L. and Haslam, R. J. (1990) *Cell Regul.* **1**, 1027–1041
- Yamanishi, J., Takai, Y., Kaibuchi, K., Sano, K., Castagna, M. and Nishizuka, Y. (1983) *Biochem. Biophys. Res. Commun.* **112**, 778–786
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990) *J. Mol. Biol.* **215**, 403–410
- Matsuzaki, F., Matsumoto, S., Yahara, I., Yonezawa, N., Nishida, E. and Sakai, H. (1988) *J. Biol. Chem.* **263**, 11564–11568
- Ogawa, K., Tashima, M., Yumoto, Y., Okuda, T., Sawada, H., Okuma, M. and Maruyama, Y. (1990) *Nucleic Acids Res.* **18**, 7169
- Moriyama, K., Nishida, E., Yonezawa, N., Sakai, H., Matsumoto, S., Iida, K. and Yahara, I. (1990) *J. Biol. Chem.* **265**, 5768–5773
- Cohen, P., Holmes, C. F. B. and Tsukitani, Y. (1990) *Trends Biochem. Sci.* **15**, 98–102
- Siffert, W., Siffert, G. and Scheid, P. (1987) *Biochem. J.* **241**, 301–303
- Yonezawa, N., Nishida, E., Iida, K., Yahara, I. and Sakai, H. (1990) *J. Biol. Chem.* **265**, 8382–8386
- Ohta, Y., Nishida, E., Sakai, H. and Miyamoto, E. (1989) *J. Biol. Chem.* **264**, 16143–16148
- Morgan, T. E., Lockerbie, R. O., Minamide, L. S., Browning, M. D. and Bamburg, J. R. (1993) *J. Cell Biol.* **122**, 623–633
- Fox, J. E. B. (1987) in *Thrombosis and Haemostasis 1987* (Verstraete, M., Vermeylen, J., Lijnen, R. and Arnout, J., eds.), pp. 175–225, Leuven University Press, Leuven
- Yonezawa, N., Nishida, E., Maekawa, S. and Sakai, H. (1988) *Biochem. J.* **251**, 121–127
- Tallant, E. A. and Wallace, R. W. (1985) *J. Biol. Chem.* **260**, 7744–7751
- Lerea, K. M. (1991) *Biochemistry* **30**, 6819–6824
- Erdödi, F., Csontos, C., Sparks, L., Murányi, A. and Gergely, P. (1992) *Arch. Biochem. Biophys.* **298**, 682–687
- Walker, T. R. and Watson, S. P. (1992) *Br. J. Pharmacol.* **105**, 627–631
- Kitazawa, T., Masuo, M. and Somlyo, A. P. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 9307–9310
- Coorssen, J. R. and Haslam, R. J. (1993) *FEBS Lett.* **316**, 170–174
- Wagner, P. D. and Vu, N.-D. (1990) *J. Biol. Chem.* **265**, 10352–10357
- Higashihara, M., Takahata, K., Kurokawa, K. and Ikebe, M. (1992) *FEBS Lett.* **307**, 206–210