Phosphorylation of Platelet Actin-Binding Protein During Platelet Activation

By Roger C. Carroll and Jonathan M. Gerrard

In this study we have followed the \(^{32}\text{P}\)-labeling of actin-binding protein as a function of platelet activation. Utilizing polyacrylamide-sodium dodecyl sulfate gel electrophoresis to resolve total platelet protein samples, we found 2–3-fold labeling increases in actin-binding protein 30–60 sec after thrombin stimulation. Somewhat larger increases were observed for 40,000 and 20,000 apparent molecular weight peptides. The actin-binding protein was identified on the gels by coelectrophoresis with purified actin-binding protein, its presence in cytoskeletal cores prepared by detergent extraction of activated \(^{32}\text{P}\)-labeled platelets, and by direct immunoprecipitation with antibodies against guinea pig vas deferens filamin (actin-binding protein). In addition, these cytoskeletal cores indicated that the \(^{32}\text{P}\)-labeled actin-binding protein was closely associated with the activated platelet’s cytoskeleton. Following the \(^{32}\text{P}\)-labeling of actin-binding protein over an 8-min time course revealed that in aggregating platelet samples rapid dephosphorylation to almost initial levels occurred between 3 and 5 min. A similar curve was obtained for the 20,000 apparent molecular weight peptide. However, rapid dephosphorylation was not observed if platelet aggregation was prevented by chelating external calcium or by using thrombasthenic platelets lacking the aggregation response. Thus, cell–cell contact would seem to be crucial in initiating the rapid dephosphorylation response.

The contractile proteins actin, myosin, and actin-binding protein comprise a large proportion of the platelet total protein. These proteins have been found to be the major components of cytoskeletal cores prepared by 1% Triton X-100 extraction of activated platelets. Certain aspects relating to regulation of contractile protein interaction and how such interactions might lead to anatomical changes remain to be clarified. Considerable evidence suggests that an elevation of cytoplasmic calcium levels is critical to initiate changes in cell anatomy occurring on activation. Recent evidence suggests that calcium regulates actin–myosin interaction by control of phosphorylation of one of the light chains of myosin (MLC1). Phosphorylation of this 20,000-dalton light chain by myosin kinase, essential for development of actin–myosin contractile tension, has been shown to be greatly stimulated by calcium and calmodulin.

It has been postulated that actin–myosin interaction might be involved in granule centralization, however, it seems likely that an alternate process is involved in pseudopod formation. Some evidence suggests myosin exists only in the body of cells and not in pseudopods.

Further, since the polarity of actin in pseudopods is such that myosin could only pull the actin toward the cell center, it is unlikely that pseudopod extension results from actin–myosin interaction. Rather, recent studies implicate platelet actin-binding protein (ABP), similar or identical to filamin in other cell types, as interacting with actin and other proteins to form filamentous gels critical to pseudopod formation. Since actin-binding protein interaction with actin to form such filamentous gels has been suggested to occur only when ABP is phosphorylated, we have investigated whether increased \(^{32}\text{P}\)-labeling of the protein occurs upon platelet activation.

MATERIALS AND METHODS

Preparation of \(^{32}\text{P}\)-Labeled Gel-Filtered Platelets

Citrated platelet-rich plasma, prepared as described, was incubated 1 hr at 37°C with 2 mCi/ml \(^{32}\text{P}\)-orthophosphate (New England Nuclear, Boston, Mass.). The plasma proteins and unincorporated \(^{32}\text{P}\)-label were then removed by gel filtration of a 5.0-mI sample of the labeled platelet-rich plasma on a 2.5 cm x 10 cm Sepharose 2B column equilibrated at 4°C with a modified Tagen-Hepes-BSA buffer, pH 7.4 (147 mM sodium chloride, 5 mM potassium chloride, 0.05 mM calcium chloride, 0.1 mM magnesium chloride, 5 mM HEPES, 5.5 mM \(\alpha\)-d-glucose, and 1 mg/ml bovine serum albumin). The platelets were eluted with the same buffer, pooled at a concentration of 1.7–2.0 \(\times\) 10\(^7\) platelets/ml, and warmed to 37°C for 15 min before the experiments.

Protein Phosphorylation Time Courses

A 1.2-mI sample of \(^{32}\text{P}\)-labeled gel-filtered platelets was allowed to warm to 37°C and after removing a 0.1-mI zero time aliquot, 1 U/ml thrombin from a stock solution of 100 U/ml purified bovine thrombin (generously provided by Dr. C. Esmon) was added with continuous stirring. Time course samples (0.1 mI) were withdrawn at the times indicated in the figures and immediately mixed at 100°C with 0.05 ml of threefold concentrated denaturing solution consisting of 6% sodium dodecyl sulfate, 6% 2-mercaptoethanol, 30% glycerol, 3 mM EDTA (ethylenediamine tetraacetic acid), 12 mM EGTA (ethyleneglycol-bis [\(\beta\)-aminoethylether] N,N'-tetraacetic acid), and 0.25 M HEPES (pH 7.5). The samples were boiled and stored at 4°C for 15 min before being boiled and stored at 4°C for 15 min before being analyzed.

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Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Aliquots (0.065–0.075 ml equal to a total of 1.25 × 10^6 cpm) of the time course samples were loaded on 6%–17.5% polyacrylamide gradient sodium dodecyl sulfate slab gel and electrophoresed as described. The gels were fixed-stained overnight at 37°C with 0.05% Coomassie blue in 25% isopropanol–10% acetic acid. After destaining for 3 days at 37°C with at least 4 changes of 10% methanol–10% acetic acid, the gels were soaked 20 min at 37°C in 50% methanol, 5% acetic acid, and 0.1% glycerol before vacuum drying on filter paper. The dried gels were then autoradiographed for 2–3 days at –70°C using Kodak BB-5 Blue Brand x-ray film.

Preparation of Actin-Binding Protein and Myosin Light Chain I Marker Proteins

The bands in the total platelet protein samples corresponding to the 250,000-dalton subunits of actin-binding protein (ABP) and the 20,000-dalton subunits of myosin (MLCI) were identified by coelectrophoresis of standard preparations. In the case of ABP protein, a preparation of actin-binding protein (filamin) from chicken gizzard smooth muscle (a generous gift from Dr. S.J. Singer, University of California at San Diego) was used as the marker standard. Myosin was partially purified from bovine platelets by a standard protocol of high salt extraction in the presence of magnesium and ATP, column chromatography on Bio-Gel A-15M, and low salt precipitation.

Platelet cytoskeletal cores were prepared by 1% Triton X-100 extraction. This protocol was modified by including 2 mM sodium molybdate and 10 mM β-glycerophosphate in the 1% Triton X-100, 50 mM Tris-HCl, and 5 mM EGTA extraction buffer pH 7.4, and performing the extraction at 0–4°C. These modifications were introduced to inhibit phosphatase activity and help maintain the 32P-labeling of the cytoskeletal proteins. 32P-labeled actin-binding protein was isolated at 4°C from cytoskeletal cores prepared as described above from 40 ml of 32P-labeled gel-filtered platelets activated 1 min with 1 U/ml thrombin. The actin-binding protein was solubilized from this cytoskeletal core material by 1-hr incubation with continuous mixing in 4 ml of a high salt extraction buffer (0.6 M sodium chloride, 50 mM sodium phosphate, 10 mM β-glycerophosphate, 2 mM sodium molybdate, 2 mM ATP, 2 mM magnesium chloride, 5 mM EGTA, pH 7.4). The residual insoluble material consisting mostly of fibrin and actomyosin was removed by centrifugation at 5000 g for 15 min, and the supernatant applied to an AcA-22 ultragel 100,000–1,200,000 molecular weight range (LKB) column (2.5 x 50 cm) equilibrated and reprecipitated by centrifugation. Fractions of 2.5 ml were collected, and 0.075-ml aliquots analyzed on SDS-slab gels. The partially purified actin-binding protein eluted as a single peak following a void volume peak of incompletely dissociated actomyosin. A corresponding peak of 32P-counts was associated with the actin-binding protein fraction.

Immunoprecipitation

Goat antisera against guinea pig vas deferens filamin, a smooth muscle actin-binding protein shown to be immunologically related to platelet actin-binding protein (a generous gift from Dr. Ira Pastan, NIH, Bethesda, Md.), was used in a direct immunoprecipitation titration of the 32P-labeled ABP isolated as described above. After incubation overnight at 4°C, the samples were centrifuged 4 min at 10,000 g and the supernatants carefully removed. The immunoprecipitates were washed by resuspension in 1.0 ml of high salt extraction buffer containing 1% Triton X-100 and reprecipitated by centrifugation as before. The supernatants were again carefully removed, the immunoprecipitates denatured for SDS-gel electrophoresis as described above in 0.05 ml of onefold concentrated denaturing solution.

Quantification of 32P-Counts/Gel Band

The 32P-label incorporated into ABP, MLCI, and a 40,000 molecular weight peptide (40P) over the time courses was quantified by cutting out the corresponding band on the dried slab gel, solubilizing the gel band overnight with 1.0 ml of 80% Protocol (New England Nuclear), and scintillation counting the label after adding 9.0 ml of Aquasol (New England Nuclear). Background counts were determined by counting gel strips from just above or below the bands of interest and the counts (20–25 cpm) subtracted from the sample counts.

Thrombasthenic Patient

32P-labeled gel filtered platelets were prepared from a well characterized patient (after obtaining informed consent) with Glanzman's thrombasthenia. This patient's platelets have been shown not to aggregate in response to activators and have little or no glycoprotein 11B and III, surface proteins believed to be involved in platelet aggregation.

RESULTS

Identification of Platelet Actin-Binding Protein as a Phosphoprotein

Detergent extraction of thrombin-activated platelets yields cytoskeletal cores (Fig. 1A, lane 3) containing...
predominantly actin-binding protein, myosin, and actin.\textsuperscript{2} The corresponding autoradiogram (Fig. 1B) clearly shows the \textsuperscript{32}P-label in association with the 250,000-dalton actin-binding protein subunit both in the total protein (lane 2) and cytoskeletal core (lane 3) samples. Quantitation of the \textsuperscript{32}P-label by scintillation counting as described in Materials and Methods for several different activated samples indicates that 90\%-100\% of the label associated with the actin-binding protein band in the total protein sample is recovered in the detergent-extracted cytoskeletal core sample. Similar extraction of unactivated control platelets yields almost no cytoskeletal material (less than 5\% of that seen in activated platelets by Coomassie blue stain intensity) and no detectable \textsuperscript{32}P-label. Virtually all of this \textsuperscript{32}P-label associated with ABP in the cytoskeletal preparation is removed in the absence of the phosphatase inhibitors \textit{\textbeta}-glycerophosphate and sodium molybdate. Even in the presence of these inhibitors, 40\%-80\% of the MLC1 subunit \textsuperscript{32}P-label is removed during the extraction in agreement with its known sensitivity to platelet phosphatases.\textsuperscript{17} The loss of label from the MLC1 band precluded any statement about its association with the cytoskeletal core. Almost all of the \textsuperscript{32}P-label associated with the 40P band is found in the solubilized material (lane 4) corresponding to previous observations that 40P is a soluble cytosolic protein.\textsuperscript{17} Confirmation that the 250,000-dalton \textsuperscript{32}P-labeled band was actin-binding protein was obtained by direct immunoprecipitation of the putative \textsuperscript{32}P-labeled ABP isolated after solubilization from cytoskeletal cores as described in Materials and Methods. The resulting immunoprecipitates were analyzed on an SDS slab gel shown in Fig. 2. It can be seen that this antibody gives quantitative precipitation of the \textsuperscript{32}P-labeled 250,000-dalton band by Coomassie blue staining (Fig. 2A) as well as quantitative precipitation of the \textsuperscript{32}P-label from the corresponding autoradiogram in Fig. 2B. The nature of the associated \textsuperscript{32}P-label was further tested as shown in Table 1. Stability to chloroform:methanol extraction, hot 10\% TCA, and hydroxylamine treatment would indicate that the \textsuperscript{32}P-label is not due to bound phospholipid or RNA and is not linked to the protein as a phosphohistidine or phosphohistidine nor as an acyl phosphate such as phosphoglutamate or phosphoaspartate.\textsuperscript{18} The \textsuperscript{32}P-label is completely removed in the absence of either phosphatase inhibitors or EGTA during detergent extraction (data not shown). In the absence of EGTA, the actin-binding protein band is digested presumably by endogenous calcium-activated proteases as previously noted.\textsuperscript{17} Addition of exogenous proteases in the presence of EGTA also causes the digestion of the ABP with a corresponding loss of \textsuperscript{32}P-label. In our hands, hot 0.5 M sodium hydroxide treatment\textsuperscript{18} also leads to the extensive digestion of total platelet protein even when the treatment is done after electrophoresis on slab gels.

\textsuperscript{32}P-Labeling Time Courses of Actin-Binding Protein

Quantification of the \textsuperscript{32}P-label associated with the ABP band in time course samples after thrombin activation is shown in Fig. 3. For comparison, the
Table 1. Nature of the $^{32}\text{P}$-Label Associated With Platelet Actin-Binding Protein

<table>
<thead>
<tr>
<th>A. Treatment of total platelet protein sample after electrophoresis associated with the ABP-band</th>
<th>Percent of $^{32}\text{P}$-cpm</th>
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<tr>
<td>10% TCA, 10 min, 95°C</td>
<td>98% ± 4%</td>
</tr>
<tr>
<td>0.8 M Hydroxylamine, pH 5.4</td>
<td>102% ± 5%</td>
</tr>
<tr>
<td>0.5 M NaOH, 10 min, 95°C</td>
<td>&lt;5%</td>
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<th>B. Treatment of cytoskeletal core samples before electrophoresis</th>
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<tr>
<td>CH$_3$Cl : CH$_3$OH (2:1) extraction</td>
</tr>
<tr>
<td>*Staphylococcal V8 protease, 1 hr, 37°C</td>
</tr>
<tr>
<td>*Trypsin, 1 hr, 37°C</td>
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*Proteases were added at a 1:50 g ratio to the cytoskeletal core protein.

$^{32}\text{P}$-labeling of 40P and MLCI, which has been demonstrated in previous studies, is also shown. The $^{32}\text{P}$-labeling of ABP in 5 separate experiments gives peak values, within 1–2 min of platelet activation, of 2.1 ± 0.5-fold the zero time value. Greater increases are observed for MLCI (3.9 ± 0.7-fold) and 40P (7.9 ± 3.0-fold). Following peak incorporation of $^{32}\text{P}$-label, a rapid dephosphorylation takes place over the next 3–5 min that reduces the levels of $^{32}\text{P}$ in ABP and MLCI to less than 1.5-fold over the zero time value and the level of 40P to about 50% of its peak level. During these time courses, there is no evidence of proteolysis. The uniform intensity of staining as well as the equivalency of total $^{32}\text{P}$-counts (±5%) in the time course samples indicates that uniform sampling is achieved. This result is probably due to the formation of small uniform aggregates in these gel-filtered platelet preparations. Unstimulated platelet control samples taken over the same time course (data not shown) indicate less than 10% variation in the basal levels with time. These basal levels are obtained with 40–60 min equilibration of the platelet-rich plasma with the $^{32}\text{P}$-label and show no further increases over an additional 2-hr incubation. When platelet aggregation is inhibited by the addition of 5 mM EGTA before thrombin activation, the resulting $^{32}\text{P}$-labeling curves (Fig. 4) show a different dephosphorylation response. Typically, over the 8-min time course, little or no decline in $^{32}\text{P}$-labeling is observed. In fact, in 5 different experiments, ABP labeling continues to increase to 2.9 ± 0.6-fold over basal levels by 8 min. No significant change in the maximum level of MLCI or 40P phosphorylation was observed under these conditions. The addition of 5 mM EGTA to an unactivated control sample showed no direct effect on basal $^{32}\text{P}$-levels over the time course (data not shown). In order to confirm that the lack of dephosphorylation was due to the absence of platelet aggregation and not simply the removal of external calcium, we examined the $^{32}\text{P}$-labeling time course of a thrombasthenic patient’s platelets lacking the aggregation response even in the presence of external calcium. The resulting $^{32}\text{P}$-labeling curves shown in Fig. 5 again indicate a lack of typical dephosphorylation response correlating with the absence of platelet aggregation.

The ratios of maximum $^{32}\text{P}$-labeling of ABP, MLCI, and 40P are shown in Table 2. Given that the specific radioactivity of the metabolic nucleotide pool doesn’t

![Fig. 3. Time course of $^{32}\text{P}$-labeling of actin-binding protein after thrombin stimulation. A 1.2-mL sample of $^{32}\text{P}$-labeled gel-filtered platelets was warmed to 37°C for 15 min. After removing a 0.1-mL zero time aliquot, 1 U/mL thrombin was added with continuous stirring at 37°C. Additional 0.1-mL samples were removed at the indicated times and denatured as described in Materials and Methods. After electrophoresis of the time course samples, the appropriate bands were cut out of the dried slab gel and the $^{32}\text{P}$-counts quantified as described in Methods. The curves shown are for ABP (△), MLCI (□), and 40P (○).]
Table 2. Comparison of Calculated Pool Sizes to Observed Phosphorylation Ratios

<table>
<thead>
<tr>
<th></th>
<th>Calculated Relative Pool Sizes</th>
<th>Observed Maximum $^{32}$P-Labeling Ratios</th>
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<tr>
<td></td>
<td>With Aggregation (n = 5)</td>
<td>Without Aggregation (n = 5)</td>
</tr>
<tr>
<td>40P</td>
<td>4.7 ± 1.7</td>
<td>3.2 ± 0.6</td>
</tr>
<tr>
<td>ABP</td>
<td>2.6 ± 0.7</td>
<td>2.9 ± 0.6</td>
</tr>
<tr>
<td>Myosin</td>
<td>2.1 ± 0.5</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>MLCI</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>40P</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>ABP</td>
<td>1.6</td>
<td></td>
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</table>

Change during activation and assuming uniform labeling and no unusual changes in turnover rates, these ratios should be comparable to the molar pool size ratios of these proteins. These pool sizes are calculated using approximate percent total platelet protein and molecular weights for ABP, of 3% and 500,000 daltons, myosin of 4% and 470,000 daltons, and 40P of 0.65% and 47,000 daltons. The percent total protein values are from our own estimates from densitometry of the Coomassie blue stained bands on these SDS-slab gels that agree fairly well with previous estimates.

It can be seen that the observed ratio without the aggregation-dependent dephosphorylation for 40P/ABP is close to the ratio of the relative pool sizes of the proteins. Even better agreement is obtained comparing ABP to MLCI phosphorylation. This result would support the significance of ABP phosphorylation, since it has been recently shown that 40P has a minimum of 0.3–0.5 mole of phosphate/mole of protein, and the MLCI pool is totally phosphorylated during platelet activation. It is possible that an unusual change in turnover rate could account for the change in ABP $^{32}$P-labeling during these time courses, so a definitive estimate of the mole phosphate/mole ABP can't be made until sufficient quantities of ABP from activated platelets are available for direct analysis.

**DISCUSSION**

That actin-binding protein in a variety of tissue homogenates can be phosphorylated in vitro by $[^{32}\text{P}]\text{ATP}$ has been previously reported. However, the functional significance of the phosphorylation has been only briefly suggested in an earlier study showing that actin interaction with actin-binding protein is inhibited by alkaline phosphatase treatment. The present paper describes for the first time a substantial increase in $^{32}$P-labeling of actin-binding protein correlated with platelet activation. The association of $^{32}$P-labeled actin-binding protein with detergent-extracted cytoskeletal cores in activated platelets provides further evidence for a requirement for actin-binding protein phosphorylation in order to attach to actin. The increase in $^{32}$P-labeling of actin-binding protein on platelet activation may therefore be involved in regulating the actin-binding protein association with actin, suggested to be critical for the formation of actin bundles in platelets pseudopods.

The rapid dephosphorylation response of actin-binding protein, myosin light chain subunit I, and a 40,000-dalton protein was found to be dependent on platelet aggregation. Thus, dephosphorylation was prevented...
using EGTA to chelate external calcium that prevents aggregation but not cell secretion or changes in cell morphology. Similarly, thrombasthenic platelets that can undergo all other aspects of the platelet response except aggregation failed to undergo dephosphorylation. The close association of platelets in large aggregates may initiate a turnoff signal for phosphorylation, such as resequestering calcium and/or activation of a phosphatase producing the dephosphorylation seen. We suggest that this process may have some similarity to the contact inhibition of cell motility in cultured cells, where cell–cell contact initiates a cell turnoff process.

REFERENCES

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