Plateslets respond to a variety of nonpene-
trating stimuli—nucleotides, enzymes, particu-
late matter. The reaction between the stimulating
agent and its specific receptor on the outer surface of
the plasma membrane initiates a signal that is trans-
mittted through the membrane and sets in motion
responses such as the actin polymerization associated
with shape change, exposure of fibrinogen receptors
necessary for aggregation, and secretion of granule
contents. Such stimulus–response coupling occurs in
many types of cell. When a ligand binds to two surface
molecules of some cells—for example, when lympho-
cytes are mixed with F(ab), fragments of anti-IgG, but
not with Fab fragments,1 or when tetrameric concana-
valin-A (Con-A) binds to Dictyostelium2 or to plate-
lets,3 an association develops between surface glyco-
proteins and Triton X-100-insoluble material (i.e., the
“cytoskeleton”). Phillips et al.4 demonstrated an
apparently similar association between 125I-labeled
surface glycoproteins and the Triton-insoluble residue
of platelets allowed to aggregate with thrombin for 30
min, but not with the residue of platelets that had been
treated with thrombin in the presence of EDTA to
prevent aggregation. Since binding of fibrinogen to the
platelet surface is necessary (though not sufficient) for
platelet aggregation,5,6 we speculated that the symmet-
rical fibrinogen molecule that is secreted from the
alpha granules of thrombin-stimulated platelets7
might bind to two sites on the platelet surface and, like
Con-A,1 cause the reported association between sur-
face glycoproteins and the Triton-insoluble residue.
This hypothesis would account for the absence of the
association in the presence of EDTA, as this chelating
agent prevents fibrinogen from binding to the mem-
brane.

The hypothesis was tested in two ways: (1) we
investigated the association of 125I-labeled glycopro-
teins with the Triton-insoluble residue of platelets that
were stimulated with thrombin or ADP under condi-
tions that caused fibrinogen binding but not aggrega-
tion, and (2) we measured the phospholipid associated with the Triton-insoluble residue of aggregated platelets to determine whether the membranes had been adequately lysed. We concluded that the surface glycoproteins do not become attached to the Triton-insoluble proteins of aggregated platelets but that the apparent association results from inadequate membrane lysis.

**MATERIALS AND METHODS**

**Preparation of Washed Platelets**

The methods were adapted from those of others. Usually about 150 ml of blood was drawn into syringes containing 0.2 ml of 0.085 M trisodium citrate, 0.065 M citric acid, 2% glucose, and 1.25 ml of 0.14 mM prostaglandin E,

(PGE),/ml of blood and was then added to 0.05 vol of 1 mM acetylsalicylic acid (stored at \(-20^\circ\)C in isotonic saline). The platelets were separated by differential centrifugation at 37°C, resuspended in a small volume of solution A (140 mM NaCl, 2.5 mM KCl, 0.1 mM MgCl₂, 0.5 mM NaH₂PO₄, 10 mM NaHCO₃, 0.35% bovine serum albumin, 0.1% glucose), with 2 mM CaCl₂, 220.35% saline). The platelets were resuspended in 1 ml of Tyrode’s solution with no added protein or CaCl₂ and labeled with 

\(1\) by the successive addition of 65 ml of lactoperoxidase (Sigma, 1 mg/ml), 0.5 mCi 

\(^{125}\)I (New England Nuclear, Boston, Mass.), and 12 ml of 1 mM H₂O₂, added every 15 sec 5 times. The labeled platelets were combined with the remaining unlabeled platelets, washed twice, and resuspended at a concentration of 1.4–2.0 \(\times 10^9/ml in solution A with 10 mM TES [N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid], pH 7.4. The TES prevented an increase in pH when the samples were shaken to produce platelet aggregation. The albumin was omitted from solution A in samples used for protein determination. In three experiments, the labeled platelets were prepared as described by Phillips et al., without the use of aspirin.

**Platelet Stimulation and Extraction With Triton X-100**

The experiments were carried out at room temperature in 1.5 ml conical plastic tubes (Eppendorf). Five microliters of one or more of the following reagents was added to the tubes, then 0.5 ml of platelet suspension was added. The final concentrations were: 1 U/ml highly purified human thrombin (kindly given to us by John W. Fenton II) with or without 1 mM CaCl₂, or 0.5 mM neutralized sodium EDTA, or 10 \(\mu\)M ADP and 1 mM CaCl₂ with or without 100 \(\mu\)g/ml human fibrinogen (Kabi, Stockholm). In some experiments, one tube contained 50 \(\mu\)l of Con-A (Sigma, Type IV) for a final concentration of 100 \(\mu\)g/ml. One set of tubes was often shaken by hand for 1–2 min and the samples observed for macroscopic aggregation. After 3 or 30 min in the experiments with thrombin, 3 min in the experiments with Con-A, and 10 min in the experiments with ADP, we added 0.5 ml of lysing solution (2% Triton X-100, hereafter referred to as Triton, 10 mM EDTA, 100 mM Tris, pH 7.4).

After 30 min at room temperature, the samples were centrifuged at 12,000 g (Eppendorf 3200 centrifuge, Brinkmann Instruments, Westbury, NY), the sediments were rinsed with the lysing solution diluted with an equal volume of isotonic saline without resuspending the pellets, the tubes were drained, 0.1 ml of 2% sodium dodecylsulfate (SDS) was added, and the tubes were stored at \(-15^\circ\)C. Frequently, one set of samples was prepared with 

\(^{125}\)I-labeled platelets and others were prepared with nonradioactive samples for determination of protein or lipid phosphorus.

In some experiments, about 0.5 \(\mu\)M \(^{14}\)C-serotonin (Amersham, Arlington Heights, Ill., 58 mCi/mmol, stored at \(-15^\circ\)C at 8 mCi/ml in 70% ethanol) was added to the platelets when they were incubating in the first wash. Triton was not added; rather, after stimulating the platelets, the samples were chilled and centrifuged. The radioactivity in an aliquot of the supernatant was measured in a liquid scintillation spectrometer after adding Aquasol (New England Nuclear, Boston, MA) and was compared to that in an equal aliquot of the initial suspension.

**Measurement of Lipid Phosphorus**

Lipid was extracted by a modification of the method of Bligh and Dyer. Platelet suspensions were prepared as described but without adding labeled platelets. The Triton-insoluble sediments were broken up by pipetting and transferred with 1 ml of water to glass tubes. Then, 3.5 ml of a 1:2.5 mixture of chloroform-methanol was added. The single phase was broken by adding 1 ml of chloroform and 1 ml of water. After centrifugation, the chloroform was transferred to a clean tube, the residue was washed with 2 ml of chloroform, and the pooled chloroform extracts were dried under nitrogen. The samples were digested with perchloric acid, and phosphorus was determined essentially as described by Chen et al.

**Polyacrylamide Gel Electrophoresis (PAGE)**

This was carried out according to Laemmli after mixing the thawed samples with an equal volume of concentrated gel buffer. Unless otherwise noted, the samples were reduced by adding 4% beta-mercaptoethanol and placing them in boiling water for 10 min. In experiments in which unreduced samples were studied, the Triton-insoluble residues were immediately boiled with buffer that contained N-ethylmaleimide. After storage, half of the sample was reduced as described above.

The gels contained a ratio of N,N′-bismethylene acrylamide to acrylamide of 0.027:1, with 7.5% acrylamide in the running gel, and 4.5% acrylamide in the stacking gel. Samples were electrophoresed for about 3 hr at 25 mA. After fixing overnight in 40% methanol–7% acetic acid, the gels were stained for 3 hr with 0.05% Coomassie Brilliant Blue G and destained with 10% methanol–7% acetic acid. For molecular weight markers, we used β-galactosidase (mol wt 120,000), phosphorylase-B (mol wt 94,000), human serum albumin (mol wt 68,000), and ovalbumin (mol wt 42,000). The molecular weights estimated in each experiment are indicated on the figures, but average values are used in the text.

For radioautography, equal volumes of samples of Triton-insoluble residues were applied; smaller samples were applied for whole platelets. The gels were dried in a slab gel dryer (Savant Instruments, Hicksville, NY) and exposed for 0.5–4 days to Cronex film using Cronex Quanta III intensification screens (DuPont, Wilmington, DE). In some experiments, the radioautographs of reduced samples of whole platelets and Triton-insoluble residues were scanned (Schoeffel Spectro-densitometer, Westwood, NJ). The area under each peak was estimated as one-half of the product of base x height for four of the labeled proteins. The amount of each protein in the Triton-insoluble residue was then expressed as a percentage of its amount in whole platelets, using the values for the area and the number of platelets represented in the material applied to the gel.

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RESULTS

Unstimulated Platelets

As shown in Fig. 1, the Triton-insoluble residue of unstimulated (control) platelets contained 10.6% of the protein found in whole platelets (2020 ± 7.65 μg/10^9, n = 11). Coomassie blue-stained SDS polyacrylamide gels of this residue always revealed a heavy band at a molecular weight of 43,000 and lighter bands at 200,000 and 250,000. These bands have been shown to be actin, the heavy chain of myosin, and actin-binding protein, respectively. Other minor bands were also present. When EDTA was added to the platelet suspension to prevent aggregation, gels of the cytoskeletons showed a marked increase in myosin. This increase was not noted when the platelet suspension contained EGTA rather than EDTA or when it contained EDTA with an excess of magnesium, but did occur if calcium rather than magnesium ions were present in excess of the EDTA (data not shown).

There were 228 ± 19 nmole of lipid phosphorus (or phospholipid) per 10^9 platelets. Less than 2% of this was insoluble in Triton (Fig. 1).

The Triton-insoluble residue contained a mean of 1.5% of the radioactivity of whole platelets in the seven experiments shown in Fig. 1; when all experiments were included, the mean was 3.5% ± 0.9 SEM (n = 12). The radioautographic pattern of reduced samples on SDS polyacrylamide gels differed strikingly from that of whole platelets; no radioactive band was seen at a mol wt of about 121,000, the location of GP IIbα, and often no labeled band was evident at mol wt 107,000 (GP III) (Figs. 2A and 3, lanes a). The distribution of the bands that were present varied considerably in different experiments, and the pattern could not be correlated with any known variable, including differences in wash solution or source of albumin. However, the radioautographic pattern was the same for the Triton-insoluble residues prepared from different samples of a single platelet suspension whether the samples were shaken or unshaken, with or without EDTA (not shown), or whether the platelets were stimulated with thrombin or adenosine diphosphate (ADP) but not aggregated (Fig. 3) (see next section).

The amounts of the labeled proteins most often detected in the Triton-insoluble residues were estimated from the radioautographs and expressed as a percentage of the amount of this protein in the same number of whole labeled platelets. The values were: 89,000 mol wt protein (GP IV), 38.5% ± 4.9%; 107,000 mol wt protein (GP III), 1.1% ± 0.45%; 132,000 mol wt protein, 8.2% ± 1.0%; and 158,000 mol

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Fig. 1. Triton-insoluble protein, radioactivity, and lipid phosphorus, expressed as percent of total amount in platelets. Control (C), thrombin-stimulated but unshaken (TU), thrombin-stimulated and shaken (TS), Con A-stimulated (Con-A), and centrifuged (CENT) platelets. Mean ± SD for 7 experiments for protein and radioactivity and 5 experiments for lipid phosphorus. Two experiments only for CENT platelets; mean and values for both experiments are shown.

Fig. 2. Radioautographs of SDS polyacrylamide gels Triton-insoluble residues of 125I-labeled platelets incubated for 3 min alone (a) or with thrombin (b), or shaken for 3 min with thrombin to produce large clumps (b). Total platelets are shown as (T). (A) Reduced samples. (B) Unreduced samples. Equal volumes representing equal numbers of platelets were applied except for T, which represents fewer platelets. The numbers indicate approximate M, in thousands.
TRITON-INSOLUBLE PLATELET COMPONENTS

Fig. 3. Radioautograph of SDS polyacrylamide gel of reduced samples of Triton residues of 125I-labeled platelets incubated for 3 min alone (a), with thrombin (b), with ADP and fibrinogen (c), and with Con-A (d). No aggregates were noted before addition of Triton. Equal volumes representing equal numbers of platelets were applied. The numbers indicate approximate M, in thousands.

wt protein, 56.2% ± 22.0% (n = 11). Other bands (e.g., mol wt 69,000 in Fig. 2) were present too infrequently to be averaged. The virtual absence of a band near mol wt 158,000 in lanes a of Figs. 2A and 3 attests to the variability of the labeled Triton-insoluble proteins in the different experiments.

In unreduced samples (Fig. 2B, lane a), Triton-insoluble radioactivity was noted at mol wt of about 90,000 and 170,000, with no bands at intermediate molecular weights. In contrast to the Triton-insoluble residues, the Triton-soluble supernatants showed a strong band at about 107,000 (GP IIb) as well as other less intense bands (not shown).

Stimulated Platelets

Thrombin

Addition of thrombin to the platelet suspension caused the platelets to change shape and secrete about 50% of the 14C-serotonin that they had incorporated. Aggregation was not observed when Triton was added 3 min after the thrombin. The amount of protein in the Triton-insoluble residue increased to 16.7% of that in whole platelets, but the amounts of Triton-insoluble lipid phosphorus and radioactivity did not change (Fig. 1), nor did the distribution of Triton-insoluble radioactivity on SDS polyacrylamide gels (Figs. 2A, 2B, and 3, lanes b). The radioactivity at the top of the gel in Fig. 2 was not seen in most experiments, but the radioautograph is shown because it provides an excellent comparison of reduced and unreduced samples.

Platelets shaken with thrombin for 3 min formed large aggregates. The Triton-insoluble residues of these samples contained much more protein and radioactivity than similar samples that did not contain aggregates either because they were not shaken (Fig. 1) or because they were shaken in the presence of EDTA (not shown). Lipid phosphorus also increased, though to a variable extent (Fig. 1). The distribution of the radioactive bands after electrophoresis resembled that seen in samples of whole platelets (Fig. 4, lane bJ, except that in a few experiments, less GP IIb was present (Fig. 2, A and B, lane bJ. Electron microscopy revealed pieces of membrane that were not seen in control samples (Fig. 5).

In three experiments (Table 1, exps. 1–3), the

Fig. 4. Radioautograph of SDS polyacrylamide gels of 125I-labeled platelets. Triton-insoluble residues of platelets shaken with thrombin for 3 min to produce large aggregates (bJ, and platelets incubated for 3 min with Con-A (d). Total platelet proteins are shown as (T). (A) Reduced samples. (B) Unreduced samples. Equal volumes representing equal numbers of platelets were applied for bJ, and d; T represents fewer platelets. The numbers indicate approximate M, in thousands.
platelets were prepared as described by Phillips et al., without the addition of aspirin, and were left at room temperature with thrombin for 30 min before the Triton was added. Small clumps were evident. Triton-insoluble radioactivity increased in two of the three experiments, and radioautographs revealed the presence of GP IIb and III in addition to the glycoproteins that were present in the Triton-insoluble residue of the control platelets. Lipid phosphorus increased in the two experiments in which it was measured. The increase was less than that often seen in shaken samples (Fig. 1), in which the clumps were much larger. Differences in the size of the clumps that formed "spontaneously" probably explain the absence of an increase in Triton-insoluble radioactivity in the labeled thrombin-treated sample in experiment 1, despite the increase in Triton-insoluble lipid phosphorus in the unlabeled thrombin-treated sample.

**ADP**

Stimulation of platelets with ADP caused them to change from discs to spiny spheres. Unshaken samples failed to release $^{14}$C-serotonin, whether or not they had been treated with aspirin, because the platelets were not aggregated. There was no change in the amount of Triton-insoluble protein (not shown) or radioactivity (Table 1), or in the distribution of radioactivity in radioautographs even in the presence of fibrinogen (Fig. 3c). Triton-insoluble lipid phosphorus usually increased slightly (Table 1).

**Table 1. Triton-Insoluble Radioactivity and Lipid Phosphorus in Platelets Incubated With Thrombin for 30 min Without Shaking (Unsh) or With ADP Plus Fibrinogen for 5 min Preceded in Some Samples by 2 min of Shaking (Sh)**

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Control Radioactivity (% of Total)</th>
<th>Thrombin Unsh*</th>
<th>ADP + Fibr</th>
<th>Lipid Phosphorus (% of Total)</th>
<th>Control</th>
<th>Thrombin Unsh*</th>
<th>ADP + Fibr</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.39</td>
<td>0.33</td>
<td>0.42</td>
<td>0.43</td>
<td>0.47</td>
<td>1.29</td>
<td>0.37</td>
</tr>
<tr>
<td>2</td>
<td>2.27</td>
<td>4.47</td>
<td>—</td>
<td>1.06</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>2.20</td>
<td>11.47</td>
<td>—</td>
<td>2.20</td>
<td>1.28</td>
<td>2.19</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>4.40</td>
<td>—</td>
<td>4.90</td>
<td>17.10</td>
<td>0.40</td>
<td>—</td>
<td>0.60</td>
</tr>
<tr>
<td>5</td>
<td>6.30</td>
<td>—</td>
<td>6.60</td>
<td>6.10</td>
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<td>—</td>
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</tr>
<tr>
<td>6</td>
<td>10.80</td>
<td>—</td>
<td>10.80</td>
<td>12.10</td>
<td>0.80</td>
<td>—</td>
<td>1.70</td>
</tr>
</tbody>
</table>

*Small-to-moderate-sized clumps were noted.*
Small-to-medium-sized clumps were always noted in samples of platelets shaken for 1–2 min with ADP plus fibrinogen. Triton-insoluble lipid phosphorus increased markedly (Table 1). Triton-insoluble radioactivity increased in some experiments (Table 1); when it did, the radioautographs of reduced and un reduced samples resembled those of whole platelets, with prominent bands for GP IIb and III (not shown).

**Con-A**

Platelets stimulated with Con-A secreted more than 50% of their 14C-serotonin, but no macroscopic aggregates were seen after 3 min in unshaken samples. The amount of Triton-insoluble protein increased to 20.7% of total platelet protein, but the amount of Triton-insoluble lipid phosphorus did not change (Fig. 1). Triton-insoluble radioactivity increased between 2- and 12-fold (Fig. 1), and the bands noted in the radioautographs were essentially the same as those in gels prepared from whole platelets (Figs. 3 and 4, lane d).

**Centrifuged Samples**

Samples of unstimulated platelets were centrifuged and an attempt was made to resuspend the pellet and extract it with Triton. A high percentage of the platelet protein, radioactivity, and phospholipid was insoluble (Fig. 1).

**DISCUSSION**

The composition of the Triton-insoluble residue of platelets is readily altered. For example, like others, we noted that the amount of Triton-insoluble myosin increased markedly when EDTA was added before or after lysis. This increase is due to the association of myosin with actin into a “contractile apparatus” when magnesium is chelated. We observed a similar increase in myosin (unpublished observations) when the platelets were lysed in the presence of apyrase, an enzyme that is often added to platelet suspension medium. This protein is prominent in Triton-insoluble residues when the platelet calcium-dependent protease is inhibited by EGTA.

According to Rosenberg et al., actin-binding protein is hydrolyzed if the protease is not inhibited and is present in the Triton-soluble supernatant if the protease is inhibited by leupeptin without depleting the ionized calcium. Because the composition of the Triton-insoluble residue depends on so many nonphysiologic variables, we have avoided the term “cytoskeleton,” with its suggestion of permanence.

When platelets are stimulated by thrombin, the amount of Triton-insoluble protein increases. An increase in Triton-insoluble actin and myosin (which was often evident in our Coomassie blue-stained gels) is partially responsible, but some of the added protein is fibrin, formed from platelet fibrinogen. Evidence for this will be presented elsewhere. Stimulation with ADP did not alter the amount of Triton-insoluble protein, whereas Con-A increased it by nearly 100%.

Only a small proportion of the surface glycoproteins of 125I-labeled platelets is associated with the Triton-insoluble residue. In our hands, the average value for Triton-insoluble 125I was 3.5% of total platelet radioactivity. The labeled proteins in this residue were not randomly selected. Thus, GP IIb (mol wt 121,000 in reduced samples) was never evident, and GP III (mol wt 107,000) averaged only 1% of the amount in whole platelets. Rotman et al. did not observe Triton-insoluble proteins that reacted with 125I-Con-A in resting platelets. The percentage of other labeled proteins varied widely in different experiments, for reasons that were not apparent, but were the same in gels of different aliquots of the same platelet suspension. The most abundant Triton-insoluble labeled protein had a mol wt of about 158,000 in reduced samples. The Israel group identified a similar protein in gels of Triton-insoluble proteins impregnated with labeled wheat germ agglutinin and termed it GP Ia. We hesitate to identify the band in our studies, since its mol wt in unreduced samples was 170,000, which does not agree with the accepted value for GP Ia.

We found that the amount of Triton-insoluble radioactivity was not altered by stimulation of platelets with thrombin for 30 min in the presence of EDTA, with thrombin for 3 min without EDTA, or with ADP with or without fibrinogen. Others made similar observations with platelets incubated with thrombin in the presence of EDTA (thrombin-activated platelets). In our hands, radioautographs were identical to those of unstimulated platelets. Thus, although the significance of the association between some of the radioabeled surface proteins and the Triton-insoluble residue is unclear, the association is unchanged by physiologic stimuli, even under conditions in which fibrinogen is bound.
In striking contrast, we, like Phillips et al., noted an increase in Triton-insoluble radioactivity in platelets aggregated with thrombin. An increase also occurred in some samples aggregated by agitation with ADP and fibrinogen. The radioautographs of these samples resembled those of whole platelets, except that occasionally GP IIb was not prominent. This protein is linked to GP III with calcium and might separate when the calcium is chelated by the EGTA in the lysis solution. According to Rotman et al., gels of Triton-insoluble material from thrombin-aggregated samples exhibited bands at mol wt 80,000, 95,000, and 120,000 that reacted with labeled Con-A; samples aggregated with ADP plus fibrinogen showed only the 80,000 mol wt band.

The Triton-insoluble radioactivity may increase after aggregation simply because the Triton does not penetrate the aggregates sufficiently to lyse the platelets adequately. We tested this possibility by measuring the amount of lipid-soluble phosphorus present in the Triton-insoluble residues. Lipid phosphorus always increased in samples with large clumps and usually in those with smaller aggregates, suggesting that membrane lysis was incomplete. Further evidence for this conclusion was the fact that membrane fragments were seen on electron microscopy of the residue prepared from a sample with large aggregates, and that large amounts of protein, radioactivity, and phospholipid were insoluble when pellets of centrifuged but otherwise unstimulated platelets were extracted with Triton.

In contrast to the results with thrombin and ADP, the dramatic increase in the association between labeled surface proteins and the Triton-insoluble residue in Con-A-stimulated platelets noted by Painter and Ginsberg and by us occurs without macroscopic aggregation or an increase in phospholipid. Presumably, crosslinking of the surface glycoproteins by the multivalent ligand Con-A results in their attachment directly or indirectly to one or more Triton-insoluble proteins. Fibrinogen is bound to platelets stimulated with thrombin or ADP even when the samples are not shaken and exhibit little or no aggregation. Yet the binding of this bilaterally symmetric molecule to the platelet surface does not cause an association between radiolabeled surface proteins and the Triton-insoluble residue as binding of tetrameric Con-A does. Physiologic agonists such as thrombin and ADP must therefore induce responses such as shape change and exposure of fibrinogen receptors in some other way—perhaps by releasing membrane calcium or increasing intracellular pH.

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REFERENCES

The Triton X-100-insoluble residue ("cytoskeleton") of aggregated platelets contains increased lipid phosphorus as well as 125I-labeled glycoproteins

MB Zucker and NC Masiello