Relocation of annexin V to platelet membranes is a phosphorylation-dependent process

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Annexins are a family of calcium-binding proteins that have been implicated in a wide range of intracellular processes. We have previously reported that stimulation of platelets with agents that increase intracellular $[Ca^{2+}]$ induces the relocation of annexin V to membranes, and that this annexin V may be binding to a 50 kDa protein located within platelet membranes. We report here, using an *in vitro* reconstitution system, that the relocation of annexin V to membranes is enhanced by ATP. We also demonstrate that when adenosine 5'-[γ -thio]-triphosphate, which can replace ATP in phosphorylation reactions, is substituted for ATP, the amount of annexin V that binds to membranes is further increased. In separate experiments using intact cells, we show that the protein phosphatase inhibitor okadaic acid mimics the action

of the physiological agonist thrombin, in that it induces annexin V to bind to membranes and that the addition of the protein kinase inhibitor staurosporine inhibits A23187-induced relocation of annexin V. In addition, alkaline phosphatase, when added to isolated membranes, was found to remove endogenous annexin V from the membranes. Furthermore, immunoprecipitation of ³³P-labelled proteins indicated that annexin V may form a multi-protein complex including phosphoproteins of 25, 50 and 83 kDa. Taken together these observations suggest that, following physiological activation, the phosphorylation of one or more proteins is responsible for the tight association of annexin V with platelet membranes and the subsequent regulation of membrane localized processes.

INTRODUCTION

Platelets are non-nucleated blood cells whose major function is to form a haemostatic plug and arrest bleeding following injury to the vessel wall. Activation by an agonist, such as thrombin, stimulates signal-transduction pathways that are mediated through an increase in intracellular calcium [1]. These calcium-dependent events include changes in internal organization, secretion of granule contents, production of thromboxane A_2 , the expression of fibrinogen receptors and the expression of phosphatidylserine on the platelet surface. It is likely that calcium-binding proteins play a major role in coupling the increase in cytosolic Ca^{2+} to the final platelet responses [1]. In platelets, mediators of the calcium signal include protein kinase C, calpain, calmodulin, phospholipase A_2 and the annexins.

Annexins are a family of at least thirteen proteins that contain repeats of a highly conserved 70-amino-acid sequence [2–6]. Annexin V is the most ubiquitous annexin and has been reported to be present in almost all mammalian cells, including blood platelets [7–12]. Although the exact physiological function of annexin V remains unclear, it has been implicated in ion-channel regulation [13–16], as an inhibitor of protein kinase C [17–21], as an inhibitor of blood coagulation [22,23] and as an inhibitor of phospholipase A_2 [24]. In addition, subcellular fractionation studies have shown that in heart, lung, liver, platelets and brain a proportion of the total annexin V remains associated with membranes even after extraction with EGTA. It is therefore likely that annexin V plays a role in the regulation of a membrane-localized process [7,8,25–28]. We have demonstrated that, following physiological platelet activation, annexin V relocates from the cytosol and binds to membranes, which implicates annexin V in coupling increases in cytosolic $[Ca^{2+}]$ to membrane-specific processes [7,8]. Subsequent studies have shown that annexin V also relocates to membranes following increases in intracellular calcium in the human osteosarcoma cell line MG-63

[29], the human SH-SY5Y neuroblastoma cell line [30] and in human fibroblasts [31].

It is now apparent that two types of binding take place: (i) binding that occurs at relatively high $[Ca^{2+}]$ and is reversed by the calcium chelator EGTA, and (ii) binding that occurs at lower $[Ca^{2+}]$ and cannot be reversed by EGTA [8]. In addition, we have demonstrated that the annexin V that can be eluted with EGTA is associated with phospholipid in the membrane, whereas the annexin V that is resistant to extraction with EGTA may bind to a 50 kDa component located on platelet membranes [8].

In the present paper we investigate the mechanism by which annexin V relocates and binds tightly to platelet membranes.

MATERIALS AND METHODS

Materials

All chemicals were purchased from BDH or Sigma unless otherwise indicated. Affinity-purified horseradish peroxidase-conjugated sheep anti-rabbit IgG was purchased from Sigma. ³³P, enhanced chemiluminescence reagents and Hyperfilm were purchased from Amersham (Amersham, Bucks., U.K.). Rabbit polyclonal antiserum specific for human annexin V was purchased from Alexis Corporation (Nottingham, U.K.). Monoclonal antibody against annexin V (clone RUU-WAC2A) was purchased from Bradsure Biologicals (Loughborough, Leics., U.K.).

Preparation and activation of platelets

Platelets from healthy volunteers were prepared as described previously [32] and suspended in Hepes-buffered Tyrode's solution (129 mM NaCl, 8.9 mM NaHCO₃, 2.8 mM KCl, 0.8 mM KH₂PO₄, 0.8 mM MgCl₂, 5.6 mM dextrose and 10 mM Hepes/NaOH, pH 7.4). The number of platelets and contaminating white blood cells were determined using a Coulter S Plus counter

Abbreviation used: ATP[S], adenosine 5'-[γ -thio]-triphosphate.

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(courtesy of The Haematology Department, The General Infirmary NHS Trust, Leeds, U.K.). The very low contamination by white blood cells was also determined by visual inspection.

Platelets (1×10^9 /ml) were then incubated at 37 °C in the presence of 1 mM Ca^{2+} for 5 min before being incubated with 5 μM A23187, 0.1 μM PMA or buffer control for 10 min. The platelets were pelleted by centrifugation at 8000 g for 3 min in a microfuge and resuspended in 0.25 ml of buffer A (100 mM KCl, 5 mM EGTA, 0.5 mM DTT, 1 mM NaN_3 and 10 mM Pipes, pH 7.4). The samples were immediately frozen and stored at -20°C .

Preparation and extraction of membranes

Platelets treated with PMA or A23187 were ruptured (as confirmed by liberation of lactate dehydrogenase) by freeze-thawing, and the membranes were sedimented by centrifugation at 200000 g for 15 min in a Beckman TL-centrifuge at 4 °C. For subcellular fractionation into EGTA-elutable and EGTA-resistant material, the supernatant (supernatant a) was removed and the pellet was resuspended again in 0.25 ml of buffer A. The resuspended pellet was again centrifuged at 200000 g for 15 min. This procedure was repeated a further five times to produce six supernatants in total (supernatants a–f), representing the EGTA-elutable material. An aliquot from each of the supernatants was taken for measurement of annexin V by quantitative immunoblotting. The washed pellet was then resuspended in 0.25 ml of buffer B [buffer A + 0.1 % (w/v) Triton X-100] and centrifuged at 200000 g for 15 min. The supernatant (supernatant g) was decanted and the pellet again was resuspended in buffer B. The extraction was repeated to produce a second supernatant (supernatant h). The final pellet was resuspended in 0.25 ml of buffer B. All samples were analysed for annexin V by Western blotting (see below).

Broken-cell reconstitution system

Resting platelets (1×10^9 cells) were ruptured by freeze-thawing in 0.5 ml of buffer A. Membranes were then separated from the cytosolic material by centrifugation at 200000 g for 15 min at 4 °C. The membrane fractions were immediately resuspended in 0.25 ml of buffer D, composed of 10 mM MgCl₂, 90 mM KCl, 5 mM EGTA, 1 mM NaN_3 and 10 mM Pipes, pH 7.4, and CaCl_2 was added to give a free $[\text{Ca}^{2+}]$ of 10 μM , as predicted by the Metlig computer program [33,34]. The membranes were then washed twice in buffer D and frozen at -20°C until ready for use.

The cytosol fractions were pooled together and dialysed overnight into buffer D. Aliquots of cytosol (400 μl) were then preincubated for 10 min at 37 °C in the presence of apyrase (0.0125 unit/ml), MgATP (0.25 mM) or adenosine 5'-[γ -thio]-triphosphate (ATP[S]) (0.25 mM). Binding reactions were initiated by the addition of 200 μl of the membrane fractions, prepared as described above. The reconstituted mixture was then incubated at 37 °C for 30 min. The binding reaction was terminated when membranes were separated from the cytosolic fraction by centrifugation at 200000 g for 15 min at 4 °C. EGTA-resistant material was prepared by washing five times in buffer A as described above. The final pellet was resuspended in 0.25 ml of buffer B, and the Triton X-100 extractable supernatant was analysed for annexin V by Western blotting.

Protein phosphorylation

Washed platelets, prepared as described above, were incubated in Hepes-buffered Tyrodes solution containing 100 $\mu\text{Ci/ml}$ $^{33}\text{P}_i$

for 3 h at 37 °C. The ^{33}P -loaded platelets were subsequently stimulated with either A23187 (5 μM) or PMA (0.1 μM) in the presence of 1 mM Ca^{2+} . The platelets were subsequently resuspended in buffer A containing 0.2 M vanadate, 0.2 mM pyrophosphate and 2 mM NaF and were fractionated as described above.

Immunoprecipitation of membrane-associated annexin V

Immunoprecipitation of annexin V was performed with a polyclonal antiserum raised against bovine annexin V. This antiserum was adjudged to be specific according to the criteria of obtaining a single immunoreactive band when various tissues were screened by Western blotting. Control experiments were performed with preimmune serum isolated from the rabbit before inoculation with annexin V. Immunoprecipitation was also performed with a monoclonal antibody specific to annexin V.

Immunoprecipitation was carried out using the method of Harlow and Lane [35] with minor modifications. A Triton X-100-soluble fraction from platelets that had been activated with A23187 was prepared as described above. This supernatant was transferred to protein G-Sepharose 4B (50 mg) beads to which the relevant IgG fraction (10 μl serum) had previously been bound by incubation overnight at 4 °C. The Triton X-100 lysate was incubated with the beads for 3 h at room temperature. The beads were then collected by centrifugation in a microfuge (12000 g for 2 min), and washed in immunoprecipitation buffer [150 mM NaCl, 10 % glycerol, 0.1 % (v/v) Triton X-100 and 20 mM Hepes/NaOH, pH 7.4]. The beads were washed a further three times, resuspended in 130 μl of sample buffer and boiled for 2 min before being centrifuged for a final time at 12000 g for 2 min. The supernatant, which constitutes the immunoprecipitated material, was then analysed for phosphoproteins as described below.

Detection of ^{33}P -labelled proteins

Samples were taken into sample buffer containing 2 % SDS and 10 % β -mercaptoethanol, and proteins were separated by SDS/PAGE using a 10 % (w/v) polyacrylamide gel and discontinuous buffer system described by Laemmli [36]. Gels were fixed and incubated with Amplify solution (Amersham International) for 30 min before being dried down. Detection of ^{33}P -labelled proteins was performed using a Fuji BAS 2000 Image Analyser (Fuji Film, Tokyo, Japan).

Identification of annexin V by electrophoresis and Western-blot analysis

Proteins were separated by SDS/PAGE as described above and by Laemmli [36].

For Western-blot analysis, proteins were transferred to nitrocellulose [37]. Complete transfer was confirmed by staining of the nitrocellulose with 0.1 % (w/v) Ponceau S in 3 % (w/v) acetic acid and destaining the background with 10 % (v/v) acetic acid. Subsequently, the blot was washed in PBS (0.15 M NaCl, 9 mM $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ and 1 mM $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, pH 7.4) containing 0.1 % (w/v) Triton X-100. The nitrocellulose membrane was then saturated with PBS containing 5 % (w/v) non-fat dried skimmed milk (Safeway).

Nitrocellulose blots were probed overnight with a 1:1000 dilution of rabbit anti-human annexin V in PBS containing 0.1 % Triton X-100. Immunoreactive bands were detected with horseradish peroxidase-anti-rabbit IgG (1:1000 dilution), and peroxidase activity was detected using the enhanced chemiluminescence detection system used in accordance with the manu-

facturer's instructions. Annexin V immunoreactivity was then quantified by densitometry as previously described [8].

Treatment of platelets with okadaic acid

Resting platelets were prepared as described above. Okadaic acid was added to a final concentration of $0-1 \mu\text{M}$, in Hepes-buffered Tyrodes solution, as indicated in Figure 2. The platelets were then incubated for 1 h at 37°C in the presence of okadaic acid. Platelets were then suspended in buffer A and lysed, and membrane-bound annexin V was assayed by Western blotting.

Treatment of platelets with staurosporine

Platelets were treated with A23187 in the presence or absence of the protein kinase inhibitor staurosporine ($1 \mu\text{M}$) in Hepes-buffered Tyrodes solution for 10 min at 37°C . Platelets were then lysed in buffer A, and membrane-bound annexin V was assayed by Western blotting.

Treatment of platelets with alkaline phosphatase

Platelets were lysed in the presence of $0.8 \mu\text{M}$ Ca^{2+} , and EGTA-washed membranes containing tightly bound annexin V were isolated as previously described [8]. The membranes were then suspended in buffer A (pH 8.0) in the absence or presence of various concentrations of alkaline phosphatase as indicated in Figure 3. The membranes were then incubated at 37°C for 30 min. The platelet membranes were then washed three times in buffer A (pH 7.4) before being solubilized in buffer B as described above. Annexin V in the Triton X-100-soluble membrane fraction was then determined by SDS/PAGE and Western blotting.

Statistical analysis

The amount of membrane-bound annexin V was measured by densitometry as described above. Combined data are presented as means $\pm$ S.E.M. from different blood donors. Corresponding means were analysed using the Student's *t* test as indicated below.

RESULTS AND DISCUSSION

When platelets are stimulated with 0.1 unit/ml thrombin or $5 \mu\text{M}$ A23187 in the presence of 1 mM extracellular Ca^{2+} , annexin V binds to the membranes in a manner that cannot be reversed by EGTA extraction (Figure 1; [7,8]). In order to investigate

Figure 1 Subcellular distribution of annexin V in platelets by Western-blot analysis

Control platelets (C) or A23187-treated platelets (A) were lysed and subfractionated as described in the Materials and Methods section. The EGTA-elutable annexin V (lanes a–f), Triton X-100-extractable annexin V (lanes g and h) and Triton X-100-resistant annexin V (lane i) were detected by SDS/PAGE and Western blotting.

whether annexin V binding to membranes is an energy-dependent process, platelets were therefore stimulated with A23187 in the presence and absence of 5 mM D-glucose. When the platelets were activated in the absence of D-glucose, the amount of annexin V that was associated with membranes was reduced to $74 \pm 3\%$ ($n = 4$; $P < 0.01$) of that observed in the presence of D-glucose.

In order to investigate further the mechanism by which annexin V binds to membranes, a broken-cell system was developed as described in the Materials and Methods section. Initial experiments using this system were designed to investigate the requirement for ATP. There are several potential mechanisms by which ATP could induce the association of annexin V with membranes. For example, the phosphorylation of one or more platelet components may be required for binding, or, alternatively, the action of an ATPase may induce conformational changes that would allow proteins to interact. In order to distinguish between these possibilities, cytosol was therefore either depleted of ATP with apyrase (see the Materials and Methods section) or supplemented with 0.25 mM MgATP or 0.25 mM ATP[S]. When apyrase-treated cytosol was incubated with membranes in the presence of $10 \mu\text{M}$ Ca^{2+} , a concentration that induces mainly EGTA-irreversible binding [8], the amount of annexin V bound to the membranes was comparable with that observed in the presence of EGTA. In contrast, when the cytosol was supplemented with MgATP, the amount of annexin V bound to membranes was found to increase to $201 \pm 15\%$ ($n = 5$, $P < 0.01$) of that observed in the control membranes. In addition, when the cytosol was supplemented with ATP[S], annexin V was also found to bind to membranes. Furthermore, following quantification it was apparent that ATP[S] increased the amount of annexin V that bound to membranes to $255 \pm 17\%$ ($n = 4$, $P < 0.02$) of that observed in control membranes.

Analogues of ATP can be utilized in some but not all ATP-dependent processes, and therefore provide a useful means of determining the mechanism by which ATP mediates a specific process. ATP[S] can be utilized as a substrate for protein kinases, but cannot be used in place of ATP in other ATP-dependent processes [38]. The observation that ATP[S] can induce annexin V to bind to membranes therefore suggests that annexin V relocation is a phosphorylation-dependent process. In addition, it has previously been shown that when ATP[S] is used as a substrate for protein kinases, the thiophosphorylated target protein shows an increased resistance to protein phosphatase activity [38]. As mentioned above, ATP[S] induces more annexin V to bind to membranes than ATP alone does. ATP[S] may therefore increase the amount of annexin V bound to membranes by maintaining the relocation machinery in a phosphorylated state. Interestingly, ATP[S] has also recently been shown by Morimoto and Ogihara [39] to support the calcium-dependent release of serotonin from permeabilized human platelets. These workers suggested that ATP[S] may promote serotonin release by maintaining the phosphorylated state of the exocytotic machinery [39].

When the ATP concentration required to induce tight binding of annexin V was investigated further, it was apparent that $25 \mu\text{M}$ ATP induced maximal binding of endogenous annexin V to the membrane fraction (results not shown). This ATP concentration has been reported to be sufficient for many ATP-dependent processes, including the activation of many protein kinases, such as the insulin receptor and cAMP-dependent protein kinase [40,41]. These experiments demonstrate that the ATP-dependent relocation of annexin V to membranes is likely to represent a physiological process. These data demonstrate that annexin V relocation is an ATP-dependent event, and that

Figure 2 Okadaic acid enhances annexin V binding to membranes

Intact platelets were treated with okadaic acid as described in the Materials and Methods section. The inset shows membranes that were subsequently isolated and assayed for membrane-associated annexin V (An V) by SDS/PAGE and Western blotting. Quantification of the amount of membrane-associated annexin V is shown in relation to the amount of annexin V bound to membranes in resting (control) platelets. Values are means $\pm$ S.E.M. ($n = 3$).

this ATP dependency probably reflects the previous observation that an energy-dependent step is necessary for maximal binding of annexin V to platelet membranes. In addition, these experiments suggest that the phosphorylation of one or more platelet components is necessary for annexin V to bind tightly to membranes. We have previously shown that annexin V is not phosphorylated following platelet activation [8]. It is, however, possible that phosphorylation of components of the relocation machinery, other than annexin V, is required for binding.

The role of phosphorylation in mediating annexin V relocation was investigated further using intact cells. The protein phosphatase inhibitor okadaic acid has been shown to increase dramatically the number of phosphorylated proteins in many cell types, including hepatocytes, adipocytes [42] and platelets [43,44]. The effect of okadaic acid on the subcellular location of annexin V was therefore investigated. The results show that okadaic acid induces annexin V to bind to platelet membranes in a concentration-dependent manner (Figure 2). Furthermore, it was apparent that 10 nM okadaic acid induced an increase of $191 \pm 14\%$ ($n = 3$) in the amount of membrane-associated annexin V when compared with the untreated control. This concentration of okadaic acid has been shown to inhibit protein phosphatase 1 and protein phosphatase 2A in intact cells [45,46], an observation that implicates inhibition of protein phosphatase 1 and protein phosphatase 2A in regulating the relocation. Interestingly, Toyoda and co-workers [47] have recently reported that following platelet activation there is a decrease in protein phosphatase 2A activity associated with the Triton X-100 soluble fraction. It is therefore possible that inhibition of protein phosphatase 2A activity, following treatment with okadaic acid, results in the phosphorylation of the relocation machinery, and the subsequent relocation of annexin V to membranes.

The role of phosphorylation in the relocation process was investigated further by stimulating platelets with A23187 in the presence or absence of the non-specific kinase inhibitor staurosporine. The cells were then assayed for membrane-associated annexin V as described in the Materials and Methods section. The amount of membrane-associated annexin V in the cells

Figure 3 Treatment of membranes with alkaline phosphatase releases annexin V (An V) from platelet membranes

Platelet membranes containing annexin V were incubated with 4.8, 34, 46, 232 and 1160 units (U)/ml alkaline phosphatase. Membrane-associated annexin V was then detected by SDS/PAGE and Western blotting (see inset), and quantified as described in the Materials and Methods section.

treated with staurosporine was found to be reduced to $75.9 \pm 5.2\%$ ($n = 3$; $P < 0.01$) of that in the maximally stimulated control cells. Inhibition of protein phosphorylation therefore reduces the amount of annexin V that binds to membranes following activation. The observation that the addition of staurosporine can inhibit annexin V relocation to membranes is consistent with the hypothesis that phosphorylation of the annexin V-binding protein induces annexin V to bind to membranes. The reason why A23187-induced relocation of annexin V is not completely attenuated by the addition of staurosporine is unclear. It is unlikely to be due to the intracellular concentration of staurosporine being insufficient to inhibit completely protein kinase activity, as when 10 μ M staurosporine was used, further inhibition of relocation was not observed (P. J. Trotter, M. A. Orchard and J. H. Walker, unpublished work). There are, however, two possible explanations why the addition of staurosporine does not completely prevent annexin V from binding to platelet membranes. First, it is possible that in intact cells a proportion of the phosphoprotein responsible for annexin V relocation is already phosphorylated. The membranes would therefore be competent for the association of annexin V following a subsequent increase in cytosolic calcium levels. A second explanation why inhibition of kinase activity does not completely attenuate annexin V relocation in intact cells is that signal-transduction mechanisms, in addition to phosphorylation, may influence annexin V binding to membranes. Multiple signal-transduction pathways are known to regulate many platelet responses, such as secretion, where both protein kinase C-dependent and GTP-dependent pathways [48,49] are believed to play a role.

The above experiments suggest that the phosphorylation of one or more membrane components is required for annexin V to bind tightly to membranes. Experiments were therefore performed to investigate whether the dephosphorylation of membrane components induces the release of annexin V from the membranes. Membranes containing tightly bound annexin V were therefore isolated from platelets, and treated with various concentrations of alkaline phosphatase as described in the Materials and Methods section and Figure 3. The results

Figure 4 A23187- and PMA-induced protein phosphorylation of platelets

Platelets were labelled with ^{33}P . Resting (C), A23187-stimulated (A) or PMA-stimulated (T) platelets were then subfractionated into cytosolic (Cyt) (lanes a–c), Triton X-100 (TX-100)-soluble (lanes d–f) and cytoskeletal fractions (lanes g–i). The radioactivity incorporated into proteins was determined by SDS/PAGE and PhosphorImager analysis as described in the Materials and Methods section. Molecular masses are indicated on the left.

demonstrate that dephosphorylation of membrane components with alkaline phosphatase does induce annexin V to be released from membranes in a concentration-dependent manner (Figure 3), and are consistent with the phosphorylation model proposed above.

In order to test directly the hypothesis that the phosphorylation of membrane-localized annexin V-binding proteins regulates binding, a series of experiments were performed to investigate whether endogenous annexin V does bind to phosphoproteins. Initial experiments were performed to investigate whether the conditions required for annexin V relocation are accompanied by changes in the phosphorylation state of membrane-localized proteins. Platelets were loaded with $[^{33}\text{P}]\text{P}_i$ as described in the Materials and Methods section. Following stimulation with A23187 or the phorbol ester PMA, it was apparent that the pattern of proteins phosphorylated differed from that of unstimulated, control cells. PMA induced the phosphorylation of several cytosolic proteins, including that of the 47 kDa pleckstrin, which has traditionally been used as a marker of protein kinase C activation in platelets (Figure 4, lane c, top arrow). More interestingly, treatment with the calcium ionophore A23187 induced changes in the phosphorylation pattern of proteins that required Triton X-100 for their extraction. For example, proteins of 37 and 20 kDa appeared to be phosphorylated in a calcium-dependent manner (Figure 4, lane e, middle and bottom arrows respectively), and longer exposure times demonstrated the existence of many phosphoproteins in this fraction (Figure 5, lanes a and b). These studies show that many Triton-soluble membrane-localized proteins are targets for calcium-dependent protein kinase activity following platelet activation, and suggest that the phosphorylation of membrane-localized components may be important in the regulation of membrane-localized processes.

Figure 5 Immunoprecipitation of annexin V-associated phosphoproteins

^{33}P -loaded platelets were stimulated with A23187, and the membrane-associated material was extracted with 0.1% (v/v) Triton X-100. Immunoprecipitated and non-precipitated materials are indicated by P and S respectively. Phosphorylated material immunoprecipitated by preimmune serum (lane c) or antiserum against annexin V (lane d) was revealed by SDS/PAGE and PhosphorImager analysis. Arrowheads indicate the positions of phosphoproteins which are immunoprecipitated by antibodies specific for annexin V. Material that was not immunoprecipitated by preimmune serum or antiserum against annexin V is shown in lanes a and b respectively.

The above experiment identifies several membrane-associated phosphoproteins that are phosphorylated in response to a stimulus that induces annexin V to bind tightly to membranes. These proteins are candidates for regulatory proteins that may play a role in the relocation of annexin V to membranes. In order to identify whether any phosphoproteins are associated with membrane-associated annexin V, the Triton X-100-extractable material was subjected to immunoprecipitation as described in the Materials and Methods section. Phosphoproteins that co-precipitated with annexin V were subsequently identified by SDS/PAGE and autoradiography. Immunoprecipitation using antibodies specific for annexin V pelleted phosphoproteins of 25, 50 and 83 kDa that were not precipitated by preimmune serum (compare Figure 5 lanes d and c). In addition, less intense phosphoproteins of 37, 45 and 110 kDa were found to be associated with annexin V in this fraction. Phosphoproteins of 50 kDa and 83 kDa were also found to co-precipitate with annexin V when a monoclonal antibody specific for annexin V, but not when a monoclonal antibody specific for annexin II, was used in the immunoprecipitation (results not shown). These results clearly demonstrate that, following platelet activation, annexin V relocates from the cytosol and binds directly or indirectly to a number of phosphoproteins in platelet membranes. The identities of these membrane-localized phosphoproteins are unclear, although it is possible that the predominant 50 kDa phosphoprotein identified here represents the annexin V-binding protein previously identified in cross-linking studies [8].

The work presented here demonstrates that annexin V relocation to membranes is an energy-dependent event, and that ATP is involved in the relocation. Furthermore, the finding that ATP[S] increases the ability of annexin to bind to membranes

and that this binding is influenced by both protein phosphatase and protein kinase inhibitors implicates protein phosphorylation in the mechanism involved in inducing annexin V to bind to membranes. The subsequent identification of phosphoproteins that interact with membrane-associated annexin V provides direct evidence that annexin V binds to phosphorylated proteins following platelet activation. Taken together these results are consistent with a model in which a calcium-dependent protein kinase phosphorylates proteins involved in binding annexin V tightly to the membranes. In this way annexin V may play a role in coupling the increase in intracellular calcium that follows platelet activation to one or more membrane-localized functions. This model is consistent with the previous demonstration that MgATP, in the presence of cytosolic proteins, enhances the binding of annexins to both chromaffin-granule membranes [50] and rat hepatocyte membranes [51], and with the study by Lin and co-workers [52], who have demonstrated that ATP stimulates annexin VI-dependent budding of clathrin-coated pits.

Interestingly, annexin V is not the only annexin that has been proposed to bind to a phosphoprotein. Following T-cell activation, Dubois and co-workers [53] have shown that annexin VI binds to a phosphoprotein with characteristics similar to p56^{lck}. Furthermore, this association was seen to be stable in the presence of EGTA, suggesting that annexin VI may be binding to p56^{lck} by a mechanism analogous to that of annexin V and its binding proteins. In addition, annexin IV has been shown to inhibit calmodulin-dependent kinase II activation of Cl⁻ channels [54]. Annexin IV neither inhibits the calmodulin-dependent kinase II nor acts as substrate for the kinase; it is therefore possible that annexin IV mediates its inhibitory effects by binding to the phosphorylated channel itself or to its regulatory subunits.

In summary, the work presented here demonstrates for the first time that both micromolar calcium concentrations and ATP are required for annexin V to bind tightly to platelet membranes in a manner where it is resistant to extraction with EGTA. In addition, the observations reported here are consistent with a model where the phosphorylation of one or more annexin-associated proteins is a prerequisite for tight binding. The demonstration that annexin V relocation to platelet membranes is tightly controlled is consistent with an important role for annexin V in platelet activation.

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