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Cap Z, a calcium insensitive capping protein in resting and activated platelets

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Abstract Capping of the barbed-ends of actin filaments is an important mechanism for control of the cytoskeleton. In platelets, a valuable model system, it has been thought that gelsolin was the major capping protein. We now report that platelets contain ~2 μM Cap Z, a calcium insensitive heterodimeric capping protein; two major and additional minor isoforms of both α and β subunits are present. In lysates from resting platelets 75–80% of the Cap Z sediments with the high speed pellet, but if the platelets are activated with thrombin for 10 s, about 15% of the Cap Z leaves the pellet fraction and is found in the high speed supernatant where it is not bound to actin. This translocation of Cap Z to the supernatant is also observed when resting platelets are lysed into buffer containing 50–100 μM GTPγS and 10 mM EGTA. Our results suggest that release of Cap Z from some actin filaments could generate free filament barbed-ends. An increase in free barbed-ends has been reported in platelet lysates prepared shortly after thrombin activation.

Key words: Blood platelet; Cytoskeleton; Cap Z; Capping protein; Thrombin activation; 2-D electrophoresis

1. Introduction

Platelets are a valuable model system for studies of the cytoskeleton and its reponse to agonists. We previously reported that the high (~500 μM) G-actin pool in resting human platelets could be accounted for by actin complexed with thymosin beta four ($T\beta_4$) only if the high affinity barbed-ends of the F-actin filaments were more than 95% capped [1,2]. The K_d of the barbed-ends for G-actin is ~0.1 μM [3], 5-fold lower than that of pointed ends or of thymosin beta four for G-actin which are both ~0.5 μM [1,3]. When platelets are activated, about half of this monomeric actin is rapidly mobilized into F-actin [4,5]; polymerization correlates with a several-fold increase in free barbed-ends of actin. However, the $T\beta_4$ that is released by this polymerization retains its ability to bind G-actin [5]; this is also true for activated neutrophils [6]. These observations suggest that the control of polymerization does not depend on a change in affinity of $T\beta_4$ for G-actin, but rather on the availability of free barbed-ends. Control of polymerization by this mechanism has recently been demonstrated in vitro [7].

We wanted therefore to determine which actin filament capping proteins occur in platelets and whether some of the new barbed-ends observed after activation come from the uncapping of existing filaments. The number of filaments in a resting platelet has been estimated as 2,000 from the rate of polymerization of pyrene-actin nucleated by cytochalasin-treated lysates of resting platelets [8]. Given the mean value for platelet volume of 7 fL/platelet [9], this corresponds to ~0.5 μM. Gelsolin has been thought to be the major capper in platelets, for Lind et al. [10] reported that about 10% of the estimated 12 μM gelsolin was complexed with actin in resting platelets. However, we found (Nachmias and Golla, unpublished) that only about 1% of the gelsolin associated with the cytoskeleton of resting platelets. Furthermore, Witke et al. [11] showed that in a mouse in which the gelsolin gene was deleted the resting platelets appeared normal, without signs of activation, and their F-actin was increased only ~10% over that in wild type resting platelets. If gelsolin were the major capper, its deletion should result in free barbed-ends, substantial actin polymerization and platelets bearing filopodia and/or lamellipodia. For comparison, cytochalasin D, an exogenous barbed-end capper, inhibits both actin polymerization and the formation of filopodia and lamellipodia in activated platelets [4,12]. The absence of the expected phenotype implies that gelsolin can account for only a small fraction of filament capping in resting platelets and that additional capping proteins are to be expected.

We now show that calcium insensitive heterodimeric capping protein (Cap Z) is present in significant amounts in human platelets and report that a fraction of this capping protein shifts into the high speed supernatant where it is not bound to actin shortly after activation.

2. Materials and methods

2.1. Preparation of cytoskeletal (CSK), high speed pellet (HSP) and high speed supernatant (HSS) fractions from lysates

Washed, resting platelets were prepared as in [13]. All donors gave informed consent using an approved protocol. For controls, 100 μl of resting platelets (2×10^9/ml at 37°C) were added to Eppendorf tubes at RT containing 2 μl of Tyrode's buffer; 4 μl of an equal mixture of: 10 mg/ml leupeptin; 1 M benzamidine; 2 mg/ml aprotinin and 20 mM 3,4-dichloro isocoumarin; 2 μl of 50 U/ml hirudin and 10 μl of ice-cold 20% Triton X-100 containing 100 mM EGTA pH 7.0; the lysate was rapidly mixed and put immediately onto ice. For GTPγS experiments the nucleotide was added to the platelets at lysis from a 1 mM stock solution to give 50 or 100 μM final concentration. For thrombin stimulated platelets, 100 μl of the resting platelets were added to 2 μl of 100 U/ml thrombin in Eppendorf tubes, briefly agitated and after 5, 10 or 15 s 4 μl of the protease inhibitor cocktail and 2 μl hirudin were added simultaneously with the lysis buffer and treated as for controls. The samples were kept on ice for 10 min, then cytoskeletons (CSKS) were pelleted by centrifugation at 12,500 × *g* at 5°C for 10 min. The upper 80 μl of this low speed supernatant was pipetted off and centrifuged in a TLA rotor at 90,000 rpm (310,000 average *g*) for 1 h to yield a high-speed supernatant (HSS). Only the upper 2/3 of the HSS was used for analysis. For HSP, the lysate was directly centrifuged at 90,000 rpm

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Abbreviations: CSK, cytoskeleton; HSP, high-speed pellet; HSS, high-speed supernatant; WPL, whole-platelet lysate; $T\beta_4$, thymosin beta four.

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and the upper 2/3 of the HSS taken as before, then the remaining HSS was removed leaving the small, dense high speed pellet. Cytoskeletons or HSPs were washed once with 0.5 ml of ice-cold buffer containing 10 mM PIPES, 140 mM NaCl, 2.7 mM KCl, EGTA 5 mM, pH 6.5 and briefly air dried.

2.2. Electrophoresis

For one dimensional SDS gel analysis, the method was as in [13,14]. For two dimensional IEF-SDS gel analysis the method of O'Farrell [15] was used with slight modifications: ampholytes were in the pH ranges of 5–8 and 3–10 and the preruns were at 200 V for 30 min, 300 V for 15 min and 400 V for 15 min. The separation run was at 500 V for 3.5 h. The urea lysis buffer contained 50 mM NaF and 10 mM sodium pyrophosphate to inhibit phosphatases. One dimensional non-denaturing gels were performed as in [16]; for the second dimension a narrow lane was excised and treated as in [15].

2.3. Immunoblotting

This was performed as described previously [13,14] with peroxidase labelled rabbit anti-goat (Sigma Chemical Co. St Louis, Mo.) or goat anti-rabbit (Boehringer Mannheim Biochemical Co. Indianapolis, IN) followed by enhanced chemiluminscence (Amersham, Arlington Heights, IL). The major antibody was affinity purified polyclonal goat anti-chicken skeletal muscle Cap Z antibody [17,18] which reacts with all isoforms. We also used affinity purified antibody to the full length human β_2 subunit (Genbank # U03271, [20]); this antibody detects both β_1 and β_2 subunits. A polyclonal antibody to chicken β_2 carboxy terminus (R-25), which differs from the carboxy terminus of β_1 [19], was a generous gift of Dr. J. Cooper and Dr. D. Schafer, Washington University St. Louis). Antibody to the α subunit of *Dictyostelium* capping protein [21], was a gift from Dr. M. Schleicher, Ludwig-Maximilians-Universitaet Muenchen, FRG. Antibody to actin (C4) was from ICN Biomedicals, Costa Mesa, CA.

Densitometry was performed as in [4]. Several loadings and exposures of standards and unknowns were used to determine the range of linearity and several measurements were made of each band.

Lower and upper estimates were made of the Cap Z in a known number of platelets using chicken muscle Cap Z as a standard and antibodies to chicken or to human Cap Z. Since purified human Cap Z was not available as a standard, the anti-human β_2 was also used to compare the Cap Z in platelets to a known amount of in vitro translated β_2 subunit of human Cap Z (Genbank accession # U03271 [20]. Incorporation of [^{35}S]methionine into the β_1 subunit of chicken muscle Cap Z was first determined by in vitro translation in reticulocyte lysate [14]; the concentration of β_1 subunit in the translation products was measured by comparing the reactivity of aliquots of the translation products with known quantities of purified chicken Cap Z on immunoblots. Results were expressed as DPM/ng protein. Based on this result, the concentration of human Cap Z β_2 in translation products was determined using the same batch of reticulocyte lysate and assuming identical methionine incorporation for the human and chicken subunits since there are the same number of methionines in the two isoforms [19,20]. The quantity of Cap Z β in platelets was then estimated by comparing immunoblots of known numbers of platelets ($0.15–1.5 \times 10^7$) to those of the human Cap Z β_2 translation product (2.8–11.3 ng). These immunoblots were scanned using an EagleEye II Still Video System (Stratagene) and densitometry with ImageQuant software (Molecular Dynamics).

2.4. Indirect immunofluorescence

To localize cap Z, washed platelets were allowed to spread on glass or fibrinogen and treated as previously described [13].

2.5. Chemicals

All chemicals were of reagent grade. Thrombin (# T4648), apyrase (A6132), hirudin (H7380), GTPγS and leupeptin were obtained from Sigma Chemical Co., St. Louis, MO.

3. Results

In whole platelet lysates (WPL) probed with antibody to chicken muscle cap Z we observed two electrophoretic bands with the same mobility as purified muscle Cap Z (Fig. 1, lanes 1–6, 9 and 10). The upper band cross-reacts with polyclonal antibody to the α chain of *Dictyostelium* capping protein (32/34) and the lower band cross-reacts with the R25 antibody (to the carboxy terminus of the β_2 isoform) as shown in lanes 7–8 and 11–12. The bands appeared unchanged in platelets incubated with thrombin for 10 s (lanes 7–12).

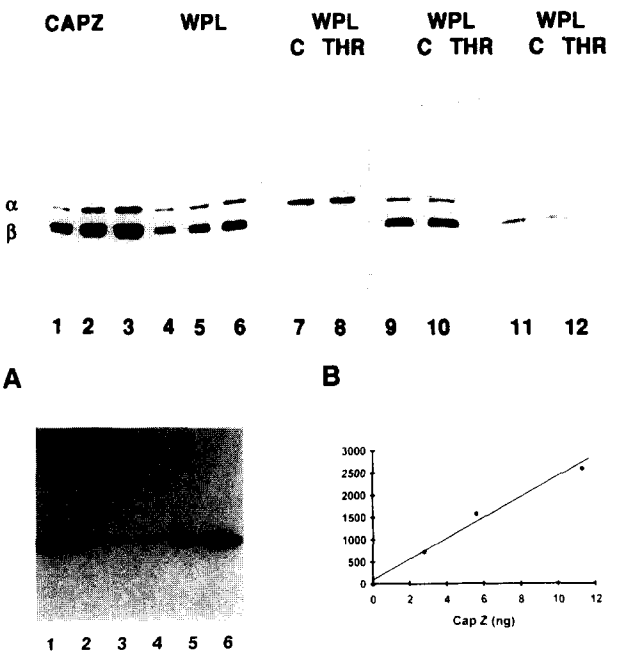


Fig. 1. A: immunoblots of Cap Z and whole platelet lysates. Lanes 1–6 show chicken muscle Cap Z and whole platelet lysates (WPL) probed with antibody to chicken muscle Cap Z; lanes 7–12 show WPL before and after thrombin treatment probed with three different antibodies. Lanes 1–3: chicken muscle Cap Z (2, 4 and 6 ng); lanes 4–6: WPL (4.08, 5.44, 8.16×10^6 platelets). Lanes 7, 9, 11 are control WPL and lanes 8, 10, 12 are thrombin treated WPL (10 s, 2 U/ml). Lanes 7 and 8 are probed with antibody to *Dictyostelium* α chain; lanes 9 and 10 are probed with antibody to chicken muscle Cap Z and lanes 11 and 12 are probed with R-25, an antibody to the carboxy terminus of non-muscle Cap Z β_2 subunit. B: quantitation of Cap Z in human platelets. Left: immunoblot using antibody to human fusion protein; lanes 1–3 are 15, 3.8, and 1.5×10^6 platelets; lanes 4–6 are human β_1 subunit translation products at 2.8, 5.6, and 11.3 ng. Right: standard curve of quantitation of the translation products by densitometry. Area (y-axis) is in arbitrary units.

We estimated the concentration of platelet Cap Z using standards of human Cap Z β_2 prepared by in vitro translation and antibody to human Cap Z β_2 fusion protein which also recognizes the β_1 subunit. This gave Cap Z as $2.2\,\mu M \pm 0.26$ (S.E.M.) on 5 determinations from two experiments. One set of results is shown in Fig. 1B. In addition, we estimated upper and lower limits with known amounts of chicken muscle Cap Z as a standard in several experiments. The lower limit, using antibody made to chicken muscle Cap Z, which will presumably react more strongly with the antigen to which it was made than to the platelet Cap Z, was 0.5–1 μM. The upper limit, using antibody made to α and β human fusion proteins was 6–8 μM.

In resting platelets, 75–80% of total Cap Z pelleted at $310{,}000 \times g$ for 1 h (HSP) as shown in Fig. 2. This sediments both cytoskeletal and membrane-associated actin [22]. Cytoskeletons sedimented at low g contained about 25% of the total Cap Z. About half the Cap Z in the cytoskeletons could be chased by incubation with excess gelsolin-actin complexes

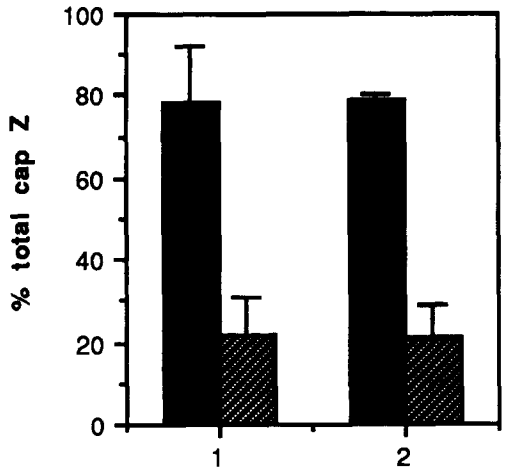


Fig. 2. Distribution of Cap Z in HSP and HSS in resting platelets from the densitometry of immunoblots; two separate experiments are shown.

which also have high affinity for barbed-ends (data not shown). When 20 μM phalloidin was added at lysis, Cap Z and actin (but not myosin) increased in the cytoskeleton by 50% and 100%, respectively (data not shown).

Free barbed-ends increase several fold in lysates of platelets made after thrombin activation [5,8]. If Cap Z were released from filament ends after stimulation and could not recombine for some period of time (uncapping), we should detect increased amounts of it in the HSS. Indeed, we found that Cap Z increased in the HSS at 5–15 s after thrombin as compared with controls (Fig. 3A). The HSP fraction made from 10 s activated platelets shows a ~15% loss of Cap Z α subunit which appears in the HSS (Fig. 3B). The relative increase in the HSS is greater since it contains only 20–25% of total Cap Z. Unlike the effect of phalloidin, Cap Z decreased while both actin and myosin increased over control levels in low speed cytoskeletons made 10 s after thrombin (not shown). Immunoblots of 1-dimensional native gels showed increased Cap Z in both low and high speed supernatants from thrombin stimulated platelets (Fig. 4 above) and two dimensional native-SDS gels showed that the Cap Z migrated separately from the actin in the HSS from stimulated platelets (Fig. 4 below). We also observed a shift of Cap Z from CSK to HSS when resting platelets were lysed in the presence of GTPγS as shown in Table 1.

Table 1
The effect of GTPγS at lysis on Cap Z in cytoskeletons (CSK) and high-speed supernatants (HSS) from resting platelets

A. Decrease in Cap Z in CSK of resting platelets lysed with addition of GTPγS as compared to controls.

GTPγS conc (mM)	% of control level in CSK	
	α band	β band
50	82.0 ± 1	63.5 ± 3
50*	86.2 ± 2.5	79.8 ± 6

B. Increase in Cap Z in HSS in resting platelets lysed with addition of GTPγS as compared to controls.

GTPγS conc (mM)	% of control level in HSS	
	α band	β band
50	130 ± 9	137 ± 15
50*	120.8 ± 2.5*	
100	124 ± 12	154 ± 40

The results are from three different experiments. The *value for HSS in B is from the same experiment as the *cytoskeletal experiment in A. The errors are standard deviations corrected for small (n from 3–5) sample size.

Fig. 5 shows that immunoblots of two-dimensional isoelectric focussing gels of platelets and fractions contain several isoforms of Cap Z; as these have relative mobilities similar to those previously identified in other cells [19] we have designated them similarly as α_1, α_2, β_1 and β_2, together with fainter spots indicated as primes. The two most prominent spots correspond in mobility to α_1 and β_2 of Schafer et al. [19]. The patterns from resting and activated platelets showed no consistent changes.

If Cap Z is involved in regulated uncapping we might expect it to be localized at the membrane, a major site for actin polymerization. Surface activated platelets, which are flat and large enough to be favorable for immunofluorescence show a distinct discontinuous rim of staining for Cap Z at the spreading margin (Fig. 6).

4. Discussion

Since the original descriptions of heterodimeric, calcium in-

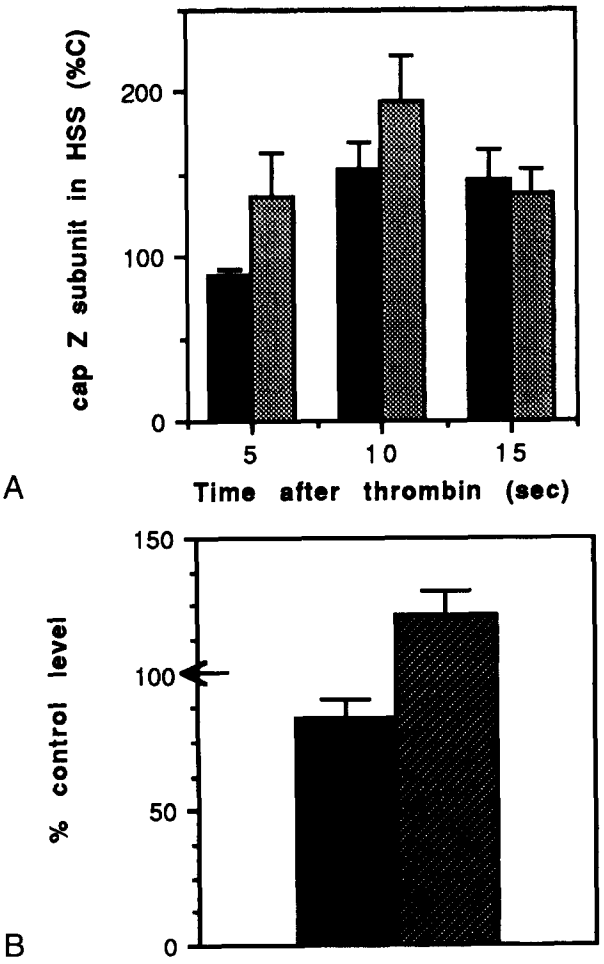
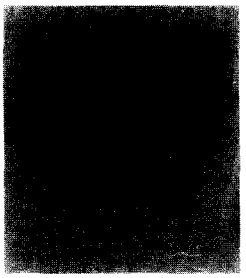


Fig. 3. Quantitative immunoblotting of control and thrombin treated platelets. A: Cap Z increases in the HSS at 5, 10 and 15 s after thrombin activation as compared to controls. Dark bars: α subunit; light bars: β subunit. B: Cap Z decreases in the HSP (black bar) and increases in the HSS (striped bar) in 10-s thrombin treated platelets as compared to controls. Pooled results from two experiments.



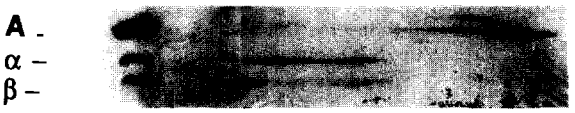


Fig. 4. Above: immunoblot for Cap Z from a native gel. Lanes 1 and 2, low speed supernatants from control and thrombin (10 s) treated platelets; lanes 3 and 4, high speed supernatants from control and thrombin treated platelets. On native gels proteins migrate as more diffuse bands than on SDS gels [16]. Below: immunoblot of two dimensional native-SDS gel of the HSS from thrombin treated platelets. It has been blotted with C4 (to actin) and with antibody to chicken Cap Z. A marks the position of actin; α and β mark the positions of the two chains of Cap Z on the second dimension SDS gel.

sensitive actin barbed-end capping activity in *Acanthamoeba* [23] and in muscle, where it was shown to be localized to the Z line [24,18], it has become clear that Cap Z is widely distributed in eukaryotic cells [19,25,26]. Two major isoforms of both α and β subunits have been observed. In brain and liver the β_2 isoform predominates, while β_1 predominates in muscle [19,25]. Platelets contain two major isoforms of both α and β Cap Z subunits similar in charge to those found in these other non-muscle cells and minor isoforms which have also been observed in muscle and gizzard [19].

We estimate total platelet Cap Z as ~2 μM. Since about 75% sediments with the washed HSP, we estimate that as much as 1.5 μM Cap Z could be associated with actin. This agrees reasonably well with the estimate of 0.5 μM filament ends [8], given the difference in experimental method. Since the affinity of non-muscle Cap Z for free actin ends has been measured as less than nanomolar under in vitro conditions [20], little Cap Z should dissociate to the HSS after lysis as our dilution is only 70 fold.

By ten seconds after activation, Cap Z in the HSP decreases about 15%. In parallel, Cap Z increases in the HSS. This shift could in principle be either free Cap Z or capper attached to very short actin oligomers. Oligomers could result if gelsolin, activated by the calcium transient after thrombin, cut some filaments very close to their ends. We therefore determined whether small oligomers would sediment under our conditions. The k value for our conditions is 6, where the time required for sedimentation in hours, $t = k/s$ where s is the sedimentation constant. In our experiments $t = 1$. Actin dimers alone have s values of ~6–6.6 [27] and the s value of Cap Z is estimated as 4 (capping protein in *Acanthamoeba*; [23]) or 4.6 (muscle Cap Z; [24]). Therefore oligomers of actin bound to Cap Z should be sedimented. Cap Z does not bind significantly to actin monomers [28]. In support of this conclusions, Cap Z and actin separated on 2-dimensional native-SDS gels of HSS from thrombin treated platelets. Finally, calcium, required for gelsolin activation, is not necessary for the shift of Cap Z since it was found when resting platelets were lysed into GTPγS and EGTA.

We conclude that thrombin treatment of intact platelets or platelet lysis in the presence of GTPγS causes some Cap Z to dissociate from filament ends. By 10 s after thrombin, the number of free barbed-ends in dilute platelet lysates increases by 70% [4], so that the timing of the loss of Cap Z from the HSP is appropriate to account for at least part of this increase. The mechanism of this release is of great interest; the effect of GTPγS suggests that a G-protein may be involved. It has been shown that actin polymerization and membrane ruffling is stimulated in resting cells by microinjecting activated G-proteins [29,30] or in permeabilized cells by GTPγS [31]. Finally, increased barbed-ends appear in permeabilized platelets treated with GTPγS or activated rac [32]. Cap Z has been localized to apposed membranes of epithelial cells and to the free margin of cultured cardiac cells [19,25]. The discontinuous localization of Cap Z at the ruffling membrane of actively spreading platelets suggests the possibility that Cap Z dissociates from fila-

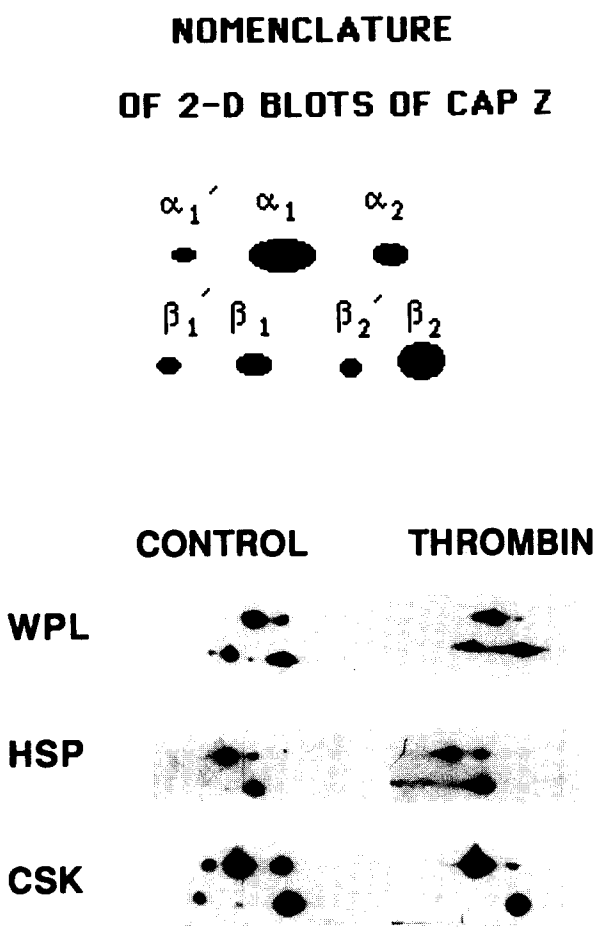


Fig. 5. Immunoblots of two-dimensional electrophoretic gels of whole platelets (WPL) high-speed pellets (HSP) and cytoskeletons (CSKS) from control and 10 s thrombin-treated platelets. Above, diagram of Cap Z isoforms (after Schafer et al., 1994). Below: all fractions are probed with antibody to chicken muscle Cap Z which probably reacts more strongly with the β_1 subunit. The pH increases to the right.

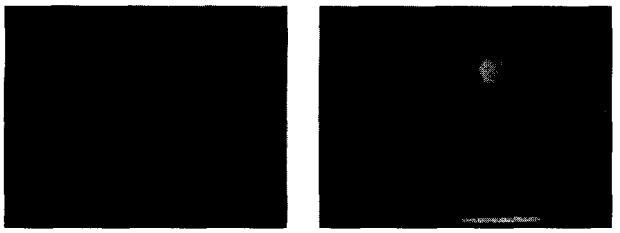


Fig. 6. Localization of Cap Z by indirect immunofluorescence in spreading platelets. Washed platelets were spread on glass for 30 min, fixed and stained with antibody R25 as described. Note the periodicities seen along the spreading margin (white bars and lines). Discontinuous staining was also observed using antibody to the human β-2 fusion protein. The fine periodicities average 0.75 μm center to center. Bar is 5 μm.

ments at specific sites along the membane. Further work will be directed at testing this hypothesis.

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