Introduction
The cytoskeleton in human platelets plays an important role in the dynamic changes that occur during platelet adhesion, shape change and clot retraction. The actin filaments in the cytoskeleton are cross-linked into three-dimensional networks by actin-binding protein (ABP or filamin). The cytoskeleton is linked to the platelet plasma membrane by an association between ABP and a transmembrane glycoprotein, GP Ib. GP Ib is the receptor for the plasma protein von Willebrand factor and is essential for adhesion of platelets to sub-endothelial structures. ABP consists of two identical subunits of 250 kDa that are linked head-to-head. The sub-units can be proteolytically split by calpain, a Ca^{2+}-dependent protease, into fragments of 190, 100 and 90 kDa. The 100 kDa fragment is a precursor of the 90 kDa fragment. The cDNA sequence of ABP was recently determined. The actin-binding region was localized to the N-terminal end of the 190 kDa fragment. GP Ib binding to ABP has previously been demonstrated in the 90 kDa region. Also actin binding was localized to the 90 kDa fragment in these studies.

Methods
Isolated platelets were extracted in 1% Triton X-100 in the presence or absence of the calpain inhibitor leupeptin and centrifuged at 8000 g for 4 min or at 100000 g for 3 h. Triton extracts were electrophoresed by crossed immunoelectrophoresis, with polyclonal antibodies to GP Ib or total platelet proteins in the second-dimension gel. Monoclonal antibodies (MAbs) TI10 or PM6/372 were incubated in an intermediate gel during electrophoresis. Co-precipitated MAbs were transferred to a nitrocellulose membrane and detected by enzyme-conjugated secondary antibodies. The corresponding gel was stained with Coomassie Brilliant Blue R for direct comparison with the nitrocellulose membrane.

Results
SDS/PAGE demonstrated that ABP was completely degraded during Triton extraction in the absence of calpain inhibitors. Western blotting after SDS/PAGE demonstrated that the monoclonal antibody MAb TI10, against ABP, bound to the 90 kDa fragment. MAb PM6/372 bound to the 190 kDa proteolytic fragment of ABP. MAB PM6/317, directed against the 190 kDa fragment of ABP, was interchangeable with PM6/372.

The MAb TI10 co-precipitated with the GP Ib immunoprecipitate obtained after degradation of ABP in platelets extracted in the absence of calpain.

Abbreviations used: ABP, actin-binding protein; GP Ib, transmembrane glycoprotein Ib; MAb, monoclonal antibody.
Fig. 2

Co-precipitation of MAb TI10 with the GP Ib immunoprecipitate on crossed immuno-electrophoresis

Triton extracts (2 × 10⁸ platelets/ml) prepared in the absence of leupeptin were electrophoresed into a second-dimension gel containing antibodies to GP Ib. Co-precipitated MAb TI10 was transferred to a nitrocellulose membrane. Bound MAb was detected by peroxidase-conjugated secondary antibodies. (a) Nitrocellulose membrane, (b) corresponding gel stained with Coomassie Brilliant Blue R.

Discussion

We have shown that MAb TI10 directed against the 90 kDa region of ABP co-precipitates with the GP Ib immunoprecipitate on crossed immuno-electrophoresis from Triton extracts prepared in the absence of calpain inhibitor. MAb PM6/317 directed against the 190 kDa fragment of ABP did not co-precipitate. The 90 kDa fragment of ABP was also adsorbed to Protein A-agarose by polyclonal antibodies to GP Ib. We conclude that the GP Ib-binding site in ABP is in the 90 kDa region of ABP. This confirms data previously reported [2].

The attempts to localize actin-binding regions of ABP were initiated by discrepancies in previous studies [1, 2]. The Triton-insoluble (cytoskeletal) fraction of platelets contain actin filaments and actin-associated proteins. We have demonstrated that the 90 kDa and the 190 kDa fragments of ABP sedimented in the Triton-insoluble fraction in approximately equal amounts. This implies that both the 90 kDa and the 190 kDa fragments are involved in actin binding. Using cell extracts, however, we cannot decide whether actin and ABP are directly or indirectly associated.

Material in this paper has been reproduced from a full-length paper accepted for publication in Thrombosis and Haemostasis with permission from the editors.


Received 28 June 1991