

# Identification and isolation of vinculin from platelets

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A vinculin-like protein was identified in chicken as well as in bovine platelets by ELISA competitive binding assay using antibodies against vinculin from chicken gizzard. By a modified procedure (J. Biol. Chem. (1980) 255, 1194–1199) we succeeded in isolating bovine platelet vinculin to apparent homogeneity. The structural identity of platelet and chicken gizzard vinculin was demonstrated by circular dichroism analysis. It was also shown that platelet vinculin induces a significant decrease in the low shear viscosity of F-actin. Vinculin, in all probability, plays an important role in the organization of actin filaments in platelets, especially in the linkages of microfilaments to the membrane.

*Platelet*

*Vinculin isolation*

*F-Actin–vinculin interaction*

## 1. INTRODUCTION

Blood platelets are highly contractile cells which display complicated morphological and biochemical changes upon activation. Platelets change shape, extend pseudopodia, secrete the content of their granules, and aggregate with each other. Contractile proteins have been implicated in most of these processes [1,2]. Resting, discoid platelets have very few actin filaments. After activation a network of microfilaments in the platelet body and bundles of actin filaments in the filopodia assemble from actin monomers or oligomers and actin microfilament associated proteins [3]. In all probability the interaction of microfilaments with membrane plays an important role in the hemostatic functions of platelets. However, only little information is available on the molecular components involved in these interactions. Although many contractile proteins – actin, myosin, high- $M_r$  actin binding protein, profilin, gelsolin,  $\alpha$ -actinin, tropomyosin, P235 protein, calmodulin, myosin kinase – have been identified and purified from platelets, the presence of these proteins is not sufficient to explain how actin filaments interact with the platelet membrane.

Recently, a new intracellular protein, vinculin ( $M_r$  130000), was found in a variety of cell types and tissues [4–7]. Vinculin is specifically associated with the cell membrane at focal contact areas as well as with different sites of actin filament-membrane association – zonula adherence of intestinal epithelium, intercalated discs of cardiac muscle and dense adhesion plaques of smooth muscle [8–10]. The intracellular location of vinculin has led to the suggestion that vinculin is involved in attachment of actin bundles to the membrane [8–10].

We report here that platelets contain vinculin-like protein. This protein may be important in the linkage of microfilaments to the platelet membranes.

## 2. MATERIALS AND METHODS

Platelet-rich plasma was separated from citrated chicken blood by centrifugation at  $100 \times g$  for 5 min at room temperature. Platelets were pelleted by centrifugation at  $2500 \times g$  for 15 min and after washing resuspended in 2 mM Tris, 1 mM EGTA, 0.5 mM PMSF, pH 9.0 (buffer A). The resuspended platelets were sonicated for 3 min at 4°C and

the suspension was stirred for 30 min at room temperature. The platelet homogenate obtained was centrifuged at  $100000 \times g$  for 45 min and the supernatant was used for identification of vinculin.

Vinculin-like protein was purified from acetone powder of bovine platelets obtained from 20 l of bovine blood. Two g acetone powder was suspended in 200 ml deionized water containing 0.5 mM PMSF and 0.5 mM EGTA. The suspension was centrifuged for 10 min at  $20000 \times g$  and supernatant was discarded. The pellet was resuspended in 200 ml of extraction buffer A and was stirred for 30 min at  $37^\circ\text{C}$  [11]. The centrifugation was repeated and the pellet was discarded. The pH in the supernatant was adjusted to 7.2. The extract was treated with 10 mM  $\text{MgCl}_2$  and then fractionated by ammonium sulfate [11]. The precipitate was dialyzed against buffer B (20 mM Tris, 20 mM NaCl, 0.1 mM EDTA, 15 mM 2-mercaptoethanol, pH 7.4) and applied to a DEAE-cellulose column ( $1.6 \times 16$ ). Fractions were analyzed on 10% polyacrylamide slab gels in the presence of sodium dodecyl sulfate [2].

Antivinculin antibodies were obtained by immunization of rabbits with chicken gizzard smooth muscle vinculin. The IgG fraction from rabbit antiserum was isolated by ammonium sulfate precipitation. Purified antibodies to vinculin were obtained by affinity chromatography on a vinculin-Sepharose column. The antibodies were characterized by standard immunological techniques and in all cases reacted only with vinculin [10].

The ELISA procedure as a quantitative competition-binding assay was used for identification of vinculin in platelet extract [3]. Antivinculin antibodies were incubated with different dilutions of platelet extract or purified vinculin overnight. Then the mixtures were transferred to Linbro 96 well plates previously incubated overnight at  $4^\circ\text{C}$  with  $2 \mu\text{g/ml}$  solution of purified chicken gizzard smooth muscle vinculin. The wells were washed and goat antirabbit IgG conjugated with horseradish peroxidase was added and incubated for 1 h. After washing the substrate for horseradish peroxidase, *o*-phenylenediamine was added. Microtiter wells were examined for development of color with an intensity well above that of negative controls.

Circular dichroism spectra were measured on a JASCO J41A spectropolarimeter. The data were expressed in terms of mean residual ellipticity (average  $M_r$  of a residue was assumed to be 115). Determination of secondary structure was performed as in [14] and published control spectra were used [15].

### 3. RESULTS AND DISCUSSION

To identify vinculin-like protein in chicken platelets we used an antibody against chicken gizzard smooth muscle vinculin. The crossreactivity of antibody with chicken gizzard smooth muscle vinculin and extract from chicken platelets was determined by a competition-binding experiment (fig.1). Chicken gizzard smooth muscle vinculin competes for the binding of antibody with vinculin-coated dishes. A similar result was obtained when extract from chicken platelets was used as competing antigen. In control experiments, where platelet-free chicken plasma or other proteins were used, no competition was observed with antivinculin antibody. These results indicate that extract

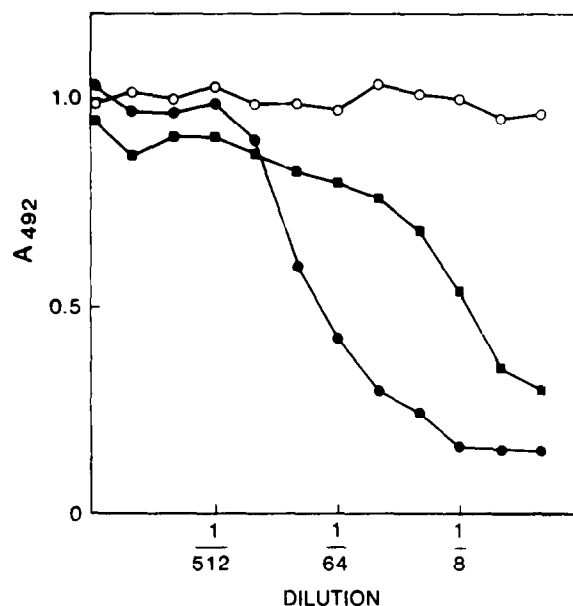


Fig.1. Identification of vinculin in extract from chicken platelets by ELISA competition-binding assay. (●—●) Vinculin from chicken gizzard smooth muscle; (■—■) extract from chicken platelets; (○—○) chicken platelet free plasma.

from chicken platelets contains a protein with the same antigenic determinants as vinculin from chicken gizzard smooth muscle. This vinculin-like protein contributed 0.1–0.3% of the total platelet proteins.

Vinculin-like protein has been purified from bovine platelets using a modification of the procedure developed for purification of vinculin from smooth muscle [11]. Chromatography through DEAE–cellulose of bovine platelet proteins extracted by buffer A separates protein of  $M_r$  130000 from other proteins (fig.2). This protein had the same electrophoretic mobility in polyacrylamide slab gel and was eluted at the same ionic strength from DEAE–cellulose as chicken gizzard smooth muscle vinculin. The crossreactivity on antibody against chicken gizzard smooth muscle vinculin with vinculin-like protein from platelets ( $M_r$  130000) was determined by two ELISA procedures: first, the direct binding of antibodies to the antigen-coated plate was examined, then a competition-binding assay was carried out (fig.3,4). It was demonstrated in both cases that pure vinculin-like protein from platelets crossreacted with the antibody against chicken gizzard smooth muscle vinculin and the value of crossreactivity was about 20%. To demonstrate

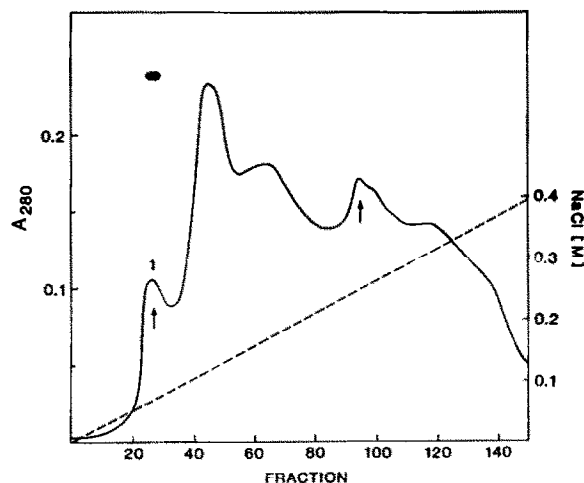


Fig.2. DEAE–cellulose column chromatography of extract from bovine platelets. Arrows show the position of vinculin and  $\alpha$ -actinin. The peak marked 1 was identified by electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulfate and contained a protein of  $M_r$  130000.

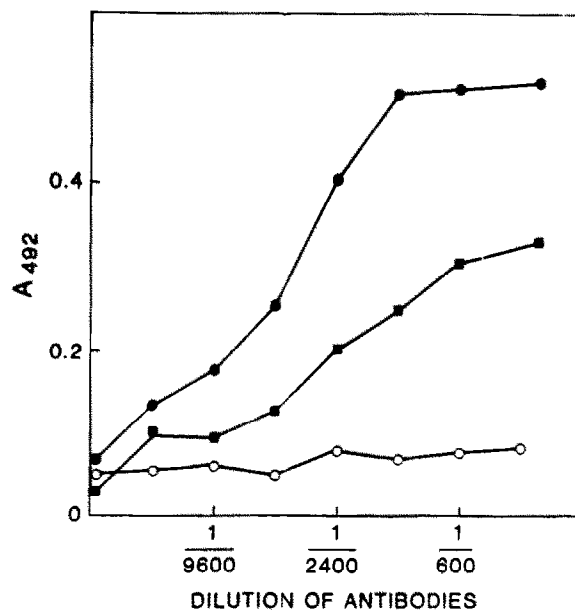


Fig.3. Binding of antibodies against chicken gizzard smooth muscle vinculin to different vinculins. The wells were coated with vinculin from chicken gizzard smooth muscle (●), vinculin from bovine platelets (■) and filamin (○).

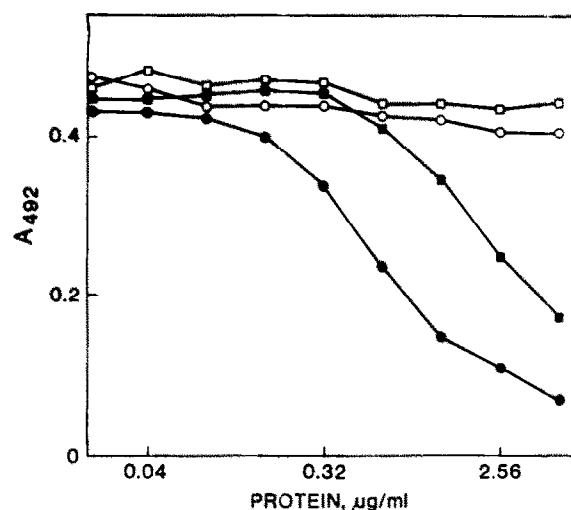


Fig.4. Competition of chicken gizzard smooth muscle and bovine platelet vinculins for the binding of antibodies. (●—●) Vinculin from chicken gizzard smooth muscle; (■—■) vinculin from bovine platelets; (○—○) filamin from chicken gizzard smooth muscle; (□—□) actin.

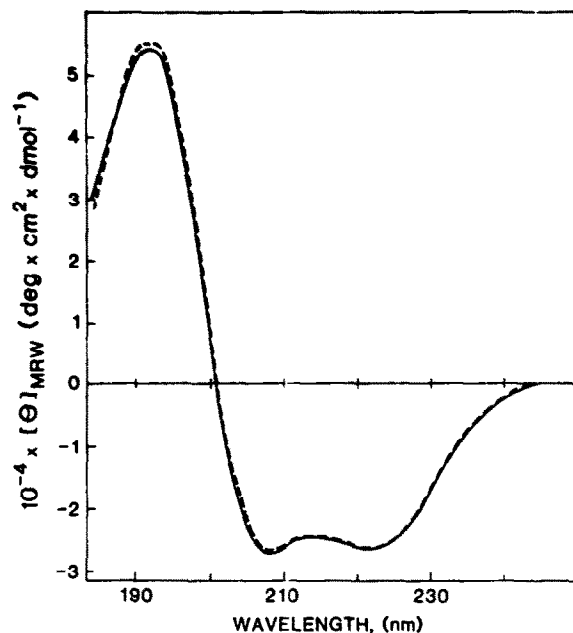


Fig.5. Circular dichroism spectra of vinculin in the far-ultraviolet region. (—) Vinculin from chicken gizzard smooth muscle; (---) vinculin from bovine platelets.

the structural similarity of chicken gizzard smooth muscle and platelet vinculin circular dichroism spectra were measured and secondary structure of both proteins was compared (fig.5). The identity of circular dichroism spectra of the two proteins in the far-ultraviolet region was demonstrated: the general contour, the extremum amount and position were shown to be identical. The spectra are typical of proteins with high content of  $\alpha$ -helices – about 80%. So, vinculin-like protein from platelets resembles smooth muscle vinculin. In addition, we have found that vinculin-like protein from platelets causes a decrease of the low shear viscosity of F-actin (fig.6).

In summary, a vinculin-like protein was identified and isolated from platelets. This protein has an apparent  $M_r$  of 130000 on sodium dodecyl sulfate polyacrylamide gel electrophoresis. It has a high content of  $\alpha$ -helices (about 80%) and common antigenic determinants with vinculin from chicken gizzard smooth muscle cells. Platelet vinculin causes a decrease of the viscosity of F-actin.

From the properties of vinculin, it appears likely that vinculin can play an important role in the

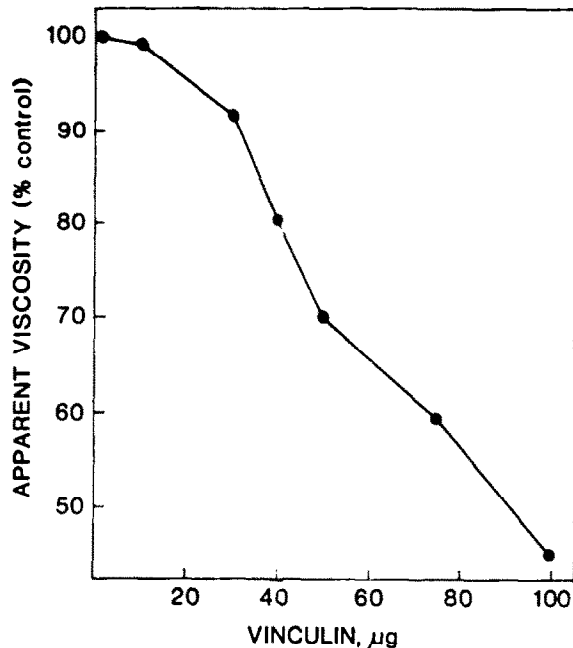


Fig.6. Effect of platelet vinculin on the apparent viscosity of F-actin. Solutions of F-actin (0.5 mg/ml) were mixed with different amounts of vinculin. The solutions were drawn into capillary tubes and after 4 h incubation their viscosities were measured by falling ball viscometry [14].

organization of actin filaments in platelets, especially in the linkage of microfilaments to the membrane.

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