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Demonstration of a Relationship Between Talin and P235, A Major Substrate of the Calcium-Dependent Protease in Platelets

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Talin is a 225,000-Dalton protein we have purified from smooth muscle. In chick embryo fibroblasts talin is found in adhesion plaques (focal contacts), areas where the cell is closely apposed to the substratum. In comparison with other cytoskeletal proteins, we found talin to be unusually susceptible to proteolysis and have identified a 190,000-Dalton proteolytic fragment of talin in the immunoblots of many tissues. These observations raised the possibility that the cleavage of talin to this fragment has physiological relevance. One system that we have investigated in which significant proteolysis occurs is platelets. During platelet activation several high-molecular-weight proteins are cleaved to lower-molecular-weight forms. Here we demonstrate that talin is closely related to one of these platelet high-molecular-weight proteins, P235. The purification of talin is comparable to that developed for P235, and the two proteins have similar biophysical properties. In addition, antibodies raised against chicken gizzard talin recognize P235 in purified form as well as in crude platelet extracts. The platelet protein also resembles smooth-muscle talin in its susceptibility to endogenous proteolysis: P235 is rapidly cleaved to a 190–200kD polypeptide by a calcium-activated protease found in platelet extracts. Moreover, partial proteolysis of P235 and talin with chymotrypsin, elastase, or trypsin also generates remarkably similar one-dimensional peptide maps. Because of their similar biophysical properties, immunological crossreactivity, and similar one-dimensional partial peptide maps, we conclude that P235 is the platelet form of talin.

Key words: actin-membrane, interactions, Ca^{++}-activated proteolysis, talin, platelets, calcium dependent protease

In order for a cell to interpret extracellular cues and respond with directed movement or tight adhesion, the actin-rich cytoskeleton must be coupled via the plasma membrane to the outside of the cell. The adhesion plaque (focal contact) of cultured fibroblasts is one model system for studying how bundles of actin filaments are attached to the plasma membrane. A number of proteins are known to be

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concentrated at these specialized regions of the cell surface [1]. Two of these, vinculin [2,3] and talin [4], have been shown to interact directly with each other [5,6]. Although in vitro studies with purified proteins initially suggested a direct association of vinculin with actin [7–9], subsequent work has shown that a contaminant in the vinculin preparation was responsible for the interaction [10–12]. As yet there is no evidence that talin binds actin. Thus, talin and vinculin are co-localized at sites of actin-membrane association and cell-substrate adhesion in fibroblasts, but their specific functions remain elusive.

One cell system in which adhesion and actin-membrane interaction is of central importance is the blood platelet. In this case the cells must adhere to a subendothelial surface and contract a membrane-linked actomyosin cytoskeleton to effect wound closure. Recently, we have begun to explore the possibility that what is known about adhesion and actin-membrane interaction in fibroblasts is applicable to the platelet. When whole, washed platelets are electrophoresed on an SDS-polyacrylamide gel, there are four major proteins that stand out in the profile; they are actin (42kD), myosin (200kD), P235 (235kD), and actin-binding protein or filamin (250kD). The functions of three of these four proteins are well-established. Actin and myosin are required for platelet contractility. Actin-binding protein is a well-characterized polypeptide that participates in cross-linking of actin filaments and could therefore be important in the filopodial extension and actin bundling that occurs during platelet activation. P235, although very abundant in platelets, has remained substantially more enigmatic. However, P235 is thought to play a significant role in platelet function because it is cleaved specifically by a calcium-dependent protease when platelets are activated [13,14].

A few years ago Collier and Wang recognized the potential significance of P235 and they purified and characterized the protein from human platelets [15,16]. While examining the purification scheme designed for platelet P235, we were struck by its similarity to the purification that we had developed for talin. Furthermore, P235's physical properties such as Stokes' radius and sedimentation coefficient were also similar to those of talin. Perhaps the most striking parallel between the two proteins was the fact that talin is also very sensitive to endogenous proteolysis, and the proteolytic fragments of smooth-muscle talin are reminiscent of the fragments generated by calcium-dependent proteolysis of P235. These similarities led us to investigate whether platelet P235 might correspond to the platelet form of talin.

MATERIALS AND METHODS

Protein Purification

Talin was purified as described previously [4] with some minor modifications to be described in detail elsewhere [Molony et al., manuscript in preparation].

Platelet P235 was purified from outdated platelet-rich plasma by the procedure of Collier and Wang [15] except we included 0.5 mM PMSF in all steps until the column chromatography and substituted a Sepharose C1-6B column for the Biogel A-5 column. Platelets were obtained from the Blood Bank at University of North Carolina Memorial Hospital.

Production and Characterization of Antitalin Antibody

A high titer antiserum directed against talin was prepared as follows. One rabbit (#A2) was immunized with purified chicken gizzard talin by multiple site, subcuta-

neous injection, the first immunization being with Freunds' complete adjuvant and the second with Freunds' incomplete adjuvant. The immunizations were spaced approximately 3 wks apart. Three wks after the second immunization, the rabbit was boosted with three successive intravenous injections of talin in PBS at 1-wk intervals. One wk after this immunization, the rabbit possessed a low titer antibody against talin as judged by immunofluorescence and immunoblotting. After bleeding the rabbit twice, it was left for a yr before being reimmunized with purified talin, the first injection (approximately 1 mg protein in a suspension of ground polyacrylamide gel) being again subcutaneous in Freunds' complete adjuvant and then followed by three intravenous immunizations of talin (200–500 μg) in PBS. At the end of this period, the rabbit serum was found to have a higher titer against talin, being able to be used at a dilution of 1:2,000 for immunofluorescence on chicken embryo fibroblasts and at 1:4,000 in immunoblots of chicken proteins transferred to nitrocellulose sheets from SDS-polyacrylamide gels.

Immunofluorescence was performed essentially as described previously [4].

Immunoprecipitation

Chick embryo fibroblasts (CEF) were seeded heavily into 60-mm-diameter culture dishes. The cells were allowed to grow until nearly confluent, were washed twice with methionine-free medium, and were then radiolabeled for approximately 18 hr with ^{35}S-methionine (from 100–250 μCi/dish) in 80% Eagle's Minimal Essential Medium with Earle's salts (methionine and glutamine-free), 10% Dulbecco's modified Eagle's Medium, 10% fetal calf serum with added antibiotics and glutamine. Before harvesting, the cells were washed two to three times in serum-free medium. The wash medium was then removed from the dish and replaced with 60 μL of Laemmli sample buffer [17] plus 60 μL deionized water. The radiolabeled cells were quickly scraped from the dish with a Teflon spatula and were passed several times through a 26-gauge needle. The resulting sample was boiled for 2 min. The remainder of the procedure was carried out exactly as described by Burridge and Connell [4].

SDS-polyacrylamide slab gels were run using the buffer system and conditions described by Laemmli [17]. Ten percent polyacrylamide gels contained 0.13% bis-acrylamide.

Immunoblotting

"Western" immunoblot analysis was carried out by a modification of the method described by Towbin et al [18]. After transferring the proteins to nitrocellulose, the nitrocellulose strips were incubated for 1 hr in Tris-buffered saline (TBS = 150 mM NaCl, 50 mM Tris-Cl, pH 7.6, 0.1% NaN_3) containing 0.2% gelatin, 0.05% Tween 20 [19], and 2.5% BSA. The nitrocellulose strips were then incubated for 90 min at room temperature in primary antibody diluted 1:2,000 in the BSA-containing buffer plus 5% v/v inactivated normal horse serum (horse serum buffer). After six 5-min washes in TBS containing gelatin and Tween 20, ^{125}I-affinity purified goat-anti-rabbit IgG in horse serum buffer containing 2% hemoglobin was applied. This antibody was radioiodinated by the chloramine T method [20] and was used at a concentration of 10^5–10^6 cpm/ml. (To reduce nonspecific interactions, the radiolabeled secondary antibody was preincubated on a rocking platform for 3 hrs at 4°C with a finely ground suspension of serum albumin-rich polyacrylamide gel prior to dilution with horse serum buffer). After a 1-hr incubation with the radiolabeled probe, the nitrocel-

lulose strips were washed, dried, taped to heavy cardboard, and exposed for autoradiography at −70°C on Kodak X-Omat AR film using an intensification screen.

Preparation and Proteolysis of Platelet Extracts

Washed platelets were prepared by a previously described method [15]. The platelets were lysed in buffer containing 1% Triton X-100 and were centrifuged at 75,000 g for 1 hr to generate a Triton-soluble supernatant and a Triton-insoluble pellet. The supernatant was enriched in P235.

The endogenous Ca^{++}-activated protease of platelets was stimulated in the Triton X-100 supernatant by raising the calcium to a level 1 mM above the EDTA concentration as described previously [15]. The proteolytic digest was performed on ice.

Partial Proteolytic Digests

Thirty micrograms of talin or P235 in 120 μL of buffer B (20 mM NaCl, 20 mM Tris-Acetate, 0.1 mM EDTA, and 15 mM β-mercaptoethanol, pH 7.6) were incubated at room temperature with 0.5 μg of protease added in 10 μL. Aliquots of the digests were withdrawn at the indicated time intervals and digestions were stopped by boiling with an equal volume of gel sample buffer. The proteolytic peptides were analyzed on a 10% SDS-polyacrylamide gel. Each gel sample contained 3.5 μg of either talin or P235.

RESULTS

Characterization of the Antitalin Antibody

An antibody was raised against chicken gizzard talin by repeated immunization of the purified protein first subcutaneously and later by intravenous injection. The resulting high titer antiserum gave a typical talin staining pattern with cultured fibroblasts (focal contacts, fibrillar streaks, and the ruffling membrane) at dilutions of 1:1,000 and 1:2,000 (Fig. 1d). The specificity of the antiserum was characterized both by immunoblot and immunoprecipitation analysis (Fig. 1a–c). By the immunoblot method the antibody binds to purified chicken smooth-muscle talin (Fig. 1b, lane 2′). In chicken embryo fibroblasts (Fig. 1b, lane 3′) the antibody recognizes a high-molecular-weight protein that comigrates with purified talin. There also appears to be some reactivity at approximately 190kD, a molecular weight that corresponds to a major proteolytic fragment of talin. The antibody also recognizes a protein of approximately 225kD in mammalian fibroblasts (data not shown). The autoradiograph shown in Figure 1c demonstrates that from total ^{35}S-methionine-labeled chicken fibroblast protein (Fig. 1c, lane 4), a single polypeptide of 225,000 daltons is immunoprecipitated by the antitalin antibody (Fig. 1c, lane 5).

Analysis of Platelet Proteins

In Figure 2, a sample of washed, unactivated platelets was subjected to electrophoresis on an SDS-polyacrylamide gel. This sample was compared with a platelet Triton-soluble supernatant (Fig. 2, lane 3) and a Triton-insoluble pellet (cytoskeleton) (Fig. 2, lane 4). The platelet protein P235 is the major component in the gels between the myosin heavy chain band and the band corresponding to platelet filamin. P235 fractionates predominantly with the soluble components, being markedly reduced in

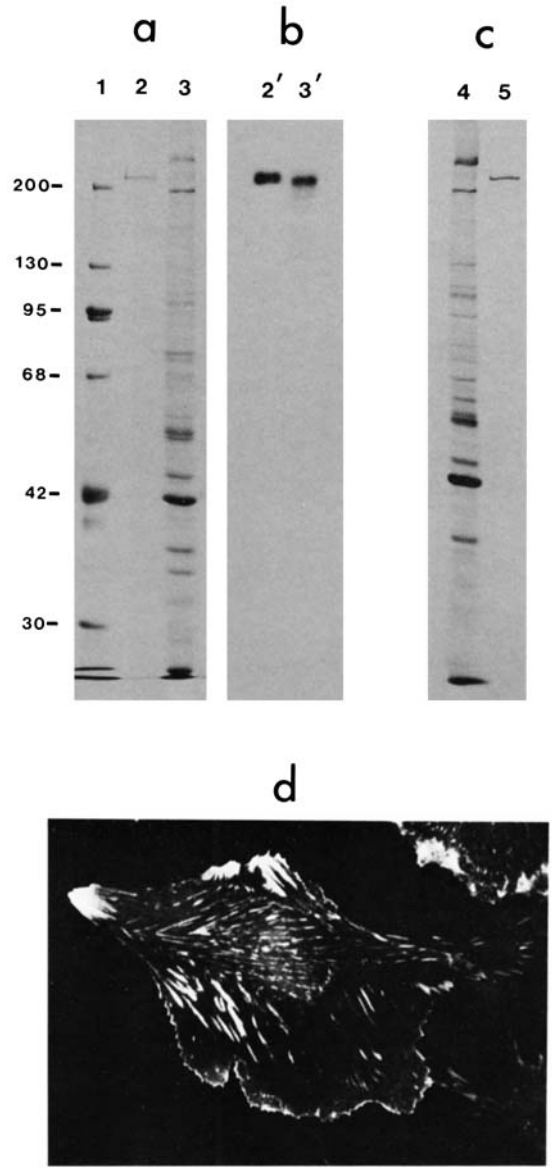

Fig. 1. Characterization of the antitalin antibody. a) A Coomassie blue-stained SDS-polyacrylamide gel of standard proteins (lane 1), purified chicken gizzard talin (lane 2), and total chick embryo fibroblast protein (lane 3). b) For immunoblot analysis, an equivalent gel was used except the amount of purified talin on the gel was reduced by eightfold. An autoradiograph of the corresponding immunoblot shows that the antibody recognizes purified talin (lane 2′) as well as a protein of equivalent molecular weight in the whole cell homogenate (lane 3′). Minor lower molecular weight species are proteolytic fragments of talin. c) Gel analysis of immunoprecipitation using the antitalin antibody. Lane 4 shows total ^{35}S-methionine-labeled protein from chick embryo fibroblasts and lane 5 shows the material specifically immunoprecipitated with the antitalin antibody. A single labeled protein at 225,000 daltons is precipitated. d) By indirect immunofluorescence the antitalin antibody labels adhesion plaques, numerous fibrillar streaks, and ruffling membranes in chicken embryo fibroblasts. The antibody was used at a dilution of 1:1,000 in this experiment. Affinity-purified antibody gave an equivalent staining pattern (data not shown).

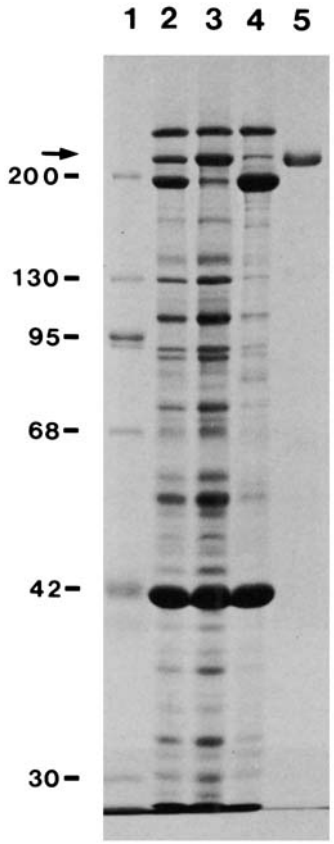

Fig. 2. Preparation of platelet extracts. A Coomassie blue-stained polyacrylamide gel of standard proteins (lane 1), total human platelet protein (lane 2), Triton X-100-soluble material (lane 3), Triton X-100-insoluble material (lane 4), and purified chicken gizzard talin. P235 (arrow) is the prominent band migrating between myosin (200,000 Daltons) and filamin (250,000 Daltons) in intact platelets (lane 2). It is greatly enriched in the Triton X-100 supernatant (lane 3) and migrates very close to the location of purified chicken gizzard talin (lane 5).

the "cytoskeletal" fraction. This is in contrast to actin, myosin, and filamin, which are the major components of the platelet cytoskeleton.

Adjacent to these platelet fractions, a sample of purified gizzard talin was electrophoresed for comparison (Fig. 2, lane 5). The purified talin migrates very close to the position of P235.

Platelet P235 and Talin are Immunologically Related Proteins

The similar biophysical properties of P235 and talin (Table I) suggested that these might be the same protein and prompted us to explore this possibility further. P235 was purified from platelets by a slight modification of the procedure of Collier and Wang [15]. The interaction of purified P235 with the antitalin antiserum was tested by immunoblot analysis (Fig. 3). The antibody against talin recognized a single polypeptide among a complex mixture of proteins found in the platelet Triton-soluble

Table I. Comparison of the Physical Properties of P235 and Talin

	Talin[a]	P235[b]
M_r by SDS-PAGE	225,000	235,000
Sedimentation value	9.3	9.8
Stokes' radius (nm)	6.5	6.7
Shape at physiological ionic strength (determined by rotary shadowing)[a]	Flexible rod	Flexible rod

[a]Molony and Burridge, unpublished results.
[b]Collier and Wang [15].

supernatant (lane 2 and 2′). This protein comigrated with purified P235, which also bound the antibody (lane 3 and 3′). As shown in Figure 1, the antibody is strongly reactive with chicken gizzard talin (lane 4 and 4′).

Immunoblot Analysis of Calcium-Dependent Proteolysis of Platelet Talin/P235

A Triton-soluble extract of platelets was made and the supernatant prepared by centrifugation at 75,000g for 1 hr. This preparation was used to examine the sensitivity of platelet talin/P235 to the intrinsic calcium-activated protease. The supernatant was incubated in the absence or presence of calcium at 0°C. Samples were withdrawn at specific time points and analyzed on an SDS-polyacrylamide gel (Fig. 4a). A similar gel was transferred to nitrocellulose and the behavior of talin/P235 analyzed by immunoblotting with the antitalin antiserum (Fig. 4b). As previously reported [15,21] two high-molecular-weight platelet proteins, filamin and P235, are particularly sensitive to calcium-induced proteolysis. In this experiment, P235 is the protein exhibiting the greatest sensitivity to the calcium-activated protease. After 5 min of incubation, P235 is almost completely cleaved whereas some intact filamin is still detected at the 10-min time point. The immunoblot probed with antitalin antiserum indicates the appearance of a major fragment of platelet talin/P235 with a molecular weight of about 190–200,000 Daltons concomitant with the degradation of the intact protein. This prominent fragment is visible in the Coomassie blue-stained gel and confirms that this protein is derived from talin and not filamin. A slightly higher molecular weight band between this component and the intact talin band appears to correspond to the major filamin fragment since its time course of appearance is slightly slower than the talin fragment and coincides more closely with the degradation of filamin. It is interesting to note that the cleavage of platelet talin/P235 induced by the calcium-activated protease generates a major fragment that is then resistant to further proteolysis over the rest of the time course of the incubation. No low-molecular-weight fragments of this protein are detected in the immunoblot.

Comparison of One-Dimensional Partial Proteolytic Maps of P235 and Talin

To investigate further the relationship between P235 and talin, proteolytic digests of the native proteins were compared. Limited proteolysis was performed using either purified P235 or talin with the proteases elastase, chymotrypsin, or trypsin (Fig. 5), as well as papain and Staphylococcal V8 protease (not shown). The most striking observation in these experiments is that both talin and P235 contain homologous domains of protease sensitivity. All the proteases tested with the exception of trypsin cleave both proteins to yield two major fragments, a high-molecular-

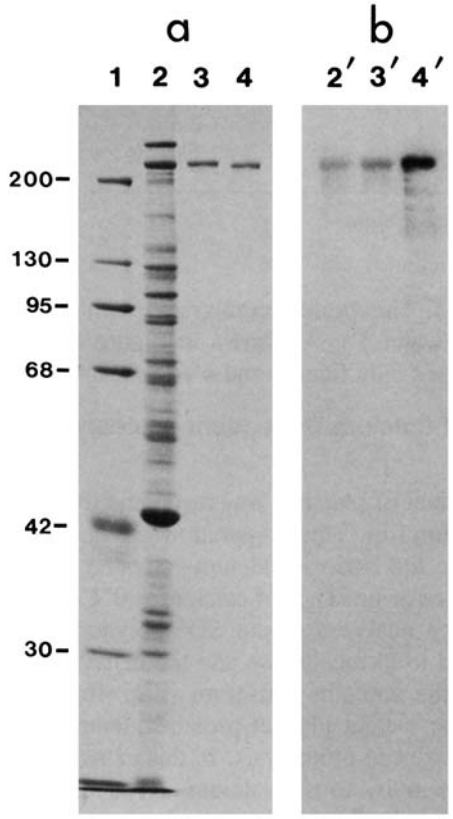

Fig. 3. Talin and P235 are immunologically related. a) A Coomassie blue-stained gel of standard proteins (lane 1), a Triton X-100 supernatant from human platelets (lane 2), purified human platelet P235 (lane 3), and purified chicken gizzard talin (lane 4). b) The proteins in a gel slice equivalent to that shown in panel a (except having a 20-fold decreased level of chicken gizzard talin) were transferred to nitrocellulose and the nitrocellulose strip was incubated first with a 1:2,000 dilution of antitalin antibody and then with an affinity-purified ^{125}I-goat antirabbit IgG. The chicken talin lane was loaded more lightly in the immunoblot because the antibody crossreacts much more strongly with chicken talin, the original antigen, than it does with talin from other sources. The autoradiograph shown in panel b demonstrates that the antitalin antibody recognizes both purified human platelet P235 (lane 3′) and chicken gizzard talin (lane 4′). Because of the high reactivity of the antibody with chicken talin, numerous minor breakdown products not visible by Coomassie-blue staining are detected in lane 4′. In more complex mixtures of platelet proteins, the antitalin antibody was judged to be monospecific in its interaction with P235 (lanes 2 and 2′).

weight fragment of 190–200kD and a similar low-molecular-weight fragment. Trypsin also generates the high-molecular-weight fragments but cleaves the low-molecular-weight domain further into polypeptides that migrate to the 20–30kD region of the gel. Although many similarities exist in the digestion patterns of P235 and talin, talin is more susceptible to proteolysis by chymotrypsin than P235 (Fig. 5b); the opposite is found with trypsin, P235 being more sensitive to cleavage than talin (Fig. 5c).

DISCUSSION

In this paper we have demonstrated a relationship between the protein talin that was isolated from smooth muscle and subsequently shown to be localized in fibroblast

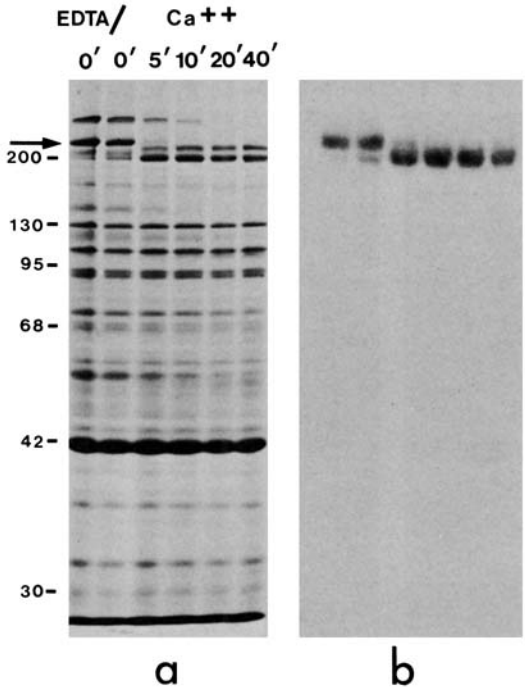

Fig. 4. Activation of the endogenous Ca^{++}-activated protease of platelets results in cleavage of P235 to a stable fragment of 190–200,000 Daltons. a) The time course of digestion of platelet proteins by the Ca^{++}-activated protease is shown. A high-speed supernatant containing Triton X-100-soluble platelet proteins was incubated for various times at 0°C in the absence (first lane) or presence (remaining lanes) of Ca^{++}. In the presence of Ca^{++}, two major high-molecular-weight proteins (filamin and P235) are reduced in abundance. The position of P235 is indicated by the arrow. It is P235 that appears to be most rapidly proteolyzed under these assay conditions. b) Because of the complex protein composition of the Triton X-100 supernatant, we used immunoblot analysis to identify the specific proteolytic products of P235 in response to the endogenous protease. As can be seen in the immunoblot of an equivalent gel to that in panel a, P235 is rapidly cleaved to a fragment of M_r = 190,000–200,000. This fragment is refractory to further digestion even with a 40′ exposure to the protease in the presence of Ca^{++}. The lower-molecular-weight "difference"peptide is not evident in this autoradiographic exposure.

adhesion plaques (focal contacts) and a major protein of platelets, P235, which is thought to be involved in platelet activation. When platelets are stimulated with thrombin, two high-molecular-weight proteins are cleaved by a calcium-activated protease to lower-molecular-weight forms [14,15]. One of these targets for proteolysis is filamin (ABP) and the other is P235. Platelet P235 has been purified [15] and we were struck by the similarity of its properties to talin. In addition to its sensitivity to proteolysis, P235 has similar, if not identical, hydrodynamic properties to those we have recently determined for talin [Molony et al., manuscript in preparation]. Moreover, the behavior of talin and P235 in their respective purifications appears very similar, with the two proteins exhibiting comparable elution properties on ion exchange and gel filtration columns. When the two proteins are run side-by-side on a 10% SDS-polyacrylamide gel, they appear to have similar molecular mass, although P235 runs slightly behind talin in our gel system. The apparent discrepancy in the

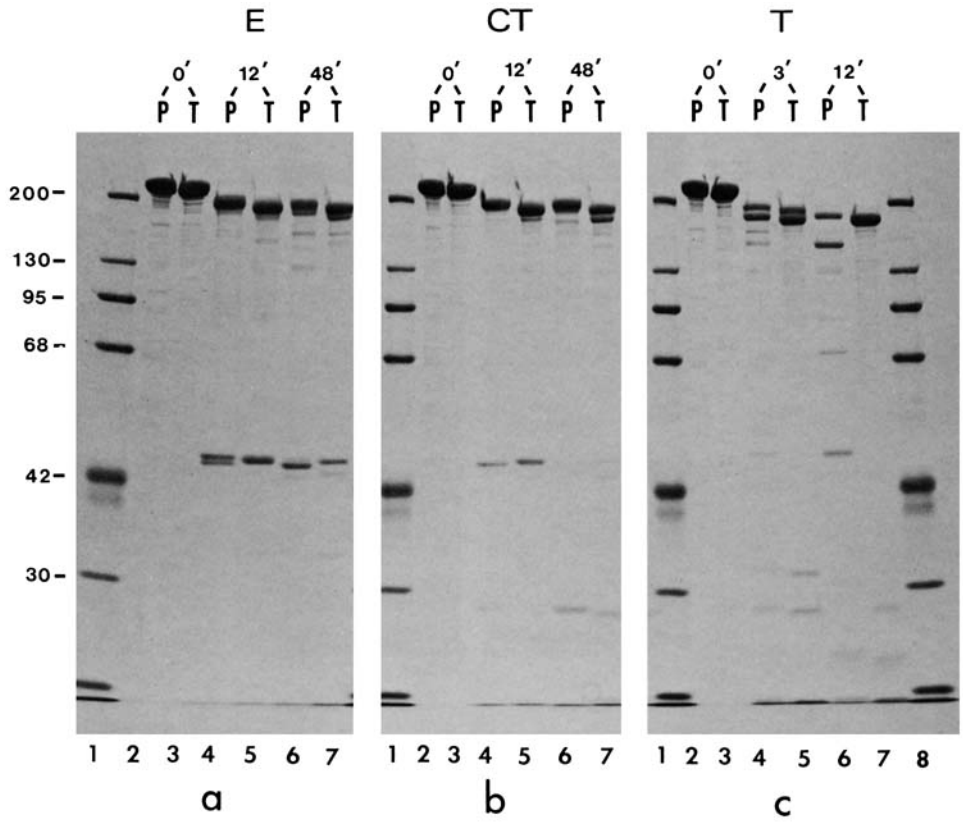

Fig. 5. Comparison of the one-dimensional partial peptide maps of P235 and talin. Photographs of Coomassie-blue-stained SDS-polyacrylamide gels are shown for digestions of P235 and talin with elastase (a), chymotrypsin (b), and trypsin (c). In each panel, lane 1 contains standard proteins, lanes 2 (P235) and 3 (talin) contain the proteins prior to proteolysis, and subsequent pairs of lanes contain the proteins (with P235 on the left) after various periods of incubation with the protease. Incubation times are indicated on the figure for each pair of lanes. Similar polypeptides are generated by digestion of P235 and talin with these proteases, including polypeptides of 190–200kD, 44–46kD, and lower-molecular-weight species in the 30 and 24kD range.

previously published values for their molecular weights on SDS gels probably resulted from the difficulty in determining molecular weights accurately in the high-molecular-weight region of gels as well as from possible differences in the gel systems of different laboratories.

In addition to their similar purification and physical properties, we have shown here that a monospecific antibody against chicken gizzard talin cross-reacts with platelet P235. Moreover, P235 and talin share common domains which exhibit similar sensitivity to proteolytic digestion. Endogenous proteases in platelet and smooth muscle generate similar polypeptides from P235 and talin: a 190–200kD fragment is generated from platelet supernatants treated with calcium (Fig. 4) and a 190kD proteolytic fragment of talin is a major component of low ionic strength extracts of chicken gizzard [22]. Limited proteolysis of purified P235 and talin with the proteases elastase, chymotrypsin, or trypsin also results in the generation of a shared 190–

200kD fragment in addition to other similarities in the one-dimensional peptide maps. In an effort to assay the functional similarity of the two proteins, we have recently demonstrated that P235, like talin, binds vinculin [23]. Taken together these observations have led us to conclude that P235 is the platelet form of talin.

The identification of this major platelet protein as talin raises several questions. Previously, because talin is localized in adhesion plaques, we considered it a strong possibility that talin functions with vinculin in the attachment of microfilament bundles to the plasma membrane. In platelets, where P235 has been estimated to represent between 3 and 8% of the total platelet protein [15], P235 has been implicated in events associated with platelet activation such as actin filament assembly [16]. Although cell adhesion is a major consequence of platelet activation and presumably an increase in attachment of microfilaments to the platelet membrane accompanies this enhanced ahesiveness, it is unclear whether that function alone would be enough to explain the abundance of P235 in the platelet. The enrichment of P235 in a very dynamic cell such as the platelet may reveal some function for talin in fibroblasts that had previously gone undetected.

The work with platelets also raises again the question of the physiological significance of the proteolytic cleavage of talin. Is the intact protein or its proteolytic fragment the "functional" form? Many enzymes are synthesized in larger inactive forms that are only activated when proteolytically cleaved. If this were the case with talin it would indicate that in fibroblasts most of the protein is in the inactive form. It will be interesting, for example, to determine whether stimulation of fibroblast motility results in an increase in proteolytic cleavage of talin. In future work it will also be very important to compare the properties of talin with those of its proteolytic fragment. This may lead to an understanding of the function of talin in fibroblasts and platelets and should also reveal the significance of its proteolytic cleavage.

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