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Isolation of a Calcium-Sensitive, 35,000-Dalton Microfilament- and Liposome-Binding Protein From Ascites Tumor Cell Microvilli: Identification as Monomeric Calpactin

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Microvilli isolated from the MAT-C1 ascites subline of the 13762 rat mammary adenocarcinoma contain a major calcium-sensitive microfilament-binding protein, AMV-p35 (ascites microvillar p35). Association of AMV-p35 with microfilament cores during Triton X-100 extraction of the microvilli is half-maximal at 0.1–0.2 mM calcium. The protein, which comprises 6% of the total microvillar protein, can be isolated from microfilament cores prepared in the presence of calcium by extraction with EGTA and purification by ion-exchange chromatography. Alternatively, the protein can be isolated from Triton extracts of microvilli prepared in the absence of calcium by precipitation with calcium, solubilization of the precipitate with EGTA, and chromatography on an ion-exchange column. AMV-p35 binds to phosphatidylserine liposomes and F-actin with half-maximal calcium concentrations of about 10 μM and 0.2 mM, respectively. Treatment of AMV-p35 with chymotrypsin yields a 33,000-dalton fragment, behavior similar to the tyrosine kinase substrates calpactins I and II and lipocortins I and II. Immunoblot analyses using antibodies directed against calpactin I, lipocortin I, and lipocortin II showed strong reactivity of AMV-p35 with anti-calpactin I and anti-lipocortin II, but little reactivity toward anti-lipocortin I. The close relationship between AMV-p35 and calpactin I was verified by amino acid sequence analyses of peptides isolated from cyanogen bromide digests of AMV-p35. By gel filtration and velocity sedimentation analyses purified AMV-p35 is a 35,000-dalton monomer. Moreover, AMV-p35 extracted directly from microvilli in Triton/EGTA also behaves as a 35,000-dalton menomer. These findings indicate that AMV-p35 is closely related to the $pp60^{src}$ kinase substrate calpactin I (p36). However, AMV-p35 occurs in the microvilli as a monomer rather than as the heterotetrameric calpactin found in several other cell types.

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Plasma membranes of animal cells are of critical importance in cellular regulation. The exterior surface of the plasma membrane contains sites for the interaction of hormones and growth factors [1]. Through the bilayer are inserted channels or transport proteins involved in the passage of ions and metabolites. The cytoplasmic surface of the plasma membrane contains sites for the synthesis of cyclic AMP, interaction of protein kinase C, and association of the cytoskeleton [1]. The submembrane microfilaments, in particular, must be involved in such processes as cell motility, cytokinesis, endocytosis, and the determination of cell morphology [2]. The dynamic organization of nonmuscle microfilaments depends on the interaction of F-actin with a number of nonmuscle actin binding proteins. Several of these have been described, and their interactions with actin have been characterized [reviewed in 2–4]. However, our understanding of the regulation of submembrane microfilament organization as well as the mechanisms of membrane-microfilament interactions remains fragmentary [2,5].

We have been developing a useful system for studying plasma membranes, submembrane microfilaments, and their associations [5]. We have isolated microvilli from 13762 ascites mammary tumor cells by a gentle shearing method which avoids disruption of the cells and provides a minimally perturbed preparation of intact microvilli [6,7]. These microvilli differ from the more commonly studied brush border microvilli in two important respects: (1) Their microfilaments are less tightly organized than those of the brush border microvilli [7,8]. (2) They contain actin binding proteins similar to those in the cortical regions of nonmuscle cells, such as α-actinin [6,8,9] and tropomyosin [10], rather than the proteins typical of brush border microvilli [8]. Indeed, the microfilament-associated proteins of the ascites microvilli are more similar to proteins of the terminal web region of brush borders than to proteins of the brush border microvilli.

The ascites microvillar microfilaments differ from other microfilament preparations in one other interesting respect: they appear to be at least as stable in the presence of calcium as in its absence [9]. In part, this apparent stability may be due to the absence of calcium-dependent microfilament-severing proteins [2–4] in the ascites microvilli. However, examination of ascites microvillar microfilament cores, prepared by Triton X-100 extraction in the presence and absence of calcium, showed that they differed in the presence of a 35,000-dalton polypeptide AMV-p35 (ascites microvillar p35), a prominent component of the cores prepared in the presence of calcium and a major microvillar protein.

Since calcium is an important cellular regulator, we have investigated this calcium-sensitive protein in greater detail to try to ascertain whether it may play a role in organization of the microfilament cores in the ascites microvilli. During the course of these investigations we noted the similarity of the properties of this protein to the submembrane tyrosine kinase substrates described in a number of studies [11–19]. In this report we describe the isolation and properties of AMV-p35 and show that it is biochemically and immunologically very similar to the microfilament- and lipid-binding $pp60^{src}$ tyrosine kinase substrate calpactin I [19]. It is less similar to the related lipocortin I [20,21]. Interestingly, AMV-p35 appears to be present in the ascites microvilli as a monomer rather than as the calpactin heterotetramer isolated from other cell types.

MATERIALS AND METHODS

Materials

The MAT-C1 ascites subline of the 13762 rat mammary adenocarcinoma was maintained [22] and cells and microvilli were isolated [7] as previously described. Bovine intestinal calpactin I (p36 protein) and antiserum directed against the calpactin were kindly provided by Dr. John Glenney, Salk Institute (La Jolla, CA). Lipocortin I and antisera directed against lipocortins I and II were generous gifts of Dr. R.B. Pepinsky, Biogen (Cambridge, MA). Horseradish-peroxidase-conjugated goat antibodies directed against rabbit immunoglobulins and 4-chloronaphthol were purchased from BioRad Laboratories (Richmond, CA) and used as directed. Chemicals were reagent-grade materials obtained from Sigma Chemical (St Louis, MO) or other sources.

Velocity Sedimentation Sucrose Density Gradient Centrifugation of Microvilli Extracted in the Presence and Absence of Calcium [9]

Microvilli (3.2 mg protein) made from cells labeled metabolically with [^{3}H]leucine (ICN, Irvine, CA) [9] were extracted at 37°C for 15 min in a total volume of 1.0 ml of 0.2% (w/v) Triton X-100, 100 mM KCl, 2 mM $MgCl_2$, 20 mM piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES) (pH 6.8), with or without 1 mM $CaCl_2$. The extracts were loaded onto 20–40% (w/v) sucrose gradients in the same buffers and centrifuged at 30,000 rpm for 3 hr in an SW40 rotor [9]. Fractions (1.0 ml) were collected in calibrated tubes and analyzed as previously reported [9].

Isolation of AMV-p35

AMV-p35 was isolated by two different procedures.

From microvillar cores prepared in the presence of calcium. Microvilli were lysed in 10 mM Tris (pH 7.5), 100 mM KCl, 1 mM $CaCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM dithiothreitol (DTT), and 0.2% (w/v) Triton X-100 at room temperature for 20 min and centrifuged at 15,000g for 15 min at 4°C. The resulting pellet (microfilament core) was extracted with 10 mM Tris (pH 7.5), 5 mM KCl, 1 mM ethyleneglycol bis(2-aminoethyl ether)-tetraacetic acid (EGTA), 0.5 mM DTT, and 0.05% Triton X-100 for 10 min at room temperature and centrifuged at 15,000g for 15 min at 4°C. The supernate was applied to a 0.5-ml DE-52 (Whatman) column; the unbound fraction contained purified AMV-p35. Because of the consistently better yield, AMV-p35 was purified by this method for the characterization studies described in this report, unless stated otherwise.

By calcium precipitation of microvillar extracts. Microvilli were extracted with 0.2% Triton X-100, 100 mM KCl, 1 mM PMSF, 0.5 mM DTT, 2 mM $MgCl_2$, and 10 mM Tris (pH 7.5) at room temperature for 20 min and centrifuged for 20 min at 15,000g at 4°C. $CaCl_2$ was added to the supernate to a final calcium concentration of 1.5 mM, and the mixture was incubated for 5 min at room temperature and centrifuged at 15,000g for 15 min at 4°C. The pellet was extracted with 10 mM Tris (pH 7.5), 5 mM KCl, 1 mM EGTA, and 0.5 mM DTT at room temperature and centrifuged at 15,000g for 15 min at room temperature; the resulting supernate was applied to a 0.5-ml DE-52 column equilibrated with the extraction buffer. The unbound fractions from the column were collected for further studies.

Immunoblot Analysis of AMV-p35

Microvilli (100 ng) and purified microvilli, calpactin, lipocortin I, and AMV-p35 (10 ng) were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) on 12% minigels (Idea Scientific, Corvallis, OR) and transferred to nitrocellulose by the method of Szewczyk and Kozloff [23] in 25 mM ethanolamine/glycine (pH 9.5) buffer in 20% methanol. The nitrocellulose transfers were then "blocked" with 0.5% (v/v) Tween 20, 20 mM Tris (pH 7.5), and 0.5 M NaCl (TTBS) for at least 4 hr at room temperature and incubated with first antibody (antibodies directed against calpactin I, 3 μg/ml; lipocortin I, 1:2,000; or lipocortin II, 1:2,000) at room temperature overnight. The bound antibodies were detected with goat antirabbit immunoglobulin conjugated with peroxidase (1:2,000), which was incubated with the transfers for 45 min. The blots were washed three times with TTBS, and the immunoreactive bands were visualized by using the BioRad GAR-HRP assay kit.

Binding of AMV-p35 to Liposomes

Phosphatidylserine liposomes were prepared by sonicating dried phospholipid films for 8 min in 50 mM Hepes (pH 7.4), 100 mM KCl, 2 mM $MgCl_2$, 0.5 mM DTT, and 2 mM EGTA, and the appropriate concentrations of calcium. The suspended aliquots of phospholipid (20 μg) were incubated with 50 μg ascites microvillar AMV-p35 in 200 μl of the same buffer containing varied concentrations of $CaCl_2$ for 30 min at room temperature, and the liposomes were pelleted in a Beckman Airfuge (18 psi, 20 min). Equal amounts of supernates and pellets were analyzed by SDS-PAGE. AMV-p35 was quantified by scanning the gels.

Analytical Methods

The Stokes radius of AMV-p35 was determined by gel filtration [10] of AMV-p35 and standard proteins in 100 mM KCl, 1 mM DTT, 1 mM EDTA, 20 mM phosphate (pH 7.4), and 0.05% Triton X-100 at 4°C on a 1 × 50 cm Sephacryl S-200 column. Standard proteins used were cytochrome c (1.87 nm), ovalbumin (3.05 nm), bovine serum albumin (3.55 nm), aldolase (4.81 nm), and fumarase (5.29 nm). The sedimentation coefficient of AMV-p35 was determined by velocity sedimentation analysis on 5–20% (w/v) sucrose density gradients in 100 mM KCl, 20 mM phosphate (pH 7.4), 1 mM EGTA, 0.5 mM DTT, and 0.05% Triton X-100 centrifuged at 20°C and 40,000g for 17 hr in an SW50.1 rotor. Standard proteins centrifuged with AMV-p35 were cytochrome c (2.1 S), hemoglobin (2.8 S), ovalbumin (3.66 S), and bovine serum albumin (4.6 S). The partial specific volume was determined from the amino acid composition, which was determined by Dr. Phillip Whitney on a Beckman 120 C amino acid analyzer after hydrolysis of the protein in 6 N HCl.

For determination of the native molecular weight of AMV-p35 microvilli were lysed in 0.2% Triton X-100 in PBS (pH 7.5) for 20 min and centrifuged in the Airfuge at 20 psi (100,000g) for 1 hr at 4°C. The supernate was dialyzed against 100 mM KCl, 1 mM DTT, 1 mM EDTA (pH 7.5), and 0.02% NaN_3 and applied to a Sephacryl S-200 (1 × 50 cm) column previously equilibrated with the same buffer. Standard proteins were the same as those used on velocity sedimentation for determining the sedimentation coefficient of purified AMV-p35. Fractions from the column were analyzed by SDS PAGE on 12% gels. Native molecular weight and frictional ratio were calculated by the method of Siegel and Monty [24].

To obtain fragments for sequence analysis, AMV-p35 (200 μg) was treated with CNBr (10 mg) in 70% formic acid (500 μl) for 18 hr at room temperature. After drying under vacuum, the cleavage products were dissolved in 50% formic acid and separated by high-performance liquid chromatography (HPLC) by using a Vydac C_4 reverse-phase column with a gradient from 10 to 60% acetonitrile in 0.1% aqueous trifluoroacetic acid over 1 hr at a flow rate of 1.0 ml/min. The effluent was monitored at 212 nm and peaks were collected manually. Aliquots of fractions were hydrolyzed with 6 N HCl at 110°C for 24 hr, derivatized with phenylisothiocyanate, and analyzed as PTC derivatives by using a Waters Pico Tag work station and a Pico Tag HPLC column with a Hewlett Packard 4084B liquid chromatograph. Two peptides and the intact protein were subjected to automatic microsequence analysis by using an Applied Biosystems 470A gas phase sequencer and standard programs supplied by the manufacturer. Phenylthiohydantoin-amino acids were identified and quantified with an Applied Biosystems narrow-bore PTH HPLC cartridge system and a Hewlett Packard 1090 HPLC [25].

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was performed on 8% minigels (Idea Scientific, Corvallis, OR), unless otherwise specified, by the method of King and Laemmli [26]. Protein was assayed by using the BCA protein assay method (Pierce Chemical Co., Rockford, IL.)

RESULTS

Identification of a Calcium-Sensitive 35,000-Dalton Microfilament-Associated Protein

When 13762 ascites microvilli are lysed in isotonic Triton X-100-containing buffers and centrifuged on velocity sedimentation sucrose density gradients, the microfilament cores sediment well into the gradients [9]. The rate at which the cores sediment on the gradients depends on the lysis conditions and how these conditions affect the stability of the microfilaments [8,9]. By adding calcium to the lysis solution, one can determine the effect of calcium on microfilament stability and identify proteins whose association with the microfilament core is calcium-sensitive. SDS PAGE gels of fractions from the gradients (Fig. 1) showed that microfilament cores from microvilli lysed in the presence of calcium (Fig. 1B) appear to sediment as rapidly as cores from microvilli lysed in the absence of calcium (Fig. 1A). When cells are metabolically labeled with ^{3}H-leucine prior to microvilli isolation, one can obtain a quantitative evaluation of the amounts of protein [9] in the peak fractions from the gradients of microvilli extracted in the absence (Fig. 2A) and presence (Fig. 2B) of Ca^{++}. In the absence of calcium the microfilament peak (fractions 8–10) contained 16% of the total microvillar protein, whereas in the presence of calcium the filament fraction (fractions 8–10) comprised 22% of the total. Thus calcium appears to stabilize the microfilaments to breakdown during extraction and centrifugation. Moreover, in several experiments the filament fraction of calcium-treated microvilli appeared more discrete and had a higher average sedimentation coefficient than that from the untreated sample, further supporting the hypothesis [9] that calcium contributes to the stability of the microfilament cores.

Examination of the SDS PAGE gels of the gradient fractions shows that the major difference in the cores from microvilli lysed in the presence and absence of calcium is the presence of a 35,000-dalton protein (AMV-p35), whose association

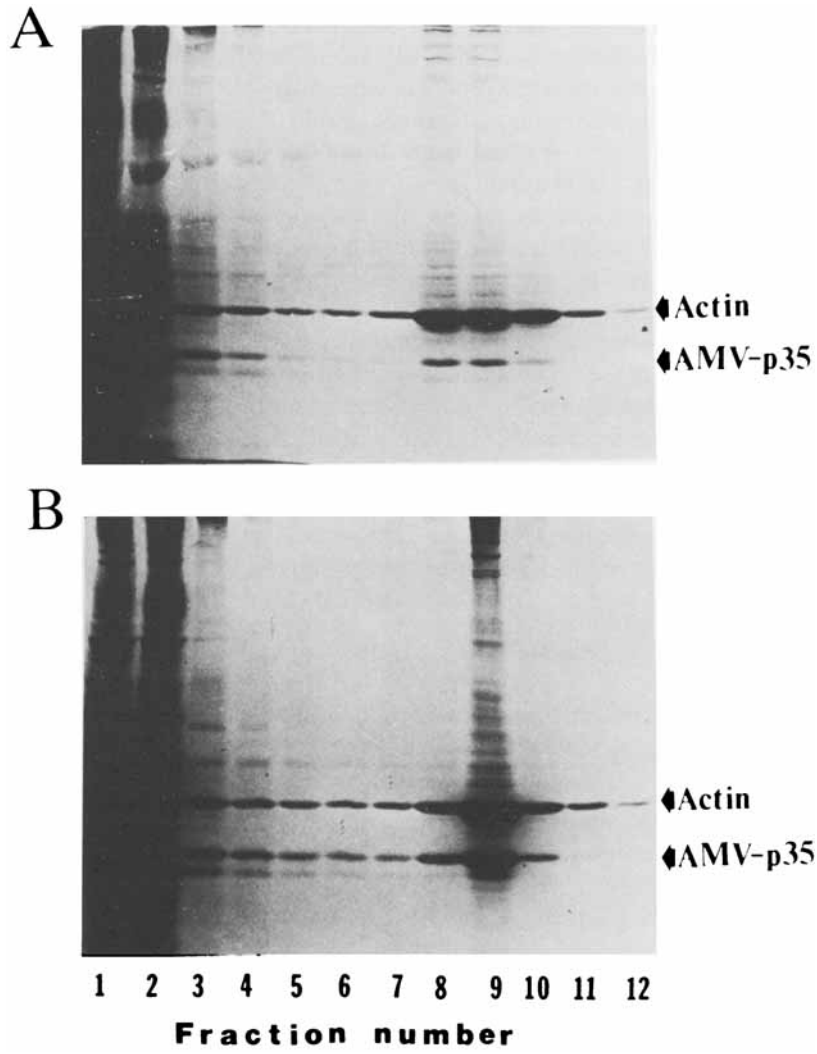

Fig. 1. SDS PAGE of fractions from velocity sedimentation analysis of microvilli extracted in 0.2% (w/v) Triton X-100, 100 mM KCl, 2 mM $MgCl_2$, 20 mM PIPES (pH 6.8) without **(A)** or with **(B)** 1 mM calcium. Analyses were performed as described by Carraway and Weiss [9].

with the core is calcium-dependent (Fig. 1). Extraction of microvilli in the presence of EGTA decreased the amount of protein having an electrophoretic mobility similar to AMV-p35; the remaining protein showed a different pI from that of AMV-p35, as shown by isoelectric focusing/SDS PAGE (data not shown). The calcium concentration dependence of the association of AMV-p35 with the microfilament core was investigated by using a pelleting assay (Fig. 3). Aliquots of microvilli were lysed in buffers containing EGTA or in different concentrations of calcium (0.01–1 mM) and centrifuged in a Beckman Airfuge. Association of AMV-p35 with the core is complete under these extraction conditions at a calcium concentration of 0.25–0.5 mM, with a half-maximal concentration of about 0.1 mM.

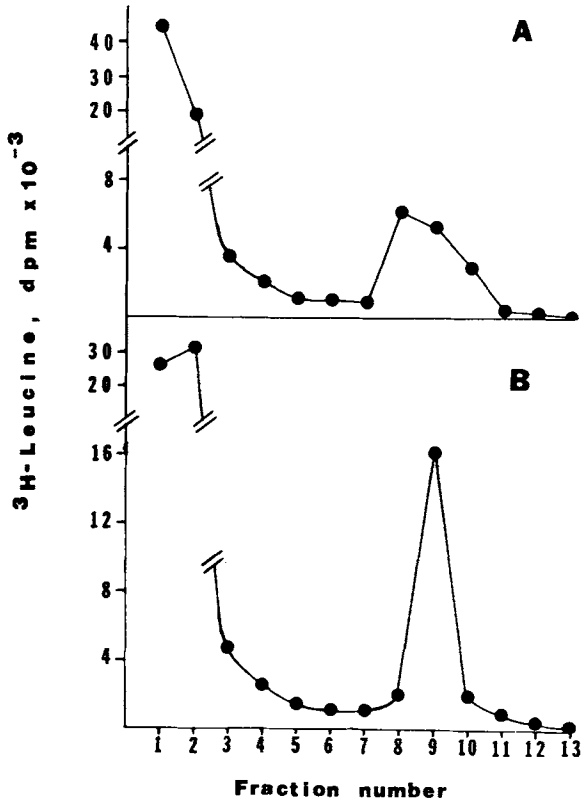

Fig. 2. [^{3}H]Leucine profiles of velocity sedimentation analyses of microfilament cores from microvilli lysed in the absence **(A)** and presence **(B)** of calcium in the experiment described in the legend for Figure 1.

Isolation of AMV-p35

Two different procedures have been used for the isolation of AMV-p35. In the first procedure (purification shown in Table I and Fig. 4) we took advantage of the calcium-sensitive association of AMV-p35 with the microfilament core. Cores were prepared in the presence of 1 mM calcium (Fig. 4, lane 1). After centrifugation, the core pellet was treated with EGTA to release AMV-p35 (Fig. 4, lane 2). When the EGTA-soluble material was chromatographed on DE-52, AMV-p35 passed through the column without binding. This unbound fraction contained predominantly AMV-p35 with a small amount of a slightly smaller polypeptide (Fig. 4, lane 4), which may be a degradation product.

The behavior of AMV-p35 during purification from the microvilli cores suggested similarities to p36 (calpactin I) [12,17]. This protein has been shown by Glenney [27] to be precipitated from cell extracts by calcium. To compare further the behavior of AMV-p35 with that of p36, we used for our second purification method a procedure based on calcium precipitation. Microvilli were lysed in the absence of calcium to release AMV-p35 from the microfilament cores (Fig. 5, lane 1). Addition of calcium to the soluble fraction of the lysate resulted in the precipitation of AMV-p35, along with small amounts of other proteins (Fig. 5, lane 4, and Fig. 6). Optimal

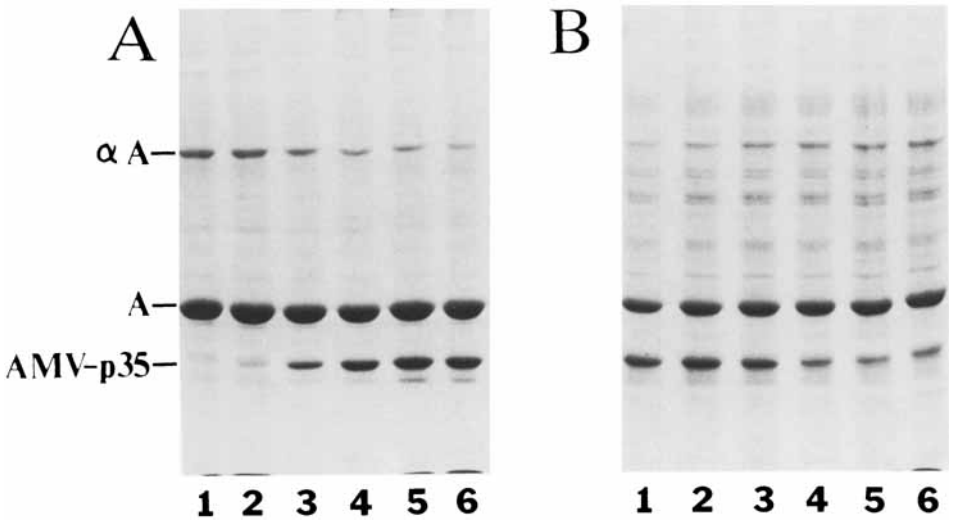

Fig. 3. Calcium dependence of the association of AMV-p35 with the microfilament core. Microvilli were lysed in 10 mM Tris (pH 7.5), 100 mM KCl, 2 mM $MgCl_2$, 0.5 mM DTT, and 0.2% (v/v) Triton X-100 with varying concentrations of calcium at room temperature for 20 min and centrifuged in an airfuge at 20 psi (100,000g) for 20 min at 4°C. The pellets **(A)** and supernates **(B)** were prepared for SDS PAGE. **Lanes 1–6** were from experiments using 0.01, 0.05, 0.1, 0.25, 0.5 and 1.0 mM calcium, respectively. α-A, α-actinin; A, actin.

TABLE I. Purification of Ascites Microvillar AMV-p35*

Purification step	Total protein (mg)	Total AMV-p35 (mg)	% as AMV-p35	Purification (-fold)	Yield (%)
Microvilli	12.0	0.70	6 (5)	1	100
Ca pellet	2.4	0.43	18	3	61
EGTA supernate	1.4	0.39	28	5	56
DE-52 flowthrough	0.25	0.24	95	16	34

*Protein was quantified by using the BCA protein assay. A second measurement of the fractional amount of AMV-p35 was made by quantification of leucine label in microvilli from metabolically labeled cells. This value is given in parentheses.

precipitation required about 1 mM calcium (Fig. 6). For purification AMV-p35 was precipitated from the soluble fraction with 1.5 mM calcium, solubilized from the precipitate with EGTA and purified by passing through a DE-52 column (Fig. 5, lane 7). This behavior of AMV-p35 demonstrated another similarity to p36.

Two comments should be made about the calcium precipitation procedure: (1) Several other proteins are precipitated by calcium with AMV-p35. (2) Purified AMV-p35 is not precipitated with calcium under the same conditions. These results suggest that other molecules are involved in the precipitation phenomenon.

Biochemical Comparisons of AMV-p35 With Calpactins

Glenney [19,28] has named the p36 [12,17] and p35 [18] tyrosine kinase substrates calpactins and defined them on the basis of two properties: their calcium-dependent association with F-actin and calcium-dependent binding to phospholipid liposomes. The first and most striking property of the AMV-p35 which we observed

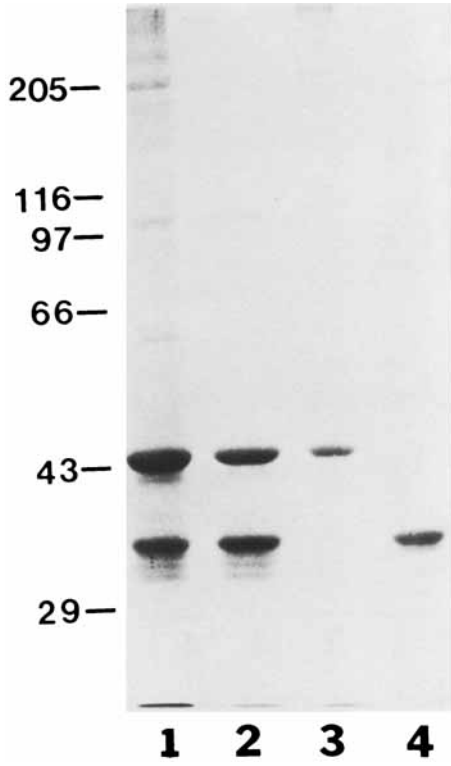

Fig. 4. Purification of AMV-p35 from microfilament cores prepared in the presence of calcium. Microvilli were lysed with 10 mM Tris (pH 7.5), 100 mM KCl, 1 mM $CaCl_2$, 1 mM PMSF, 0.5 mM DTT, 2 mM $MgCl_2$, and 0.2% Triton X-100 at room temperature for 20 min and centrifuged at 15,000g for 20 min at 4°C. The resulting pellet (Ca-microfilament core) was incubated in 10 mM Tris (pH 7.5), 5 mM KCl, 1 mM EGTA, 0.5 mM DTT, 0.05% Triton X-100, for 20 min and recentrifuged at 15,000g for 15 min at 4°C. The supernate was passed through a DE-52 column equilibrated in the same buffer and the flow-through volume was collected. **Lane 1:** Ca-microfilament core. **Lane 2:** Supernate from EGTA extraction of Ca-microfilament core. **Lane 3:** pellet from EGTA extraction of Ca-microfilament core. **Lane 4:** Flow-through from DE-52 column. Molecular weights of standard proteins (kDa) are shown on left.

was its calcium-dependent association with microvillar microfilaments. To determine whether AMV-p35 is related to the calpactins, we have examined four properties: binding to F-actin, binding to phospholipid vesicles, fragments derived from limited chymotryptic digestion, and amino acid composition.

To evaluate the ability of AMV-p35 to bind directly to microfilaments, purified AMV-p35 was incubated with F-actin. Sedimentation of the mixtures in the Airfuge showed that AMV-p35 binds to F-actin in a calcium-dependent manner, requiring mM calcium concentrations for maximal binding (Fig. 7). This high calcium requirement is similar to that reported for calcium binding to p36 in the absence of phospholipid [29].

Since the calpactins may be membrane-associated proteins, we examined the ability of AMV-p35 to bind to phospholipid liposomes. As shown in Figure 8, microvillar AMV-p35 binds to phosphatidylserine liposomes in a calcium-dependent

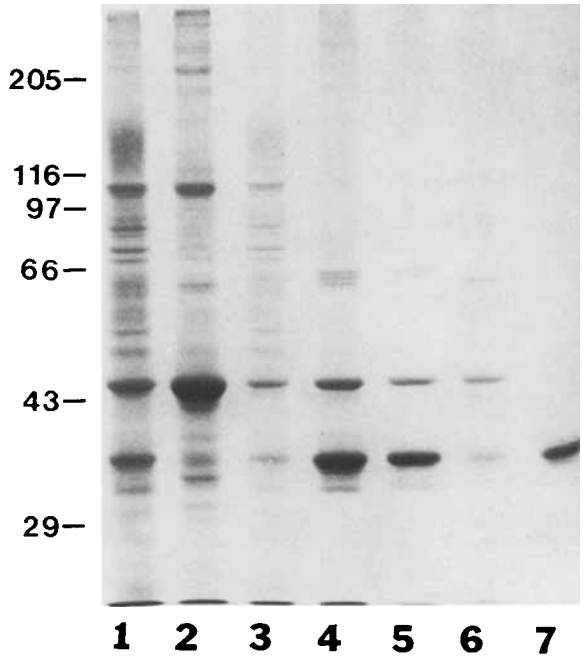

Fig. 5. Purification of AMV-p35 by calcium precipitation from microvillar lysate supernates prepared in the absence of calcium. Microvilli were lysed with 10 mM Tris (pH 7.5), 100 mM KCl, 1 mM PMSF, 0.5 mM DTT, 2 mM $MgCl_2$, 0.2% Triton X-100 at room temperature for 20 min and centrifuged at 15,000g for 20 min at 4°C. $CaCl_2$ was added to the supernate to a final calcium concentration of 1.5 mM. After 5 min at room temperature the calcium-treated sample was centrifuged at 15,000g for 15 min at 4°C. The pellet was resuspended in 10 mM Tris (pH 7.5), 5 mM KCl, 1 mM EGTA, 0.5 mM DTT, 0.05% Triton X-100, and applied to a 0.5 ml DE-52 column equilibrated with the same buffer. The flow-through volume was collected. **Lane 1:** Supernate of Triton lysate. **Lane 2:** Pellet from Triton lysate. **Lane 3:** Supernate after calcium precipitation. **Lane 4:** Pellet after calcium precipitation. **Lane 5:** Material resolubilized from calcium pellet. **Lane 6:** Material insoluble during resolubilization. **Lane 7:** DE-52 flow-through.

reaction when analyzed by a sedimentation assay. Half-maximal binding occurs at about 10 μM calcium.

AMV-p35 is very similar to calpactin I with respect to its cleavage by α-chymotrypsin (Fig. 9). Chymotrypsin cleavage of ascites AMV-p35 yields a fragment of 33,000 daltons, just as previously reported for intestinal brush border p36 (calpactin) [27]. Since AMV-p35 gave the same chymotrypsin cleavage pattern as calpactin, it was of interest to compare the amino acid compositions of the two. For amino acid analysis, AMV-p35 was purified by solubilization microvilli in the absence of calcium, precipitation from the low-speed supernate with calcium, solubilization of AMV-p35 from the pellet with EGTA, and chromatography on a DE-52 column, as in the experiment depicted in Figure 5. This AMV-p35 fraction was further purified by chromatography on hydroxyapatite to a purity of >95%, as assessed by SDS-PAGE. A comparison of microvillar AMV-p35 with p36 of calpactin I shows very similar amino acid compositions (Table II).

Immunological Comparisons of AMV-p35 With Calpactin I and the Lipocortins

Recent studies suggest that the calpactins are members of a family of calcium-sensitive proteins with different functions in the sub-plasma membrane region of

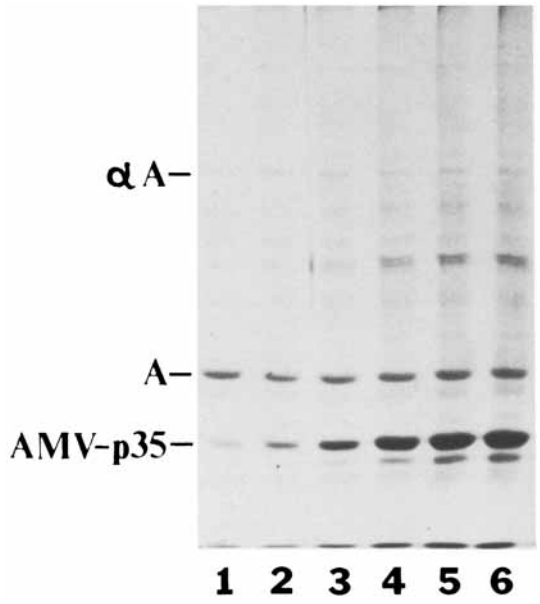

Fig. 6. Calcium dependence of AMV-p35 precipitation. Microvilli were extracted with 0.2% Triton X-100, 80 mM KCl, 0.5 mM dithiothreitol, 10 mM TES (N-tris[hydroxymethyl]methyl-2-aminoethane sulfonic acid), pH 7.2, at room temperature for 20 min and centrifuged in the Airfuge at 12 psi for 30 min at 4°C. Calcium was added to the final concentrations indicated, and the samples were incubated for 10 min at room temperature. After centrifugation at 12 psi for 30 min in the Airfuge at 4°C, the pellets and supernates were prepared for SDS PAGE. **Lanes 1–6** are from samples containing 0.05, 0.1, 0.25, 0.5, 1.0, and 2.0 mM calcium, respectively. α-A, α-actinin, A, actin.

cells. Also included in this family are the lipocortins. Lipocortins I and II have been shown to be identical or highly related to calpactins II (EGF receptor kinase substrate) and I ($pp60^{src}$ kinase substrate), respectively. Therefore, we examined the reactivity of AMV-p35 with antisera directed against bovine intestinal brush border calpactin I and human placental lipocortins I and II. The anti-calpactin serum has been shown to react with both calpactin I and calpactin II [19]. Anti-lipocortin I has been shown to react strongly with lipocortin I and weakly with lipocortin II [21]; anti-lipocortin II reacts strongly with lipocortin II and weakly with lipocortin I [21].

As shown by immunoblot analysis in Figure 10, in which equivalent amounts of all purified proteins were loaded onto the gels, anti-calpactin reacts strongly with purified AMV-p35 (panel B, lane 3) and a corresponding band from the ascites microvilli (panel B, lane 1). As expected, this antiserum also reacts strongly with purified intestinal calpactin I (panel B, lane 4), the protein against which it was raised. It reacts very weakly with purified lipocortin I (panel B, lane 2). In contrast, anti-lipocortin I reacts strongly only with lipocortin I (panel C). Only a very weak reaction is observed with ascites microvilli or purified AMV-p35, and no reaction was detected with intestinal calpactin I. Anti-lipocortin II reacted with AMV-p35 (panel D, lane 3), but the reaction appeared weaker (based on equivalent protein amounts) than reaction with calpactin I (panel D, lane 4). These combined results suggest that ascites AMV-p35 is more similar to calpactin I (lipocortin II).

Sequence Analysis of Regions of AMV-p35

To clarify relationships among AMV-p35, calpactin I, and lipocortin I, sequence studies on AMV-p35 were performed. When the intact protein was subjected to direct

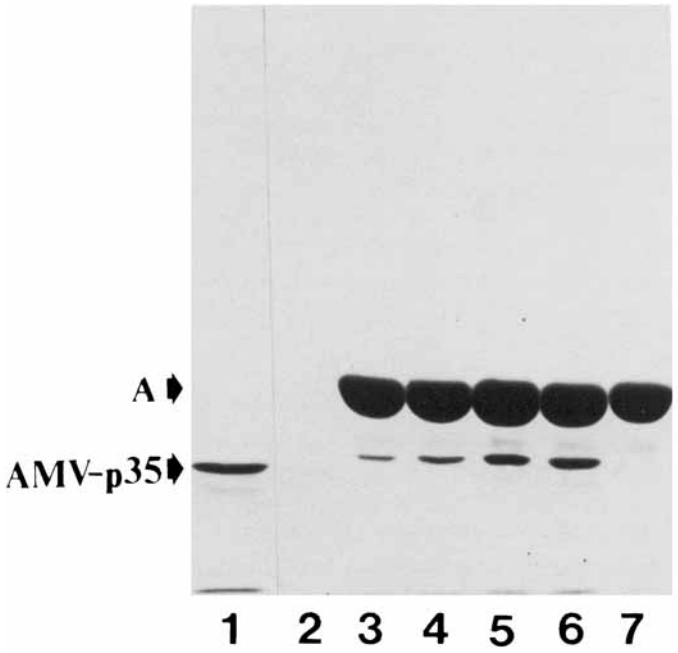

Fig. 7. Binding of AMV-p35 to F-actin. F-actin (55 μg/ml final concentration) and AMV-p35 (40 μg/ml) were incubated in 10 mM HEPES, 50 mM KCl, 4 mM $MgCl_2$, 0.5 mM DTT, 0.05% Triton X-100 and various concentrations of calcium for 30 min and centrifuged at 12 psi (50,000g) for 20 min at 4°C in the Airfuge. The pellets and supernates were prepared for SDS PAGE. **Lane 1:** Supernate from sample containing only AMV-p35, 1.2 mM calcium. **Lanes 2–6:** Samples containing actin and AMV-p35 with calcium concentrations of 0.06, 0.12, 0.6, and 1.2 mM, respectively. **Lane 7:** Sample containing actin and 1.2 mM calcium.

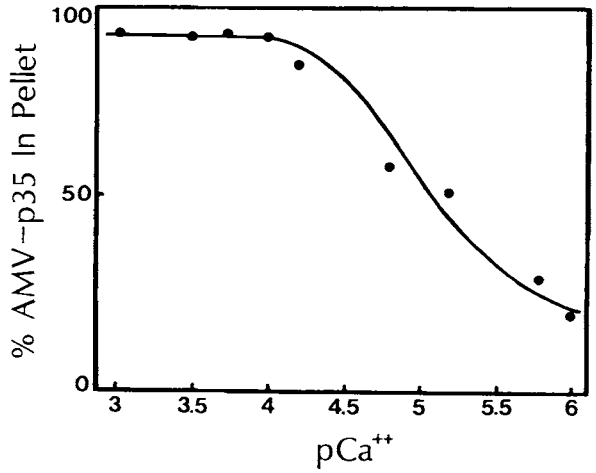

Fig. 8. Binding of AMV-p35 to liposomes. Calcium dependence of binding of AMV-p35 to phosphatidylserine liposomes was determined as described in Materials and Methods. EGTA-buffered calcium concentrations were determined by the method of Robertson and Potter [52].

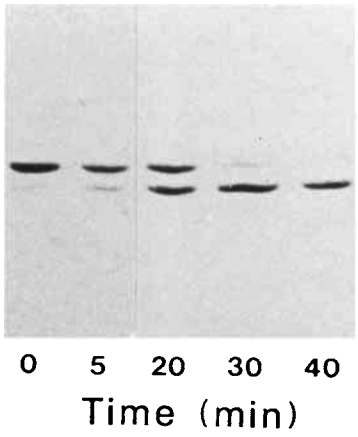

Fig. 9. Cleavage of AMV-p35 to a 33,000-dalton fragment by α-chymotrypsin. Purified AMV-p35 (0.15 mg/ml) and 0.11 units/ml of α-chymotrypsin were incubated in 100 mM NaCl, 1 mM $CaCl_2$, 10 mM Tris (pH 7.5) at room temperature for 0–40 min. The reaction was stopped by adding SDS PAGE sample buffer and boiling for 2 min.

TABLE II. Amino Acid Comparisons Between AMV-p35 and Intestinal p36 Proteins

	Mole %	
Amino acid	AMV-p35	Intestinal p36
Aspartic	10.4	11.4
Threonine	4.8	5.1
Serine	9.6	10.0
Glutamic	13.4	12.1
Proline	3.6	3.1
Glycine	10.7	8.4
Alanine	6.6	6.6
Valine	5.2	4.5
Methionine	0.8	2.3
Isoleucine	4.3	4.4
Leucine	8.9	8.8
Tyrosine	3.7	4.3
Phenylalanine	2.4	3.5
Histidine	0.8	1.2
Lysine	9.5	9.5
Arginine	5.4	4.8

sequence analysis, no PTH derivatives were obtained for the first four Edman degradation cycles, suggesting the presence of a blocking group at the amino terminus. Following CNBr cleavage, fragments of AMV-p35 were separated by HPLC, and two peptides, CN1 and CN2, denoted by arrows in Figure 11, were selected for sequence analysis. Table III shows a comparison of the sequences of these peptides with those of corresponding sequences previously reported for calpactin I [30] and lipocortin I [21]. AMV-p35 peptide CN2 is identical with the homologous region of calpactin, with the exception of the presumed presence of methionine preceding the sequence, which corresponds to an isoleucine (residue 98) in calpactin. AMV-p35 peptide CN1 is identical to the corresponding sequence of calpactin I, including the presumed methionines at either terminus. These AMV-p35 sequences also show

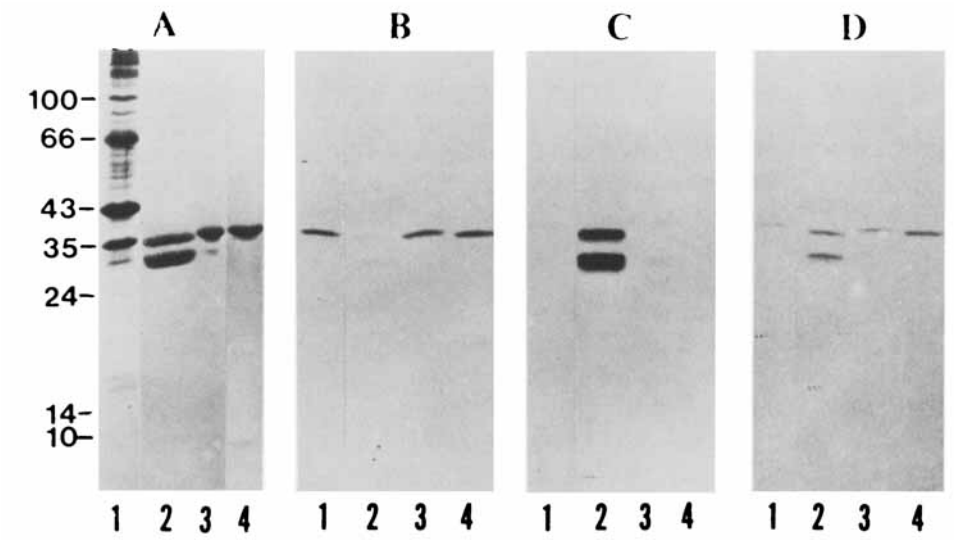

Fig. 10. Reaction of AMV-p35 with antisera directed against intestinal calpactin (B), lipocortin I (C), and lipocortin II (D). **Panel A:** Coomassie blue stain. **B:** Anti-calpactin. **C:** Anti-lipocortin I. **D:** Anti-lipocortin II. **Lane 1:** Ascites microvilli. **2:** Lipocortin I. **3:** AMV-p35; **4:** Calpactin I.

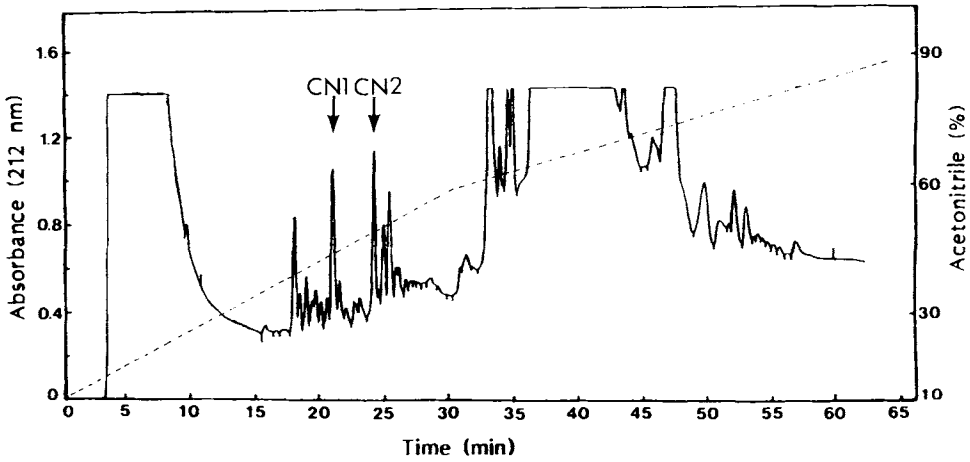

Fig. 11. HPLC separation of cyanogen bromide peptides from AMV-p35. The peptides used for sequence analysis are marked with arrows.

strong homology to equivalent regions in lipocortin I, as does calpactin. In lipocortin I there are different amino acids at three sites in each region. These results support and extend the biochemical and immunological findings of the close similarities between AMV-p35 and calpactin I.

Biophysical Properties of AMV-p35

Calpactin (p36) has been reported to have a native molecular weight of 80,000 daltons and to be composed of 36,000- and 10,000-dalton subunits [17,27]. In contrast, the related 35-kDa protein, which is apparently the same protein as lipocortin I [21], has been reported to behave as a monomer [18]. When purified ascites AMV-

TABLE III. Amino Acid Sequence Comparisons of Calpactin I and Lipocortin I With Cyanogen Bromide Peptides of AMV-p35*

Peptide CN1																
AMV-p35	(met)	lys	gly	lys	gly	thr	arg	asp	lys	val	leu	ile	arg	ile	(met)	
Calpactin	met	lys	gly	lys	gly	thr	arg	asp	lys	val	leu	ile	arg	ile	met	
	277														291	
Lipocortin	met	lys	gly	val	gly	thr	arg	his	lys	ala	leu	ile	arg	ile	met	
	286														300	
Peptide CN2																
AMV-p35	(met)	leu	gly	leu	leu	lys	thr	pro	ala	gln	tyr	asp	ala	ser	glu	leu	—
Calpactin	ile	leu	gly	leu	leu	lys	thr	pro	ala	lys	tyr	asp	ala	ser	glu	leu	lys
	98																114
Lipocortin	val	leu	ala	leu	leu	lys	thr	pro	ala	gln	phe	asp	ala	asp	glu	leu	leu
	107																123

*Amino acid sequences of AMV-p35 CNBr peptides compared to homologous sequences of calpactin I [30] and lipocortin I [51]. Peptide CN1 corresponds to endonexin fold region 4 of both calpactin I (residues 276–334) and lipocortin I (287–302) [31]. Peptide CN2 corresponds to a sequence outside of any endonexin fold region [31] in either calpactin I or lipocortin I.

p35 was examined by gel filtration and velocity sedimentation under nondenaturing conditions, its molecular weight was found to be 35,000, with a frictional ratio of 1.27 (Table IV). To insure that this monomeric form did not result from changes in the protein during purification, detergent extracts of microvilli were chromatographed on Sephacryl S-200. An SDS PAGE gel of the column fractions is shown in Figure 12. The 35-kDa species from the microvilli was eluted predominantly at a volume corresponding to a molecular weight of a monomer, suggesting a monomeric structure for AMV-p35 in the microvilli. No evidence was found in these studies for a subunit of 10,000 daltons associated with AMV-p35.

DISCUSSION

The results of this study show that one of the major proteins (AMV-p35) of ascites tumor cell microvilli is a calcium-sensitive microfilament and liposome binding protein. AMV-p35 was isolated from microfilament cores prepared in the presence of calcium by elution with EGTA and passage over a DEAE column. This isolation procedure and the properties of the protein were strongly reminiscent of a family of proteins which had been described as tyrosine kinase substrates associated

TABLE IV. Physical Properties of Ascites Microvillar AMV-p35

Native molecular weight	35,100
Molecular weight by SDS PAGE	35,000
Stokes radius	2.75 nm
Sedimentation coefficient	3.1 S
Partial specific volume	0.724 cm^3/g
Frictional ratio	1.27

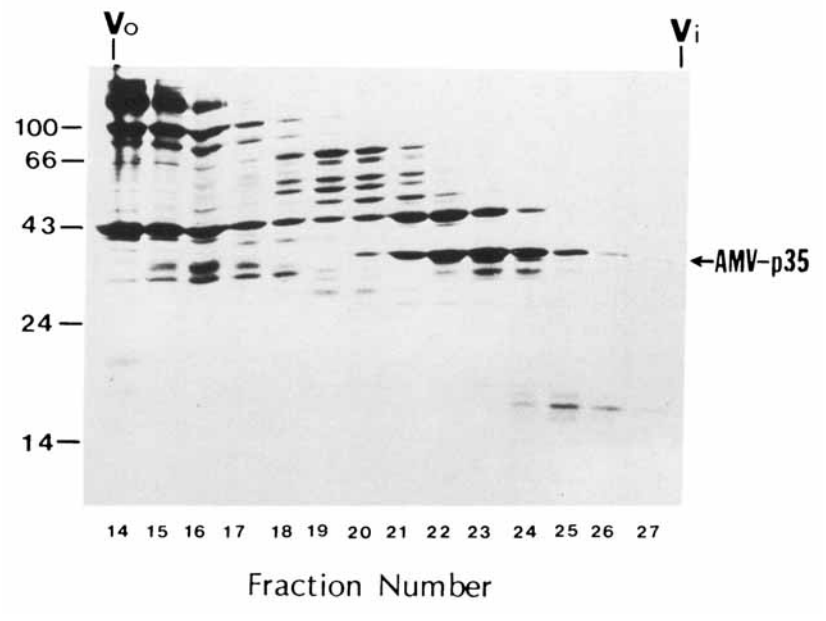

Fig. 12. Molecular size determination of AMV-p35 by gel filtration of microvillar extracts. Molecular weight (kDa) markers on left; fraction numbers indicated at the bottom.

with the plasma membrane or submembrane cytoskeleton (calpactins, p36, p35). More recently, these proteins have been shown to be related to the lipocortins; lipocortins I and II have been shown to be identical or very similar to calpactins II and I, respectively [21]. Lipocortin I is identical or very similar to the EGF receptor kinase substrate p35 [20,21].

AMV-p35 resembles both calpactin (calpactin I) and lipocortin (lipocortin I) in its calcium-dependent binding to F-actin and liposomes. Although related to both, as shown by the immunological assays and by the sequence comparisons, it is clearly more closely related to calpactin. It is not surprising that cyanogen bromide peptide 1 is identical to a sequence in calpactin, since the homologous sequence comprises most of the fourth endonexin fold region (calpactin residues 277–294) [31]. These endonexin fold regions appear to be highly conserved in annexin-like proteins [31], of which calpactin is a member. However, peptide 2, which lies completely outside any of the endonexin regions of calpactin [30,31], also shows a very high degree of homology with calpactin.

Despite the strong homology between AMV-p35 and calpactin, the two differ in one significant parameter, ie, molecular size of the native molecule. Like lipocortin I [18], AMV-p35 is a 35,000-dalton monomer, while calpactin I, as originally described in brush border microvilli and other cells, is an 80,000-dalton multimer [17,27]. Calpactin I (p36) has been shown to be a tetramer of two 36-kDa subunits and two 10-kDa subunits [28,29]. However, lipocortin II, the analog of calpactin I, has also been reported to be a monomer [21]. Moreover, Glenney and co-workers have recently found a monomeric native form of calpactin I as well as the heterotetramer in bovine lung and human placenta [19]. In our ascites tumor cells the 10-kDa subunit appears to be absent in purified AMV-p35. This observation is consistent with the fact that AMV-p35 is present as a monomer rather than the tetramer in the microvilli. The absence of the 10-kDa subunit may result from the failure of the ascites cells to synthesize this protein; attempts to identify a 10-kDa polypeptide in either isolated microvilli or intact ascites cells have been unsuccessful. Interestingly, mRNA for p36 and p11 (the 10-kDa subunit), are differentially expressed in different tissues [32]. We will address this question by looking for mRNA for the 10-kDa subunit. Alternatively, the absence of the 10-kDa subunit may result from a difference in AMV-p35 which prevents or reduces its binding to the 10-kDa subunit. Studies are in progress to examine this possibility.

In considering a function for AMV-p35, two factors are important: the concentration of AMV-p35 in the microvilli and the calcium dependence. The amount of AMV-p35 in the microvilli is extremely high, approximately 6% of the microvillar protein. Thus the molar concentration of AMV-p35 is more than half that of actin, the major microvillar structural protein. The possibility that AMV-p35 acts as a phospholipase inhibitor, as previously proposed for the lipocortins [33], would appear to be unlikely, since the concentration of AMV-p35 must be several orders of magnitude higher than the concentration of the phospholipase. Moreover, recent studies suggest that calpactins and lipocortins do not inhibit the phospholipase by interaction with the enzyme, but rather, by binding to the substrate phospholipid [34]. Therefore, one possible function for AMV-p35 might be to act as a membrane-protecting agent, preventing the action of phospholipases which might modify the membrane in the presence of increased calcium concentrations.

The localization of p36 analogs in the submembrane region of cells [15] suggests that these proteins are bound to the membrane or submembrane microfilaments or

both. The high concentration of AMV-p35 would also permit it to occupy a sizable fraction of binding sites on microvillar microfilaments or membranes. Thus AMV-p35 could serve as a structural or stabilizing agent on the microfilaments. Preliminary crosslinking studies with cleavable chemical crosslinkers suggest that a sizable fraction of the AMV-p35 is associated with the microfilament core of the microvilli [see Fig. 2. of ref. 8]. How these observations can be reconciled with the calcium requirements for binding to microfilaments or membranes is unclear, since these calcium concentrations are well above the normal concentrations of free calcium found in cells. Glenney has also shown binding of calpactins I and II to microfilaments in the presence of high (1 mM) but not low (< 1 μM) concentrations of calcium [19]. Other factors may play a role in the association of calpactins with microfilaments and membranes. Glenney has shown that calcium binding [27] and the calcium dependence of phosphorylation [35] of calpactin I are shifted to the micromolar range in the presence of phosphatidylserine. Perhaps other effectors cause a similar shift in the calcium requirement, which could allow AMV-p35 to associate with microfilaments at normal cellular calcium levels.

Recent studies have shown that the calpactins or lipocortins can be classified with a larger family of calcium-sensitive, membrane-binding proteins. Included in this family along with the calpactins [19,27] or lipocortins [20,21,31] are proteins I and II [29,36], p36 [12–14,17], p35 [18], endonexin [31], calelectrins [37–42], chromobindins [43,44], lymphocyte membrane proteins [45,46], mammary epithelial cell proteins [47,48], and phenothiazine binding proteins called calcimedins [49]. AMV-p35 appears to be related to the calcimedins in that it binds phenothiazine [50]. Recently, Geisow and co-workers [31] have reported common, consensus sequences from endonexin, calelectrin, p36 (calpactin I), lipocortin II, and protein II. The calelectrins, originally isolated from electroplax tissue [37], have been implicated in exocytosis [31]. They are present at high concentrations in secretory tissues, bind to membranes at micromolar calcium concentrations, and cause aggregation of membranes, although only at high calcium concentrations [41]. The liposome binding of the calpactins and AMV-p35 might suggest that they are involved in similar membrane functions. Alternatively, one might postulate that a class of structurally related, but different, proteins could act as antagonists of these functions, in the manner of drug analogs. Clearly, further studies are necessary to understand the relationships among these various classes of proteins and their functions in the different types of cells in which they are found.

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