Eur. J. Biochem. 178, 543 – 553 (1988)

Characterization of an inhibitor of actin polymerization in vinculin-rich fraction of turkey gizzard smooth muscle

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(Received June 10/September 1, 1988) – EJB 88 0693

We report here on the purification and characterization of a new 25-kDa inhibitor of actin polymerization from turkey gizzard smooth muscle. The protein was purified by chromatography on DEAE-cellulose and hydroxyapatite, as well as by affinity chromatography on an immobilized-antibody column. The purified polypeptide reduced the low-shear viscosity of actin, apparently due to its inhibitory effect on actin polymerization. We demonstrate that this protein is largely responsible for the apparent inhibitory activity previously reported to be associated with smooth muscle vinculin preparations. Three independent monoclonal antibodies prepared against the 25-kDa inhibitor of actin polymerization can effectively adsorb the inhibiting activity of actin polymerization from the crude vinculin preparation or inhibit it. We also show here that the 25-kDa inhibitor of actin polymerization tends to undergo dimerization when maintained in non-reducing buffers, concomitant with the loss of its inhibitory activity. Immunohistochemical labeling of frozen sections, as well as immunoblotting analyzes, indicated that the 25-kDa inhibitor of actin polymerization is particularly enriched in smooth muscle cells and that its distribution is apparently homogenous throughout the cytoplasm showing no apparent enrichment in the vinculin-rich dense plaques located along the endofacial surface of the plasma membrane.

Shortly after the discovery of vinculin and the characterization of its subcellular and tissue distribution [1 – 4], attempts were made to study its putative interactions with the various components of the microfilament systems and, predominantly, with actin. Early studies along these lines have shown that purified vinculin preparations considerably reduced the low- and high-shear viscosity of F-actin solutions [5 – 7]. However, further studies indicated that this actin-capping or actin-bundling activity was attributable not to vinculin itself but rather to 'contaminating' polypeptide(s) of lower molecular mass which were copurified with vinculin. The putative 'contaminant(s)' could be separated from vinculin by chromatography on carboxymethyl cellulose [8, 9] or hydroxyapatite [10] columns. These studies disclosed a group of polypeptides ranging in molecular mass from 20 kDa to 45 kDa [9, 10] which were consistently present in the active fraction, but the interrelationship between the various polypeptides present in this fraction and the exact identity of the actin-capping component(s) in this mixture remained unclear. Furthermore, Wilkins et al. [11] suggested that the low-molecular-mass polypeptides present in the fraction (denoted HA-1) were, at least partially, proteolytic products of polypeptides of higher apparent molecular mass (200 kDa and 150 kDa). The latter was recently isolated and named tensin [12].

In the present study we purified from a vinculin-rich fraction of turkey gizzard smooth muslce a protein which is an inhibitor of actin polymerization (IAP). Several monoclonal antibodies prepared against the IAP-rich fraction reacted with a single polypeptide of 25 kDa (denoted here as 25-kDa IAP). These monoclonal antibodies adsorbed the 25-kDa IAP and some of them inhibited its effect on actin viscosity. These antibodies were immobilized and were used for the purification of the inhibiting protein as well as for its localization in gizzard tissue. Under non-reducing conditions the 25-kDa IAP tends to form dimers concomitantly with the loss of its inhibitory activity. This inactivation could be inhibited either by carboxymethylation or by the maintenance of the purified protein in sulfhydryl-containing buffers. The general properties of the 25-kDa IAP and its relationships to several actin-capping proteins and to vinculin are discussed.

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Abbreviations. BSA, bovine serum albumin; IAP, inhibitor of actin polymerization; 25-kDa IAP, 25-kDa polypeptide which inhibits actin polymerization; IAP-9, IAP-11, IAP-28 are monoclonal antibodies against 25-kDa IAP; $NaCl/P_i$, 140 mM sodium chloride, 10 mM sodium phosphate pH 7.4.

MATERIALS AND METHODS

Purification of 25-kDa IAP

The 25-kDa IAP was extracted from fresh (or freshly frozen) turkey gizzards following the procedure described for vinculin purification by Feramisco and Burridge [13]. Further purification of the 25-kDa IAP was carried out on a DEAE-cellulose column (DE-52, Whatman, Maidstone, UK) equilibrated with buffer A (20 mM Tris, 10 mM NaCl, 15 mM 2-mercaptoethanol, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.6). Crude IAP extract (175 ml containing about 1 g protein) was dialyzed against buffer A and applied to a DE-52 column (2.6 × 23 cm). The column was washed with buffer A (7.5 bed volume) and elution carried out with a linear NaCl gradient (0 – 0.25 M NaCl in buffer A, 400 ml each). Absorbance at 280 nm, salt concentration, low-shear viscosity and characterization in SDS/PAGE were determined for every second fraction (4.9 ml/fraction). The

active fractions were pooled and dialyzed against buffer A, adjusted to 15 mM phosphate, and loaded on a hydroxyapatite column (1.3 × 11 cm; Bio-gel HTP, Bio-Rad, Richmond, CA, USA), pre-equilibrated with 50 mM phosphate buffer pH 7.0. A total of 24 mg protein was loaded and the column was washed with 50 mM potassium phosphate pH 7.0 until no protein was detected in the effluent. Subsequently a linear phosphate gradient (50 – 250 mM, pH 7.0, 100 ml each) was used to elute adsorbed proteins. Fractions of 2.8 ml were collected and analyzed for their absorbance at 280 nm, salt concentration and their capacity to reduce actin viscosity. Under these conditions most of the 25-kDa IAP did not bind to the hydroxyapatite column whereas vinculin was bound to the column and could be eluted from it by a linear phosphate gradient (50 – 250 mM, pH 7.0).

Immunochemical methods

Monoclonal antibodies to the 25-kDa IAP were prepared following the procedure described in detail by Eshhar [14]. For the preparation of monoclonal antibodies we injected mice with either the DEAE-cellulose or the partially purified hydroxyapatite fractions of the 25-kDa polypeptide. The mice were boosted twice and their spleen cells fused with NSO/1 myeloma. Positive wells were selected by radioimmunoassay and further analyzed by multiple-slot immunoblotting. Affinity purification of goat anti-[mouse $F(ab)'_2$] was carried out on mouse IgG coupled to *p*-nitrophenyl-carbonate – agarose (5 mg protein/g gel) according to Wilchek and Miron [15]. The column was loaded with immune goat serum, rinsed with phosphate-buffered saline ($NaCl/P_i$), and the antibodies eluted with 0.2 M glycine/HCl, pH 2.7. For the affinity purification of monoclonal antibodies, purified goat anti-(mouse antibodies) [or their $F(ab)'_2$ fragments] were coupled to polyacrylhydrazido-agarose (15 – 25 μmol/ml, Sigma, St Louis, MO, USA) according to Miron and Wilchek [16] with the following modifications: 10 g wet gel was suspended in 20 ml 0.3 M HCl at 4 °C and freshly prepared (1 M, 4 ml) $NaNO_2$ solution was added under magnetic stirring. After 7 min at 4 °C, the gel was rinsed with cold 0.3 M HCl followed by cold water. After neutralization of the gel, the goat anti-mouse antibodies (3 – 4 mg/g wet gel) were added in 0.1 M $NaHCO_3$ and coupling proceeded at 4 °C for 16 h. The conjugated gel was rinsed with 0.2 M glycine/HCl, pH 2.7, followed by 0.15 M NH_4OH and finally with $NaCl/P_i$. Purification of monoclonal antibodies was carried out as described above. Screening of hybridoma supernatants was carried out in a locally produced multiple-slot blotting apparatus, which enables the use of 0.1 – 0.3 ml antibody sample/slot and simultaneous labeling with 20 antibody samples.

Affinity purification of the 25-kDa IAP

Purified anti-25-kDa IAP monoclonal antibodies (2 mg/g wet gel) were coupled to 6-aminohexanoyl-Trisacryl (Trisacryl Gf 2000, LKB, Bromma, Sweden) after activation with *p*-nitrophenol according to Miron and Wilchek [17]. Partially purifed 25-kDa IAP was applied to the column, rinsed with $NaCl/P_i$ and eluted with 0.15 M NH_4OH.

Distribution of the 25-kDa IAP in chick tissues

A preliminary qualitative analysis was performed by dot blotting. Several tissues (including heart, gizzard, kidney, liver, brain, and intestine) were excised from 16-day-old chick embryos, and small slices were immediately boiled for 15 min in SDS sample buffer. Samples at appropriate dilutions were subsequently subjected to electrophoresis on 12% SDS/PAGE and blotted onto nitrocellulose paper, or directly applied to nitrocellulose paper. The papers were quenched with 0.1% milk in $NaCl/P_i$, treated with anti-(25-kDa IAP) and/or anti-vinculin. After rinsing the unbound antibodies, the blots were treated with radioiodinated goat anti-(mouse IgG), washed, and examined by autoradiography. Quantitative analysis for the content of the 25-kDa IAP was carried out using solid-phase radioimmunoassay. Serial dilutions of the purified 25-kDa IAP were used in order to construct a standard calibration curve. Several dilutions of each extract were examined.

Measurement of actin polymerization

Actin was extracted from acetone powder of rabbit skeletal muscle according to Spudich and Watt [18] and stored in filamentous form at 4 °C. F-actin was converted to G-actin by exhaustive dialysis against 'G-actin buffer' (2 mM Tris/HCl, 0.2 mM $CaCl_2$, 0.2 mM 2-mercaptoethanol, 0.2 mM ATP, pH 8.0) followed by centrifugation at 80000 × *g* for 2 h at 4 °C. Actin was purified by gel filtration on Sephadex G-150 equilibrated with G-actin buffer according to McLean-Fletcher and Pollard [19]. Pyrenyl-actin was prepared by reacting actin in 1 mM $NaHCO_3$, 0.1 mM $CaCl_2$, 0.2 mM ATP, pH 7.6 with *N*-(pyrenyl)iodoacetamide (purchased from Molecular Probes, Roseville, MN, USA) according to Kouyama and Mihashi [20] and was further purified on a Spherogel TSK-3000 SW (Beckman Instruments, Geneva, Switzerland) column (7.5 × 30 mm). Kinetic studies of actin polymerization were performed according to Pollard [21] at 25 °C, with 10% pyrenyl-actin of actin monomers and 0.5 μM actin nuclei. Actin polymerization was examined in the presence of 0.2 – 0.6 μM purified 25-kDa IAP. Gel-filtered actin monomers containing 10% pyrenyl-labeled actin were preincubated for 2 – 5 min with 25-kDa IAP (0 – 0.6 μM) in G-actin buffer. A freshly mixed solution of actin nuclei, KCl and $MgCl_2$ was added to the actin solution to a final concentration of 0.5 μM nuclei, 50 mM KCl and 2 mM $MgCl_2$. The fluorescence intensity was measured by using a Perkin Elmer MPF-3L fluorimeter. The excitation and emission wavelengths were 366 nm and 407 nm, respectively. The effect of several monoclonal antibodies on the activity of 25-kDa IAP was determined after preincubation with 25-kDa IAP for 30 min at 25 °C.

Low-shear viscosity measurements were carried out using the falling-ball microcapillary-viscometer assay as described by MacLean-Fletcher and Pollard [19]. All measurements were carried out at room temperature (22 – 25 °C) and at an angle of 50°. The microcapillaries were loaded with actin solution 30 min prior to measurement.

Electrophoretic methods

SDS/PAGE was carried out on 8%, 12% or 10 – 20% gradient slab gels in Laemmli buffer system [22]. Immunoblotting analysis was carried out essentially according to Towbin et al. [23]. The transfer buffer consisted of 50 mM Tris, 50 mM glycine, 1 mM $MgCl_2$, pH 8.3 and the electrotransfer was performed at 65 V for 3.5 h at room temperature. Two-dimensional gels were prepared according to O'Farrel

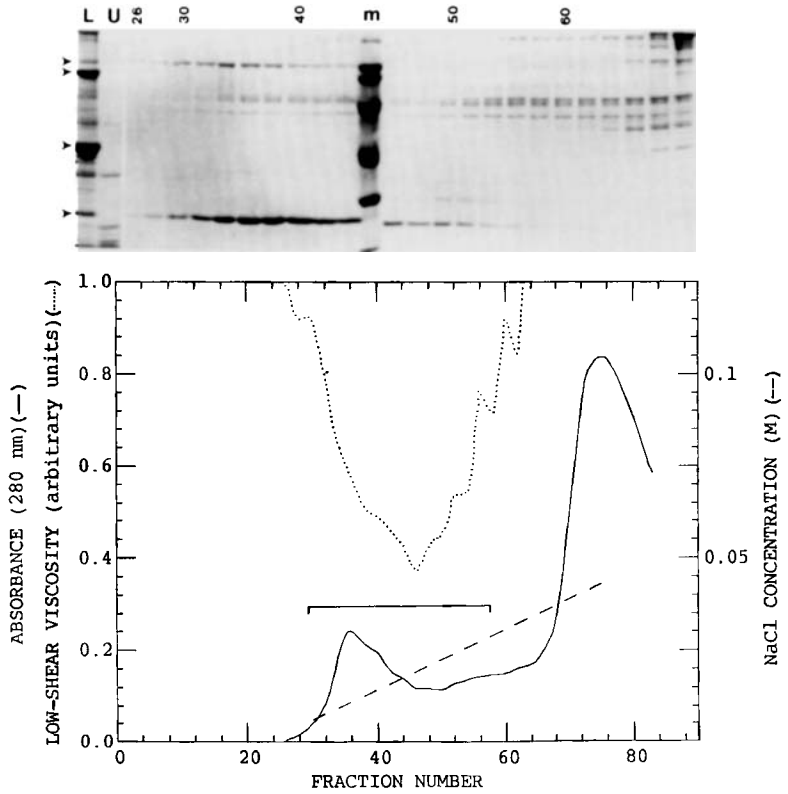

Fig. 1. *Purification of the 25-kDa IAP by DEAE-cellulose chromatography.* Upper part: SDS/PAGE (8%) of every second eluted fraction from DEAE-cellulose column (fractions 26–70, corresponding to lower part); L, crude extract loaded onto the column; U, unbound fraction; m, molecular mass markers [from top to bottom: myosin from rabbit muscle (205 kDa), β-galactosidase from *E. coli* (116 kDa), phosphorylase *b* from rabbit muscle (94 kDa), albumin from bovine serum (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa)]. Arrows from top to bottom indicate vinculin, α-actinin, actin and the 25-kDa polypeptide. Lower part: elution profile of turkey gizzard 25-kDa IAP from DEAE-cellulose column. The bracket indicates the fractions which were collected for further purification

[24]. Non-equilibrium electrofocusing was used as first dimension. The second dimension was 12% SDS/PAGE. After the second run, the gel was blotted onto nitrocellulose paper and characterized by reaction with antibodies.

Biochemical analyzes

Amino acid analysis was carried out in a Durrum D-550 amino acid analyzer (Durrum Instruments, Palo Alto, USA) following acid hydrolysis according to Spackman et al. [25]. Carboxymethylation was performed according to Crestfield et al. [26]. Determination of free sulfhydryl groups was done using Ellman's reagent [5,5-dithiobis-(2-nitrobenzoic acid), Sigma, St Louis, MO, USA] using $\varepsilon_{412} = 13600\ M^{-1}\ cm^{-1}$ [27]. Protein concentration was determined according to Gornall [28], Lowry [29] or Bradford [30]. Actin concentration was measured by absorbance at 290 nm using $\varepsilon_{290} = 26600\ M^{-1}\ cm^{-1}$. The concentration of pyrenyl-actin was measured by absorbance at 344 nm with $\varepsilon_{344} = 22000\ M^{-1}\ cm^{-1}$ according to Pollard [21]. Solid-phase radioimmunoassay was performed on polyvinyl microtiter plates. Serial dilutions of protein extract solution were adsorbed to the wells. After 2 h at room temperature the wells were rinsed with NaCl/P_i containing 1% bovine serum albumin (BSA). A monoclonal-antibodies solution was then applied and incubation proceeded for 2 h. The wells were rinsed again (×4) with NaCl/P_i/BSA and incubated with ^{125}I-labeled goat anti-(mouse IgG) (10^5 cpm/well). After 2 h the wells were rinsed again (×5) with NaCl/P_i/BSA and counted in a γ scintillation counter.

Immunofluorescence localization of the 25-kDa IAP

Immunofluorescence labeling of a 7-μm frozen section of gizzard tissue was carried out as previously described [31]. Hearts of 7-days-old chick embryos were dissected and suspended with trypsin/versene in Dulbecco's modified Eagle medium containing 10% fetal calf serum. Cell suspension were seeded onto sterile glass coverslips. After 24 h at 37°C, the cell were washed with NaCl/P_i, fixed with methanol (10 min, −20°C) and acetone (5 min, −20°C), and then transferred to NaCl/P_i. Cells were stained according to Avnur and Geiger [31].

Electron microscopy

Actin was polymerized at 1.5 μM final concentration, in the presence of 0.5 μM actin nuclei either with or without 25-kDa IAP. After 5 min, aliquots of 10 μl were placed on a carbon-coated, glow-discharged, copper-formvar grid. Negative staining was done with 0.75% uranyl formate according to Cooper and Pollard [32]. Electron microscopy pictures were taken with a Philips 410 electron microscope at an accelerating voltage of 80 kV.

RESULTS

Purification of 25-kDa IAP from turkey gizzard

The main source used for the purification of the 25-kDa IAP was the low-salt extract of turkey gizzard. When added

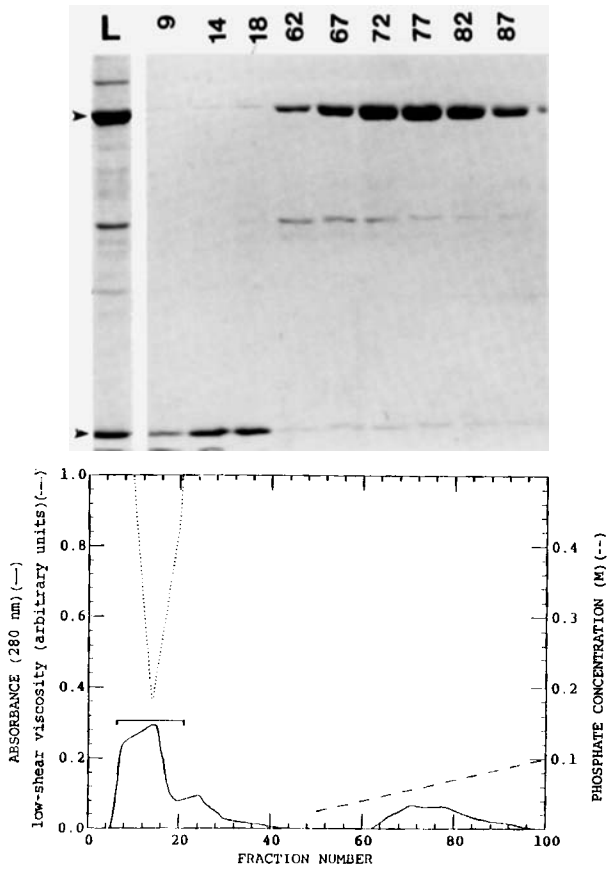

Fig. 2. *Chromatography of DEAE-cellulose-purified 25-kDa IAP on a hydroxyapatite column.* Upper part: SDS/polyacrylamide gel (8%) pattern of various fractions (the numbers refer to fractions in the lower part of this figure) after hydroxyapatite chromatography. L, the solution loaded on the column. Arrows at top and bottom indicate vinculin and the 25-kDa IAP respectively. Lower part: elution profile of 25-kDa IAP from hydroxyapatite column (1.3 × 11 cm)

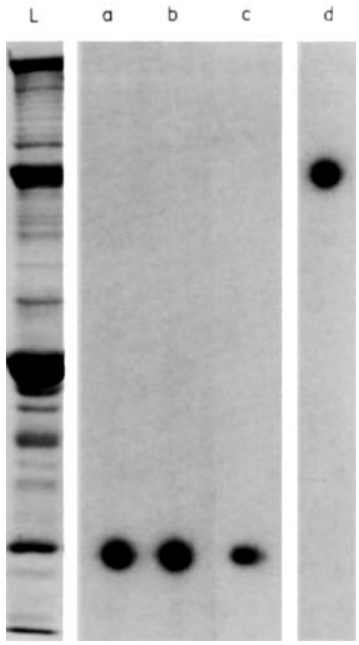

Fig. 3. *Immunoblot analysis of the hybridoma clones.* SDS/PAGE (8%) pattern of DEAE-cellulose-purified 25-kDa IAP stained by Coomassie blue is given in lane L. The gel was run under reducing conditions, then transferred onto nitrocellulose paper. The different monoclonal-antibody-producing hybridomas were assayed by multiple slot immunoblotting. Three antibodies, reactive with the 25-kDa IAP fraction were detected: (a) IAP-9 monoclonal antibody; (b) IAP-11 monoclonal antibody; (c) IAP-28 monoclonal antibody; (d) anti-vinculin monoclonal antibody isolated in the same assay

to polymerizing actin solution, this rather crude fraction induced a considerable reduction in the low-shear viscosity of actin, in spite of the fact that it contained a large number of proteins, the major of which were actin, vinculin, α-actinin, and filamin (Fig. 1, upper part, lane L). It is noteworthy that the latter two are potent actin-cross-linking proteins. Ion-exchange chromatography on DEAE-cellulose column (10 mg protein/ml bed volume) followed by low-shear viscosity assay revealed a fraction which lowered the low-shear viscosity of actin (Fig. 1, lower part) and contained a major polypeptide with an apparent molecular mass of 25 kDa, as well as vinculin and a few minor bands. The active fractions (marked by bracket in Fig. 1, lower part) were pooled, concentrated, dialyzed against buffer A containing 15 mM phosphate and applied to hydroxyapatite column (1.7 mg/ml bed volume). Following a wash with 50 mM phosphate buffer pH 7.0, the bound material was eluted using a linear phosphate gradient (50 – 250 mM, pH 7.0). Examination of each fraction by the actin-viscosity assay disclosed a peak of actin-inhibiting activity in the unbound fraction (Fig. 2, lower part), coinciding with the 25-kDa polypeptide (see corresponding fractions in Fig. 2, upper part). It should be pointed out that loading of the protein on an hydroxyapatite column could also be carried out in the absence of phosphate ions. Under these conditions the 25-kDa protein was first bound to the column and subsequently eluted by the phosphate gradient. We have, nevertheless, noticed that the actin-inhibiting activity of the protein eluted by the latter procedure was relatively low and tended to deteriorate upon storage, the reason for which is not clear (see Discussion).

Further purification of the 25-kDa polypeptide was carried out by immunoaffinity chromatography on an immobilized anti-(25-kDa IAP) column. As shown in Fig. 3, and as discussed below, we have prepared and characterized three independent hybridoma clones which produce antibodies that react specifically and exclusively with a 25-kDa polypeptide of a crude gizzard extract. For their use as immunoaffinity reagents, we initially isolated monoclonal antibodies to the 25-kDa IAP by affinity chromatography on goat anti-(mouse IgG) coupled to a polyacrylhydrazido-agarose column. Subsequently, the hydroxyapatite or the DEAE-cellulose fractions containing the 25-kDa polypeptide were loaded on the monoclonal antibodies column which was then extensively rinsed with NaCl/P_i. The 25-kDa polypeptide was eluted with 0.15 M NH_4OH (Fig. 4). The eluted protein was apparently over 95% pure, as determined by densitometry; minor bands of lower molecular mass were shown by immunoblotting analysis to be the proteolytic breakdown products of the 25-kDa protein.

When we applied a partially purified preparation of 25-kDa IAP, after DEAE-cellulose, onto either a TSK-3000 SW column (beads inner diameter of 25 nm) or to TSK-4000 SW (beads inner diameter 500 nm) two major protein peaks were obtained (Fig. 5). When subjected to SDS/PAGE, the first peak, which eluted with the void volume, was found to contain the 25-kDa IAP whereas the second peak contained vinculin. The chromatographic pattern indicated that 25-kDa IAP,

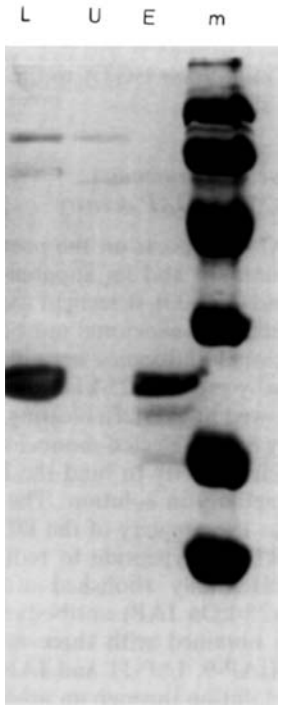

Fig. 4. *SDS/PAGE analysis of immunoaffinity purification of 25-kDa IAP on Trisacryl-bound monoclonal antibody (TA-IAP-28).* Lane L represents the fraction loaded on the affinity column. Lane U represents the unbound effluent of the affinity column. Lane E represents the 25-kDa IAP eluted from the affinity column. Lane m contains molecular mass markers (from top to bottom): phosphorylase *b* from rabbit muscle (94 kDa), albumin from bovine serum (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), α-lactalbumin (14.4 kDa)

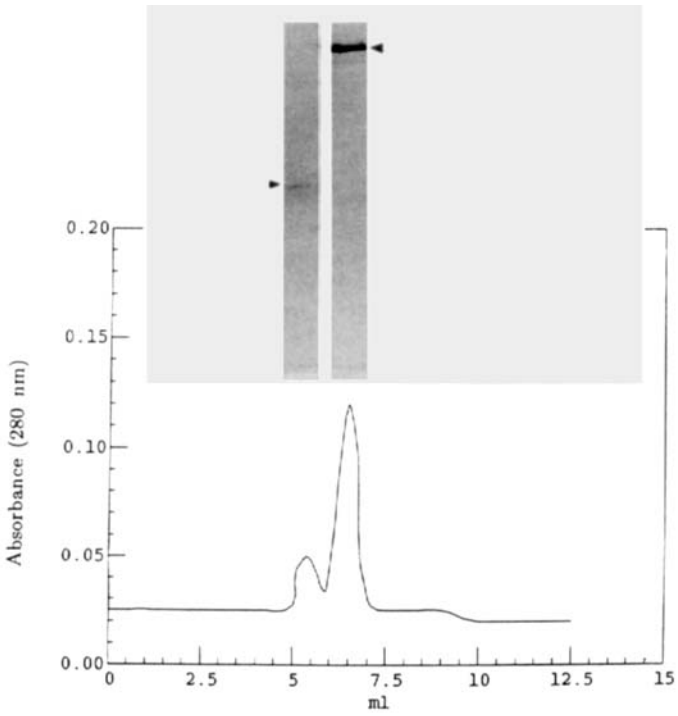

Fig. 5. *Gel filtration of crude 25-kDa IAP, after chromatography on DEAE-cellulose, on TSK-3000 SW column (7.5 × 30 mm).* The proteins were eluted by buffer A at 30 ml/h. The SDS/PAGE pattern of the corresponding fractions is shown at the top. Upper arrow indicates vinculin. Lower arrow indicates the 25-kDa IAP

Table 1. *Amino acid composition of 25-kDa IAP from turkey gizzard smooth muscle*

Amino acid	Relative content/ 25-kDa molecule	No. residues/ 25-kDa polypeptide
	mol %	
Asx	8.1	20
Thr	6.0	15
Ser	10.5	26
Glx	10.9	27
Pro	10.9	27
Gly	8.9	22
Ala	9.7	24
Cys[a]	1.2	3
Val	5.3	13
Met	2.0	5
Ile	2.8	7
Leu	6.5	16
Tyr	1.6	4
Phe	3.2	8
His	2.0	5
Lys	4.9	12
Arg	4.0	10
Trp[b]	1.2	3
Total	100	247

[a] Cysteine was determined as cysteic acid.
[b] Content of tryptophan was determined spectrophotometrically.

under non-denaturing conditions but in presence of reducing agent, tends to form aggregates with a high apparent molecular mass (> 130 kDa), suggesting that at least six monomers assemble into these complexes or that the 25-kDa IAP binds to an additional protein(s) (see Discussion). The distinct migrations of the 25-kDa IAP and vinculin suggest that there is no tight association between the two proteins.

Two-dimensional gel electrophoresis of the pure fraction revealed two isoelectrophoretic variants of the 25-kDa IAP with pI values of 6.5 and 6.8, both in fresh and old preparations of 25-kDa IAP.

Amino acid analysis of the purified 25-kDa protein is shown in Table 1. It is notable that this polypeptide displays a high content of Glx, Ser, Ala, Gly and Pro.

Kinetics of actin polymerization in presence of 25-kDa IAP

As pointed out above, both crude and pure 25-kDa IAP considerably reduce the low-shear viscosity of F-actin (Fig. 6 B and F). The effect was concentration-dependent and reached about 80% inhibition at a molar ratio of 25-kDa IAP to actin of 1:4. To further characterize the molecular mode of action of the 25-kDa IAP, we examined its effect on the initial rate of elongation of polymerizing pyrenyl-derivatized actin in the presence of F-actin nuclei. As shown in Fig. 7, the addition of the 25-kDa IAP had a marked effect on the rate of actin elongation, causing 50% inhibition at molar ratio (25-kDa IAP to monomeric actin) of 1:4 – 1:5. The 25-kDa IAP had no significant effect on the association constant (K_a). Analysis of the result also indicated that the 25-kDa IAP has a marked effect on the apparent critical concentration of actin monomers (in the presence of 0.5 μM nuclei) changing it from 0.28 μM (for actin alone) to 0.63 μM in presence of 0.2 μM 25-kDa IAP or to 0.95 μM in presence of 0.4 μM 25-kDa IAP. Moreover, addition of the 25-kDa IAP to preformed F-actin

filaments resulted in reduction of viscosity which was not significantly different from that obtained when the protein was added before or after the initiation of actin polymerization (Fig. 8). Addition of the 25-kDa IAP to actin monomers in the absence of nuclei did not provide nucleation sites for actin polymerization. The reduction in viscosity induced by the 25-kDa IAP was also obtained when the latter was added to F-actin/α-actinin gels. Those solutions, which were highly viscous before the addition of the 25-kDa IAP, were essentially solated by the 25-kDa IAP (Fig. 8F and H).

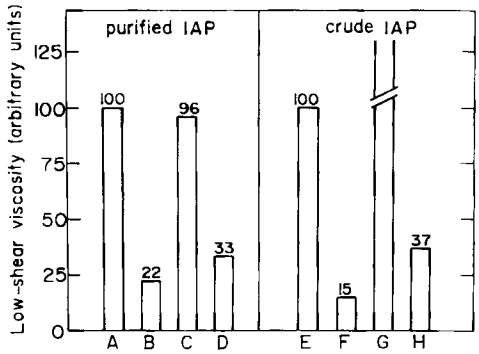

Fig. 6. *Immunoadsorption of the polymerization inhibitory activity of the 25-kDa IAP.* The histograms depict the low-shear viscosity of F-actin (0.5 mg/ml) (A and E) to which purified IAP (66 μg/ml) (B, C, D) or crude IAP (before DEAE-cellulose purification) (F, G, H) were added at the time of initiation of polymerizations. B and F are controls of low-shear viscosity of F-actin in presence of purified IAP or crude IAP respectively. (C) Purified IAP, pre-adsorbed on immobilized IAP-28 in an Eppendorf tube. (D, H) IAP and crude IAP, respectively, pre-adsorbed on an irrelevant immobilized antibody (anti-vinculin) in an Eppendorf tube. (G) Crude IAP following pre-adsorption on immobilized IAP-28

Furthermore, we found that the IAP activity was apparently Ca^{2+}-independent since it was not significantly affected by the addition of millimolar EGTA to the actin/25-kDa IAP solution (data not shown).

Specificity of monoclonal antibodies and their effect on 25-kDa IAP activity

In view of previous reports on the protease sensitivity of the vinculin contaminant and its apparent association with several protein bands [11, 33], it seemed useful to identify the IAP protein by specific monoclonal antibodies with defined specificity. Monoclonal antibodies were induced by repeated injections of partially purified 25-kDa IAP and screened by immunoassay followed by Western blotting. In order to determine the specificity of the selected monoclonal antibodies, we have examined their capacity to bind the IAP component(s) or to block their activity in solution. The results, shown in Fig. 6, indicate that the capacity of the DEAE-cellulose partially purified 25-kDa polypeptide to reduce actin viscosity (B and F) was essentially abolished after adsorption on immobilized anti-(25-kDa IAP) antibodies (C and G). The same results were obtained with three independent monoclonal antibodies (IAP-9, IAP-11 and IAP-28), Chromatography of the same solution through an unmodified Sepharose column, or through Sepharose to which a monoclonal anti-vinculin antibody was bound, did not affect significally the capacity of the IAP solution to reduce actin viscosity (Fig. 6D and H). The crude low-salt extract which, in addition to 25-kDa IAP and vinculin, also contained α-actinin and filamin (see Fig. 1, upper part, lane L), was also chromatographed through the antibody column. As shown in Fig. 6G, addition of the unbound fraction to actin caused a marked increase (rather than reduction) in the low-shear viscosity. These results suggest that the 25-kDa IAP is capable not only of reducing

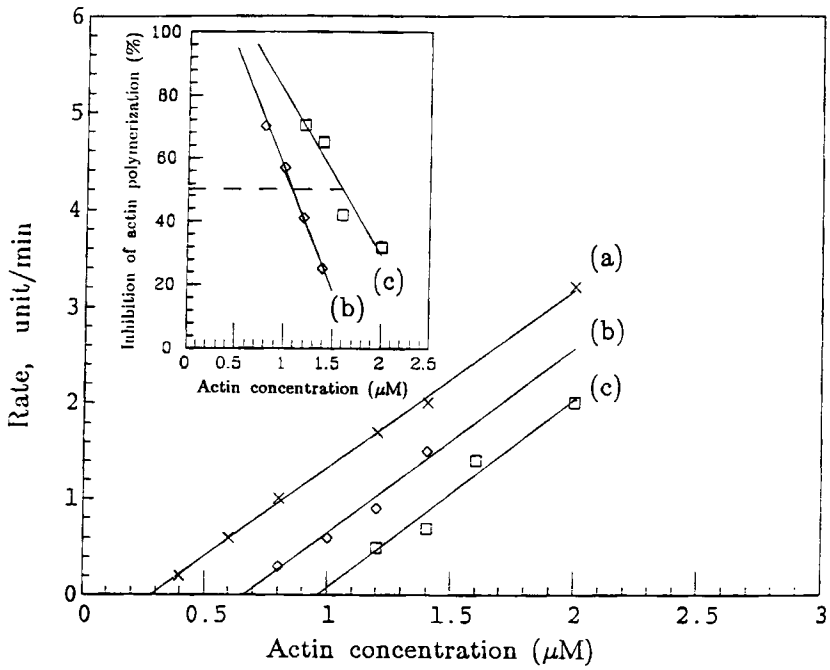

Fig. 7. *Effect of 25-kDa IAP on the critical concentration of actin.* The data show the dependence of initial rate of actin polymerization on the concentration of actin monomers in presence of pyrenyl-actin (10%), actin nuclei (0.5 μM), KCl (50 mM), and $MgCl_2$ (2 mM). (a) Actin alone. (b) Actin in presencce of 25-kDa IAP (0.2 μM). (c) Actin in presence of 25-kDa IAP (0.4 μM). The insert represents the same experimental result expressed as percentage inhibition of actin polymerization induced by the 25-kDa IAP

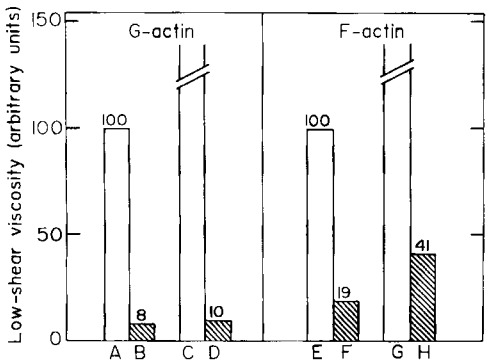

Fig. 8. *Effect of 25-kDa IAP on the low-shear viscosity of gels of F-actin and F-actin copolymerized with α-actinin.* G-actin (0.5 mg/ml) was polymerized by KCl (50 mM) and Mg^{2+} (2 mM). (A) Actin polymerized without 25-kDa IAP. (B) Actin polymerized in the presence of 25-kDa IAP. (C) Actin polymerized in presence of α-actinin. (D) Actin polymerized in presence of α-actinin and 25-kDa IAP. (E) The same as A, low-shear viscosity was measured after 60 min at room temperature. (F) The 25-kDa IAP was added to the F-actin (30 min after initiation of polymerization at room temperature) and measured 30 min later. (G) F-actin prepared in presence of α-actinin. (H) The 25-kDa IAP was added to F-actin polymerized in the presence of α-actinin

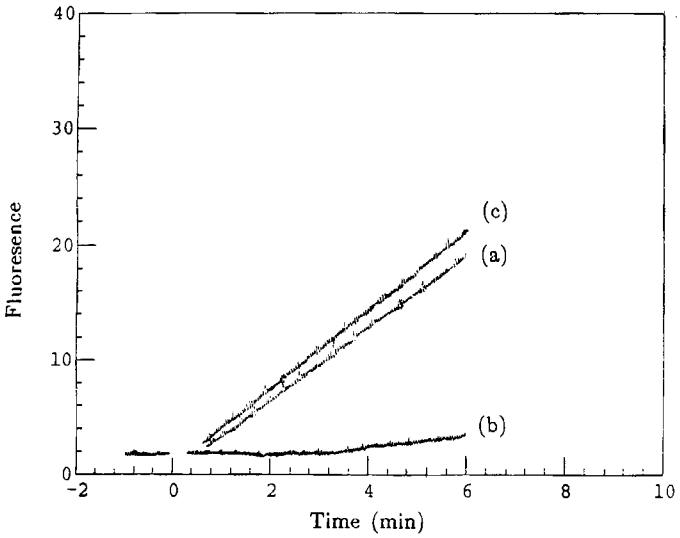

Fig. 9. *Polymerization of pyrenyl actin in the presence or absence of the 25-kDa IAP.* Actin monomers (1 μM) containing 10% pyrenyl actin were mixed with short actin nuclei (0.5 μM) and the changes in fluorescence were measured. (a) Actin alone. (b) Actin in presence of 25-kDa IAP (0.4 μM.) Preincubation with antibody IAP-11 yields the same results. (c) Actin polymerized in presence of 25-kDa IAP (0.4 μM) which was preincubated with monoclonal antibody IAP-28. Preincubation with antibody IAP-9 yields the same result

the viscosity of polymerizing actin solutions but also of preventing subsequent gelation, brought about by actin cross-linking proteins such as α-actinin and/or filamin.

Preincubation of 25-kDa IAP with the various antibodies, applied in a soluble form, pointed to some interesting differences between their respective reactivities: the monoclonal antibodies IAP-28 and IAP-9 both abolish the inhibition of actin elongation induced by the 25-kDa IAP, whereas incubation of the latter with IAP-11 did not affect the IAP activity (Fig. 9).

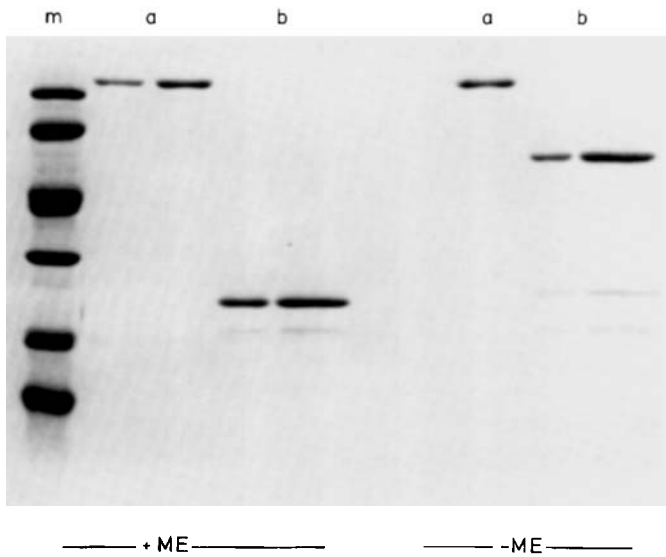

Fig. 10. *SDS/PAGE (10–20% gradient slab gels) of vinculin (a) and the 25-kDa IAP (b) under reducing conditions with 2-mercaptoethanol (+ME) and also under non-reducing (−ME) conditions.* Molecular mass markers, as in Fig. 4, are shown in lane m. Three of the samples are shown in duplicate

Formation of inactive disulfide-linked dimers by the 25-kDa IAP

Examination of hydroxyapatite-purified 25-kDa IAP/vinculin mixture by SDS/PAGE under non-reducing conditions indicated that the migration of the latter was not altered but a new band of about 58 kDa became apparent (Fig. 10). Since this molecule was also detected in fractions containing pure 25-kDa IAP, we considered the possibility that the ≈ 58-kDa molecule is a dimer of the 25-kDa molecule. The formation of the ≈ 58-kDa molecule was most pronounced in fractions stored for prolonged periods prior to the electrophoretic analysis. The reason for the slight disagreement between the molecular mass values obtained for the presumptive dimer (≈ 58 kDa) and twice the molecular mass of the monomer ≈ 50 kDa) is not clear at present and may be attributed to altered migration of the reduced and non-reduced 25-kDa IAP in the gel. Dimer formation could also be monitored by immunoblotting analysis (Fig. 11). Such analysis indicated that two out of the three monoclonal antibodies examined (IAP-11 and IAP-28) reacted strongly both with the monomer and the dimer while one antibody (IAP-9) reacted exclusively with the monomer (Fig. 11) suggesting that the specific epitope recognized by it became masked or modified upon dimer formation.

To determine whether in its original state the 25-kDa IAP is a monomer or S-S dimer, freshly excised tissue samples were extracted with SDS sample buffer, subjected to electrophoresis under non-reducing conditions and immediately examined by immunoblotting. Such examination indicated that nearly all the protein in the fresh specimens was in the monomer state (data not shown).

As pointed out above, the appearance of dimers occurred concomitantly with loss of inhibiting actin-polymerization activity. We have, moreover, found that this loss of activity could be partially reversed by reduction with 2-mercaptoethanol and could be prevented either by storage of the protein under mild reducing conditions or by carboxymethylation (data not shown).

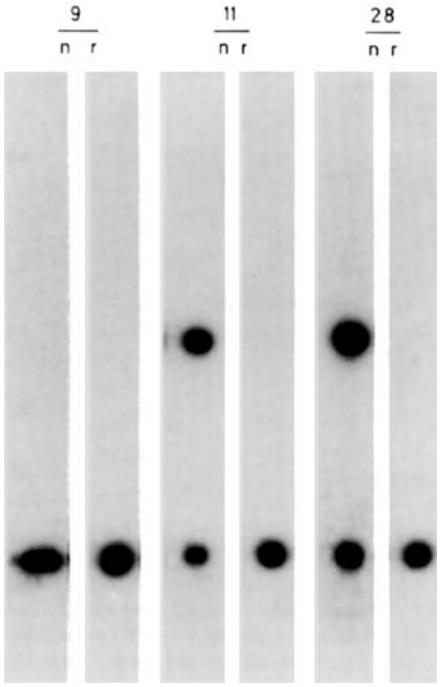

Fig. 11. *Immunoblot analysis of the monoclonal antibodies (9, 11, 28) produced against 25-kDa IAP.* DEAE-cellulose purified 25-kDa IAP was subjected to SDS/PAGE under reducing (r) or in non-reducing (n) conditions and subsequently to immunoblotting with the different monoclonal antibodies

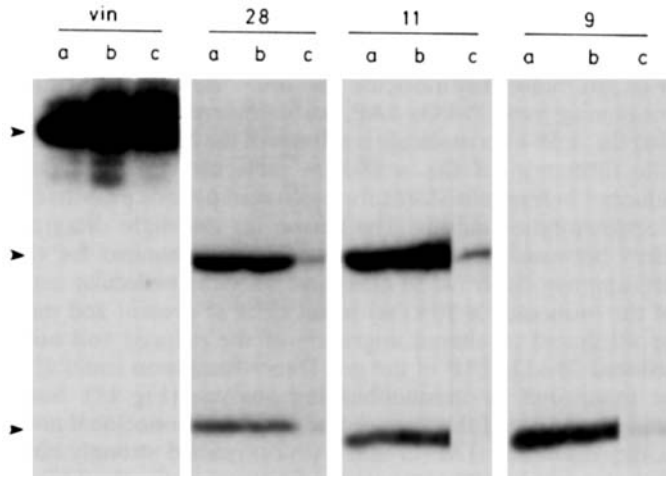

Fig. 12. *Interrelationships between the various anti 25-kDa IAP monoclonal antibodies.* Immunoblots of SDS/PAGE, 12%, non-reducing conditions, of (a) unfractionated DEAE-cellulose-purified 25-kDa IAP; (b) DEAE-cellulose fraction after adsorption on an irrelevant column [Sepharose – goat anti-(mouse IgG)]; (c) DEAE-cellulose fraction after adsorption on Trisacryl-bound anti-IAP-28. Antibodies used for the immunoblotting were: anti-vinculin (vin), IAP-28 (28), IAP-11 (11), and IAP-9 (9). Notice that adsorption on antibody IAP-28 drastically reduced the amount of antigenic material recognized by all three anti-IAP antibodies. This treatment did not affect the level of immunoreactive vinculin

In view of differences in the reactivities of the three antibodies prepared to the 25-kDa IAP (see below), it was important to verify that they all indeed react with the same polypeptide. In order to study this aspect, we passed DEAE-cellulose-purified, 25-kDa-IAP solution (which contains also vinculin) through an immobilized antibody column (IAP-28 bound to Trisacryl) and examined the unbound fraction by immunoblotting with all three monoclonal antibodies. As shown in Fig. 12, the immobilized monoclonal antibody IAP-28 effectively removed the polypeptide recognized by the other two antibodies (IAP-11 and IAP-9). The levels of vinculin in the unbound fraction was not effected by this adsorption.

Table 2. *Distribution of 25-kDa IAP in chicken organs*
Quantitative determination was carried out by solid phase radioimmunoassay. The sample of intestine contained submucosal smooth muscle and the levels of 25-kDa IAP detected in the tissue may be related to that tissue

Organ	25-kDa IAP/ organ protein	Quantity of total protein as 25-kDa IAP
	ng/mg	%
Brain	70	0.007
Gizzard	2100	0.21
Heart	1024	0.10
Intestine	292	0.03
Kidney	39	0.004
Lens	undetectable	undetectable
Liver	12	0.001
Skeletal muscle	420	0.04

Tissue distribution of the 25-kDa IAP in chicken

Immunoblotting analysis with the different anti-(25-kDa IAP) monoclonal antibodies of various chicken tissues indicated that the 25-kDa IAP was predominantly present in muscle tissues (gizzard, heart and skeletal muscle). In the qualitative dot-blot tests, in which increasing amounts of extracts were loaded onto the nitrocellular paper, we detected low but significant levels of the 25-kDa IAP also in kidney, intestine and brain. It was not detected in lens and peripheral blood. By quantitative solid-phase radioimmunoassay, we found that the 25-kDa IAP levels in muscle tissues amount to about 0.2 – 0.04% total extractable proteins. The levels in other tissues are apparently 10 – 100-fold lower (for details see Table 2).

Immunofluorescent labeling of frozen sections with the anti-(25-kDa IAP) monoclonal antibodies revealed intense staining of chicken gizzard smooth muscle cells (Fig. 13a). The fluorescence, often displaying a punctate or fine fibrillary pattern, was detected throughout the cytoplasm and showed no apparent enrichment along the plasma membrane. Overall, the gross distribution of the 25-kDa IAP was quite similar to that of actin in this tissue (Fig. 13b) and clearly discernable from that of vinculin. The labeling of formaldehyde-fixed Triton-permeabilized cultured chicken fibroblasts was essentially negative. Localization of the 25-kDa IAP in cultured heart cells showed that the protein was predominantly cytoplasmic (data not shown).

DISCUSSION

Studies on the functional organization of actin in non-muscle cells have often suggested that a large battery of actin-associated proteins can coordinately modulate microfilament organization in a variety of ways. These include the induction of monomer/polymer transitions, gel/sol interconversions,

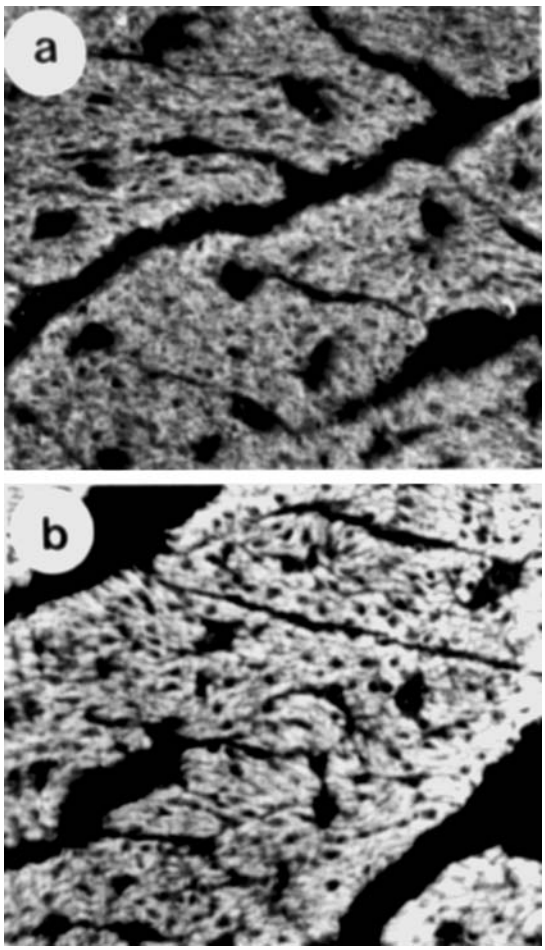

Fig. 13. *Immunofluorescent microscopic localization of the 25-kDa IAP (a) and actin (b) in a 7-μm frozen section of chicken gizzard smooth muscle.* Notice that the labeling of the 25-kDa IAP is found predominantly in the smooth muscle cells displaying fine fibrillary patterns throughout the sarcoplasm. Magnification: ×255

bundling, severing, etc. (for reviews see [34–36]). Certainly one of the interesting and functionally important aspects of actin organization in cells is its specific spatially regulated association with the plasma membrane. A common form of actin-membrane interaction present in a wide variety of cell types are adherens-type cellular contacts. These contacts may be formed with diverse surfaces and exhibit variable shapes and topology, but they all contain vinculin along their cytoplasmic interfaces [2, 4, 37]. While the complex molecular interactions in these sites are still poorly understood, suggestions have been made in the past that vinculin might interact with actin filaments and link them to specific integral transmembrane protein(s) [37] or even anchor them to the lipid bilayer directly [37, 38].

Preliminary reports attempting to demonstrate a direct association of vinculin with actin indicated that the DEAE-purified vinculin preparations induced a considerable reduction in the low-shear viscosity of F-actin [5–7, 39]. Incidentally, this activity was readily apparent in vinculin-containing extracts prepared by the rapid procedure [13] and were detected to a much lower extent in preparations prepared according to original purification procedure [1]. Subsequent studies indicated, however, that this activity was not attributable to vinculin itself but rather to contaminating molecule(s) of apparently lower molecular mass which copurify with vinculin. It has also been indicated that the reduction in actin viscosity is largely attributable to actin-capping activity of the contaminant [8–10]. Several investigators have recently studied this molecule but to date little information is available concerning its exact molecular identity, mechanism of action, relationship to vinculin and cellular distribution.

A major obstacle in identifying unequivocally the native inhibitor was its apparent sensitivity to proteolysis and the absence of well-defined non-specific immune reagents reacting with the native molecule. Our goal in the present study was to identify the specific component responsible for the actin-capping activity in the vinculin contaminant preparation from avian smooth muscle and to determine its molecular properties and tissue distribution. Our experimental strategy included partial purification of the contaminant, preparation of highly specific monoclonal antibodies against it and the use of these antibodies for the identification of the native protein and its purification to homogeneity. SDS/PAGE indicated that the active IAP is a polypeptide with an apparent molecular mass of about 25 kDa both in reducing and non-reducing conditions. In its native extraction buffer the protein migrates as a high-molecular-mass component just like an aggregate.

Previous results with crude or semi-purified vinculin contaminant suggested that it has an actin-capping activity which was relatively sensitive and progressively diminished upon storage [40]. We have confirmed these observations and showed that this loss of activity may be partially attributed to proteolysis by residual endogenous proteases present in the preparation and to the formation of inactive disulfide-linked dimers. A major proteolytic fragment of 22 kDa was often detected in purified 25-kDa IAP preparations and proteolysis apparently proceeded upon prolonged storage. The disulfide-linked dimers, which were formed upon storage of the purified 25-kDa IAP under non-reducing conditions, were not isolated in pure forms but we have shown that dimer formation was accompanied by loss of their inhibitory activity. Their effect on actin could be preserved or be restored by reduction with 2-mercaptoethanol. It was, however, concluded that the presence of free sulfhydryl groups on the 25-kDa IAP molecule was not essential for its effect on actin viscosity since carboxymethylation of the monomers had no detrimental effect on their activity and, in fact, stabilized it. Another factor which apparently affected the long-range stability of the 25-kDa IAP was the exact procedure employed for the purification of the protein. We have used here two alternative purification procedures on an hydroxyapatite column. In the first, the 25-kDa polypeptide was adsorbed on the column and then eluted by phosphate gradient, while in the second, phosphate ions were added to the sample prior to chromatography and the 25-kDa IAP recovered in the flow-through fraction. Comparison of the subsequent stabilities of the purified protein indicated that the one purified by the latter procedure was considerably more stable. This is consistent with the notion that adsorption on charged columns may alter protein conformation and even induce denaturation [41].

In the present study several approaches were taken to verify that the 25-kDa polypeptide recognized by our monoclonal antibodies is indeed the specific actin-capping protein, previously referred to as the vinculin contaminant [8]. This notion seems to be substantiated by several lines of evidence: (a) the purified 25-kDa polypeptide isolated by immunoaffinity chromatography exhibited marked inhibition of actin viscosity; (b) this activity, exerted by crude or partially purified contaminant preparations, could be completely re-

moved by chromatography on the immobilized IAP-specific monoclonal antibodies; (c) all three monoclonal antibodies presented here reacted with the same polypeptide; (d) two of the monoclonal antibodies could effectively inhibit the 25-kDa IAP in solution.

Several studies carried out over the last few years were directed towards the isolation and characterization of the polymerization-inhibiting protein originally detected in vinculin preparations [9–12, 33]. These studies have pointed to a battery of polypeptides which were associated with IAP activity. Following the demonstration that the inhibitory activity is not directly associated with vinculin [8], several reports have indicated that it was attributable to low-molecular-mass peptides in the range of 20–85 kDa [9, 10]. Moreover it was reported that multiple protein bands present in the inhibitory fraction were derived from higher-molecular-mass polypeptide(s) of 150–200 kDa [12] or 85 kDa [33]. The interrelationships between the IAP proteins reported by these two laboratories are not clear yet and it appears possible that the precursor for the 150–200-kDa polypeptide (tensin) described by Wilkins et al. [12] and the 85-kDa polypeptide described by Ruhnau et al. [33] are identical.

Several lines of evidence indicate that the 25-kDa IAP described here is, in fact, a different protein, distinct from the high-molecular-mass polypeptides mentional above. (a) The highest polypeptide molecular mass detected on SDS/PAGE was 25 kDa. The same value was obtained for the pure 25-kDa IAP and for crude chicken gizzard homogenate. (b) Tensin was reported to exhibit actin-capping activity while the 25-kDa IAP seems to show no capping capacity and to be effective in considerably higher stoichiometric ratios. (c) Antibodies reactive with the 25-kDa IAP were sent to S. Lin and coworkers and were found not to react with crude or purified chicken tensin. These antibodies did react with a 25-kDa polypeptide in the crude chicken gizzard extract.

Taken together, these results suggest that the crude gizzard extract may contain several different components which exert a generally similar effect on actin. Immunoblot analysis of our 25-kDa IAP preparation indicated that it was devoid of tensin and thus we assume that the latter was lost during purification. Moreover, it appears that the entire inhibitory activity found in these preparations is associated with the 25-kDa IAP since the monoclonal antibodies described here could completely adsorb the inhibitory activity and some of them could effectively inhibit it.

It should be mentioned that, in its general properties, the 25-kDa IAP bears some similarly to the protein described by MacLean-Fletcher and Pollard [19] and by Maekawa et al. [42]. The former protein was detected in actin preparations from skeletal muscle of rabbit or chicken and inhibited actin polymerization in a mode similar to that of the 25-kDa IAP. The 21-kDa protein described by Maekawa et al. [42] was isolated from pig brain (whereas chick brain contains only a little of the 25-kDa IAP) and the relationships between the two are not clear.

The mechanism of action of 25-kDa IAP on actin seems to involve primarily inhibition of polymerization. This activity was demonstrated by both viscosimetric assay (low-shear viscosity) and by direct fluorimetric analysis of pyrenyl-actin polymerization. In the former, the 25-kDa IAP was also effective when applied in 1:20 molar ratio to monomeric actin, while the effect on the initial elongation stages of actin (in the presence of nuclei) was obtained at 1:4–1:6 molar ratio. Moreover, the 25-kDa IAP did not show any nucleating activity, when mixed with monomeric actin without added nuclei. These results taken together suggest that the 25-kDa IAP does not have a potent capping activity. Electron microscopy analysis of F-acin formed in the presence or absence of the 25-kDa IAP did not point to major differences in the appearance of actin filaments (not shown). It is also noteworthy that the inhibitory effect of the 25-kDa IAP was very pronounced when added to preformed actin filaments or to F-actin/α-actinin gels.

As described above, the 25-kDa IAP migrates as a single band of 25 kDa on reducing SDS/polyacrylamide gel, yet examination of its migration profile on a gel filtration column indicated that it exhibits a higher molecular mass (> 130 kDa) in non-denaturing buffers. It is not entirely clear what is the basis for the high native molecular mass. However, since such a migration profile was also observed with pure preparations of the 25-kDa IAP, it is conceivable that the protein undergoes self-aggregation in solution rather than interacting with another high-molecular-mass protein. This conclusion is further corroborated by the tendency of the 25-kDa IAP to undergo disulfide-mediated dimerization upon storage in non-reducing solution. This process occurred at relatively low concentration of the protein, both in the presence or absence of additional proteins, in line with the notion that the 25-kDa IAP molecules are aggregated. It should be noted, however, that the 25-kDa IAP did not co-migrate with vinculin on the same column, indicating that the two molecules are not associated with each other.

The tissue survey in the adult chicken showed a high content of the 25-kDa IAP in muscles: highest relative contents were detected in smooth muscle; somewhat lower levels were found in cardiac and skeletal muscles. Tissues such as brain and liver exhibited lower, yet significant, levels of the protein. Immunohistochemical analysis of 25-kDa IAP distribution in chick tissues was largely in line with the quantitative analysis and indicated that in muscle tissue the protein was rather uniformly distributed throughout the sarcoplasm without showing any special enrichment near the membrane-bound dense plaques.

In conclusion, the results presented here suggest that the 25-kDa polypeptide is a muscle-specific inhibitor of actin polymerization. Its antigenic properties, size and mode of interaction with actin are distinct from that of other proteins which inhibit a similar activity in the crude vinculin preparation. It is conceivable that the major inhibitory component of the crude vinculin extract recovered from DEAE-cellulose column is the 25-kDa IAP.

This study was supported by a grant from the Muscular Dystrophy Association. B.G. is incumbent of the Prof. Erwin Neter Chair in Cell and Tumor Biology. M.W. is incumbent of the Marc R. Gutwirth Chair of Molecular Biology. The authors would like to thank Ida Oren for her help in running the HPLC, Rachel Samuel for typing the manuscript, and Dvorah Ochert for editorial assistance.

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