Studies on Proteins That Co-purify with Smooth Muscle Vinculin: Identification of Immunologically Related Species in Focal Adhesions of Nonmuscle and Z-lines of Muscle Cells

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Abstract. Membrane extracts from chicken smooth muscle contain, along with filamin, vinculin and alpha actinin, a group of polypeptides that have the ability to interact with the "barbed end" of actin filaments. These low molecular mass polypeptides were designated as HA1 (Wilkins, J. A., and S. Lin, 1986, *J. Cell Biol.*, 102:1085–1092). In this study, polyclonal antibodies raised against the HA1 preparation were used to study the cellular localization and tissue distribution of these polypeptides. Immunofluorescence experiments revealed a primary localization of staining at the ends of stress fibers on the ventral surface of cultured chicken embryo fibroblasts, i.e., those areas known as the focal adhesions. Specific staining was also seen at the Z-lines of both skeletal muscle myofibrils and cultured embryonic heart cells. Immunoblotting analyses of proteins from different tissues prepared to avoid proteolytic degradation showed a much different pattern than that of HA1 itself. Immunoreactive polypeptides with reduced molecular masses of 200,000 and 150,000 D were found in smooth muscle and fibroblasts while 200 and 60 kD polypeptides were found in cardiac muscle tissue. The antibodies recognized 60- and 31-kD polypeptides on immunoblots of chicken breast muscle. The results from this study strongly suggest that the polypeptides in HA1 arose from proteolysis of high molecular mass molecules. The studies also raise the possibility that immunologically related proteins in muscle and nonmuscle cells may be involved in linking actin filaments to Z-lines and membranes, respectively.

THE molecular mechanism of the association of actin filaments (F-actin) with membranes of smooth muscle and nonmuscle cells, and with the Z-line in striated muscle has been the subject of intense interest in recent years (1, 14). In this regard vinculin, a 130,000-D protein, has attracted considerable attention due to its localization in cultured cells at the ends of stress fibers that make contact with the cytoplasmic side of the ventral cell surface, i.e., the focal adhesions (6, 13). Initially, vinculin was thought to be capable of inhibiting F-actin elongation by interacting with the "barbed" end of filaments in a manner consistent with its proposed function as a filament–membrane linkage protein (7, 25). However, this inhibitory activity was subsequently found to be absent from highly purified vinculin (18) or to be due to the presence of contaminants in the vinculin preparations (11, 20, 26). These highly active contaminants, a group of polypeptides with molecular masses in the 20,000–45,000-D range, can be separated from vinculin preparations by hydroxylapatite chromatography and were collectively designated as HA1 (26). Similar results regarding these contaminants were reported by Schroer and Wegner (20).

In this report, we describe the cellular localization and tissue distribution of polypeptides immunologically related to those in HA1. Using indirect immunofluorescence, we found that polyclonal antibodies made against HA1 bound to cultured chicken embryo fibroblasts (CEFs)[1] and PtK2 cells at focal adhesions. The HA1 antibodies also stained the Z-lines of sarcomeres in chicken embryo cardiac myocytes and in chicken skeletal muscle myofibrils. On immunoblots, the antibodies bound to major bands of 200,000 and 150,000 D from cultured CEFs and from adult chicken smooth muscle, and to 60,000-D polypeptides from adult chicken cardiac and skeletal muscle tissue. In addition, a 200,000-D band was detected in cardiac muscle tissue and a 31,000-D band was found in skeletal muscle. Because the HA1 polypeptides were not detectable in tissues carefully prepared to minimize proteolysis, our present view is that they are proteolytic fragments of a larger protein(s). The results of the present study are discussed with regard to the possible role of the above proteins in the interactions of actin filaments with membranes of nonmuscle and smooth muscle cells as well as with the Z-line structures of striated muscle cells.

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1. *Abbreviations used in this paper*: CEF, chicken embryo fibroblast; TBS, tris-buffered saline.

Materials and Methods

Preparation of HA1

The vinculin preparation described by Feramisco and Burridge (12) was the source used to prepare the HA1 fraction. As described in detail in our recent paper (26), vinculin-containing fractions from DEAE cellulose chromatography were pooled, concentrated by ultrafiltration, and loaded onto a column of hydroxylapatite. Fractions flowing through the column contained proteins that had the ability to lower the "low-shear" viscosity of F-actin as measured by falling ball viscometry. These fractions were pooled, concentrated, and designated HA1. This preparation was used as antigen for the production of antibodies in rabbits.

A modification of the above protocol was found to increase the yield of the polypeptides of interest to this study. All procedures were performed at 0–4°C. Proteins precipitated by ammonium sulfate between 25 to 35% saturation from the 37°C extract of 160 g of chicken gizzard myofibrils (12) were resuspended in and dialyzed extensively against buffer B (20 mM Tris-acetate, 20 mM NaCl, 15 mM 2-mercaptoethanol, and 0.1 mM EDTA [pH 7.6]). After dialysis, this solution was batch-adsorbed with 100 ml (wet volume) of Whatman DE52 (Whatman Chemical Separation Inc., Clifton, NJ), which had been pre-equilibrated to pH 6.7 in buffer B by addition of 6 N HCl. This suspension was allowed to stir for 1 h. The DE52 was then sedimented by centrifugation in a rotor (model JA20; Beckman Instruments Inc., Fullerton, CA) at 3,000 g for 10 min. The supernatant fraction was loaded onto a 2.5 × 50-cm column of Whatman DE53 that had been pre-equilibrated to pH 10 in buffer B by addition of 6 N NaOH. The column was rinsed with ~200 ml of buffer B (pH 7.6) and proteins were eluted with a 300-ml linear gradient of 0–370 mM NaCl in buffer B. Fractions that lowered the low-shear viscosity of F-actin as measured by falling ball viscometry (26) were pooled and dialyzed against buffer B. The pooled active fractions (containing ~8 mg of protein) were then subjected to hydroxylapatite chromatography as described above for preparation of HA1. The active fractions flowing through this column were collected, pooled, and concentrated and designated as HA1a. The yield of HA1a by this procedure was ~1 mg of protein as measured by the Bradford assay (3), using BSA as a standard.

Preparation of Antibodies to HA1

Antibodies to HA1 were raised in a New Zealand white rabbit weighing ~7 pounds. The rabbit was given an initial injection of 90 µg HA1 mixed with an equal volume of Freunds' complete adjuvant. This was followed by two 45-µg boosts at 2-wk intervals, each in Freunds' incomplete adjuvant. After the boosts, the animal was bled from the marginal ear veins and the sera were shown to contain antibodies that recognize the proteins in HA1 in immunoblotting assays (see below).

Affinity Purification of Anti-HA1 IgG

Sera collected after the first and second boosts were pooled, dialyzed, and loaded onto a 1.6 × 30-cm column of DEAE-Affigel Blue (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's directions. Fractions containing IgG were pooled and the antibodies were precipitated by adding solid ammonium sulfate to 50% saturation. The precipitated protein was suspended in and dialyzed overnight against 0.1 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, and 1 mM NaN₃ (pH 7.4; IgG buffer). IgGs that crossreact with HA1 were further purified by affinity chromatography on an HA1a-agarose column as follows. 1 mg of HA1a prepared as described above was mixed with 1 ml (wet volume) of CNBr-activated agarose (Sigma Chemical Co., St. Louis, MO) and the mixture was allowed to incubate for 2 h with shaking at room temperature. Unoccupied sites were blocked by addition of 0.1 M glycine (pH 8.0) and incubation at room temperature for 2 h. This procedure resulted in ~90% coupling of HA1a to the agarose. The HA1a-agarose was poured into a column and equilibrated with IgG buffer. 8 mg of IgG prepared as described above was loaded onto the column and the column was washed and eluted as described by Bennett and Stenbuck (2). Fractions eluted from the column in 1 M acetic acid were immediately neutralized with concentrated NH₄OH. HA1-specific IgG was dialyzed overnight against 1 liter of IgG buffer. The protein concentration of the preparation was estimated to be in the range of 200–250 µg/ml, using an extinction coefficient of 1.5 mg⁻¹cm² at 280 nm. This affinity-purified preparation, referred to as HA1 antibodies, was used for all of the experiments described in this paper unless otherwise specified.

Further Purification of HA1 Antibodies on Nitrocellulose Sheets

Lyophilized chicken gizzard tissue (200 mg) was solubilized by boiling for 20 min in 6 ml of SDS sample buffer (0.0625 M Tris-Cl, 10% [vol/vol] glycerol, 2% [wt/vol] SDS, 5% [vol/vol] 2-mercaptoethanol [pH 6.5]) containing 5 mM EGTA. After the insoluble material had been removed by centrifugation, equal volumes of the solubilized proteins were loaded onto two separate 10% SDS polyacrylamide slab gels with 5% stacking gels. The gels were run and transferred to nitrocellulose as described below. After transfer, the sheets were incubated in 25 mM Tris-HCl, 0.9% (wt/vol) NaCl, 0.01% (wt/vol) NaN₃, 3% (wt/vol) BSA (pH 7.4; Tris-buffered saline (TBS)-BSA) for 1 h at room temperature, then incubated for 2 h at room temperature in the same buffer containing 40 µg/ml of HA1 antibodies that had been affinity-purified on the HA1a-agarose column as described above. After washing, the sheets were incubated for 1 h at room temperature in alkaline phosphatase-conjugated goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA) diluted 3,000-fold with TBS-BSA. The sheets were again washed and bands were detected as described by Smith and Fisher (21). Antibodies were eluted from excised bands by incubation in 1 ml of 1 M acetic acid containing 0.1% gelatin with shaking for ~1 min. Samples were neutralized by addition of 0.1 ml of a 50 mg/ml solution of BSA in 2 M Tris base. Eluted antibodies were dialyzed overnight against 1 liter of IgG buffer and insoluble material was removed by centrifugation at 8,000 g for 15 min.

Electrophoretic Techniques

SDS polyacrylamide gels were run as described by Laemmli (17). Some gels were transferred to nitrocellulose paper for antibody staining by a method similar to that described by Burnette (4). Gels were transferred in an electrophoretic transfer unit (model TE42; Hoefer Scientific Instruments, San Francisco, CA) at an initial current of 0.3 A for ~12–15 h. After transfer, the nitrocellulose sheet was immersed in TBS-BSA and incubated for 1 h at room temperature. All incubations and washes were performed with shaking. The sheet was then incubated with appropriate dilutions of various antibody solutions in TBS-BSA at room temperature for 1.5 h (see figure legends), washed as described by Burnette (4), incubated in 5 × 10⁵ –1 × 10⁶ CPM [¹²⁵I] protein A or [¹²⁵I] goat anti-rabbit IgG/ml of TBS-BSA for 30–60 min, and washed again. The sheet was dried and autoradiographed for 12–36 h at −70°C using Kodak XAR film and DuPont Hi Plus intensifying screens (E.I. DuPont de Nemours & Co., Inc., Sorvall Instruments Div., Newton, CT).

Radioactive Labeling of Proteins

Protein A (Genzyme, Boston, MA) or goat anti-rabbit IgG (Cappel Laboratories) was labeled by the chloramine T method (15). 20 µg of either protein was labeled using 2 mCi of Na [¹²⁵I] (Amersham Corp., Arlington Heights, IL). The final specific activity was 10⁸ dpm/µg for protein A and 5 × 10⁷ dpm/µg for goat anti-rabbit IgG.

Immunoprecipitation of HA1a Proteins and Measurement of Activity

HA1a (0.37 µg in 2 µl) was mixed with various amounts of either affinity-purified HA1 antibodies or equivalent amounts of nonimmune rabbit IgG (Cappel Laboratories) in a total volume of 0.2 ml of IgG buffer. After an overnight incubation at 4°C, samples were mixed with 22 µl of Sepharose-conjugated goat anti-rabbit IgG (from Cappel Laboratories, with a binding capacity of 0.9 mg rabbit IgG/ml of gel). The suspensions were incubated with shaking for another 2 h at 4°C and the gel was removed by centrifugation for 5 min at 8,000 g. Aliquots of the supernatant fractions were tested for their ability to decrease the low-shear viscosity of actin solutions measured by falling ball viscometry as previously described (26).

Cell Cultures

CEF cultures were prepared essentially as described by Vogt (23). Some cultures were transformed by the addition of Rous sarcoma virus (Schmidt-Ruppin A) at the time of the initial plating. Secondary or tertiary cell cultures were used for experiments. Chicken heart cell cultures were prepared by trypsinization of hearts from 11-d-old embryos and grown essentially as described by De Haan (10). Kangaroo rat kidney epithelial cells (PtK2 cells)

were grown in Eagle's minimum essential medium with nonessential amino acids (Gibco, Grand Island, NY) and 10% fetal bovine serum (HyClone Laboratories, Logan, UT).

Immunofluorescence Studies

Myofibrils prepared from chicken breast muscle according to the method of Knight and Trinick (16, Fig. 5) or the method of Wang and Ramirez-Mitchell (24, Fig. 9) were spread on circular 25-mm acid-cleaned cover glasses and washed twice with PBS (Dulbecco's without divalent cations, [pH 7.4]). Trypsinized cells were seeded on cover glasses at least 24 h before use. Cover glasses with attached myofibrils or cells were fixed for 15 min in fresh 3.7% (wt/vol) paraformaldehyde (Sigma Chemical Co.) in PBS. Fixation and all subsequent steps were performed at room temperature unless otherwise noted. The cover glasses were washed in PBS, quenched in 0.1 M glycine in PBS, washed again in PBS, and those with cells were treated with 0.2% Triton X-100 in PBS to make cellular membranes permeable to the labeling reagents. After a wash in PBS containing 1 mM EGTA and 1% BSA, samples were incubated overnight at 37°C in a humidified atmosphere in the same buffer containing HA1 antibodies (40–80 μg/ml for

CEFs, chicken heart cells, and chicken myofibrils, and 120–150 μg/ml for PtK2 cells). The samples were then incubated in 50–100 μg/ml affinity-purified fluorescein or rhodamine-conjugated goat anti-rabbit IgG (Cappel Laboratories), followed by several washes in PBS with 1 mM EGTA and 1% BSA. After additional washes in PBS, the myofibrils or cells were mounted in 90% glycerol/10% PBS with 0.1% p-phenylenediamine (pH 8.0). Control samples were prepared with omission of the incubation with primary antibodies, substitution of the same concentration of affinity purified nonimmune rabbit and/or mouse IgG for the primary antibodies, preabsorption of HA1 antibodies with purified vinculin, or omission of the permeabilization step.

In some experiments a mouse monoclonal anti–vinculin antibody, VIN-11-5 (ICN Immunobiologicals, Lisle, IL), was added at a dilution of 1:500 or 1:1000 to the primary incubation with HA1 antibodies and 100 μg/ml affinity-purified rhodamine-conjugated goat anti–mouse IgG (Cappel Laboratories) was added to the secondary incubation with fluorescein-conjugated goat anti–rabbit IgG. Only secondary antibodies that failed to show species cross-reactivity were used in these experiments. In other experiments rhodamine- or fluorescein-conjugated phalloidin (Molecular Probes Inc., Junction City, OR) was added to the incubation with secondary antibody to label F-actin.
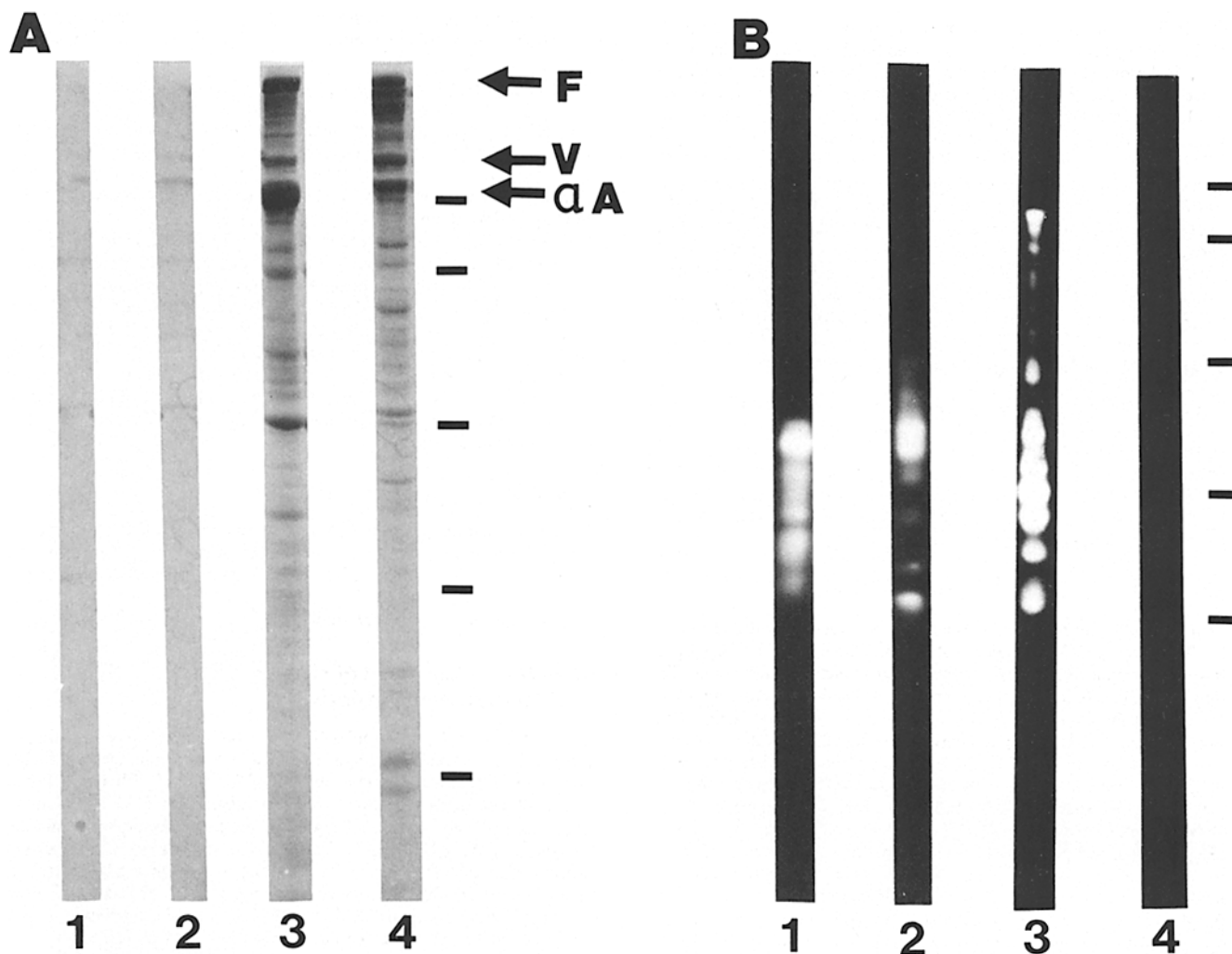

Figure 1. Immunoblots of proteins in HA1, HA1a, and ammonium sulfate precipitates probed with HA1 antibodies. 10% SDS polyacrylamide gels were run and stained with Coomassie Blue (A) or transferred to nitrocellulose (B). The nitrocellulose transfers were incubated in a 40-μg/ml solution of the antibodies and their binding to specific bands was detected by incubation with radiolabeled Protein A followed by autoradiography as described in Materials and Methods. Lanes 1, HA1, 2 μg; lanes 2, HA1a, 2 μg; lanes 3, 25–35% ammonium sulfate precipitate from 37°C chicken gizzard extract prepared as described (12); lanes 4, 35–40% ammonium sulfate precipitate from the above extract. Marks to the right of A and B show the positions of molecular mass standards: 92.5, 66.2, 45, 31, and 21.5 kD, from top to bottom. Arrows indicate the positions of the following proteins: F, filamin; V, vinculin; A, alpha actinin.

Myofibrils or cells were viewed with a Zeiss standard microscope equipped with epifluorescent illumination, a fluorescein filter set with a BP 515/565 barrier filter, and a standard rhodamine filter set for double-labeling experiments. Single-labeled specimens as well as double-labeled specimens were examined. The labels were reversed (fluorescein-conjugated secondary antibody and rhodamine-conjugated phalloidin vs. rhodamine-conjugated secondary antibody and fluorescein-conjugated phalloidin) in some samples to rule out the possibility that filter "bleed through" was responsible for any of the staining patterns observed. Myofibrils or cells were photographed using a Nikkormat El camera and Kodak Tri-X Pan film. The location of staining with HA1 antibodies within sarcomeres was determined by comparing images obtained with phase-contrast images, and with the location of fluorescently labeled F-actin.

Results

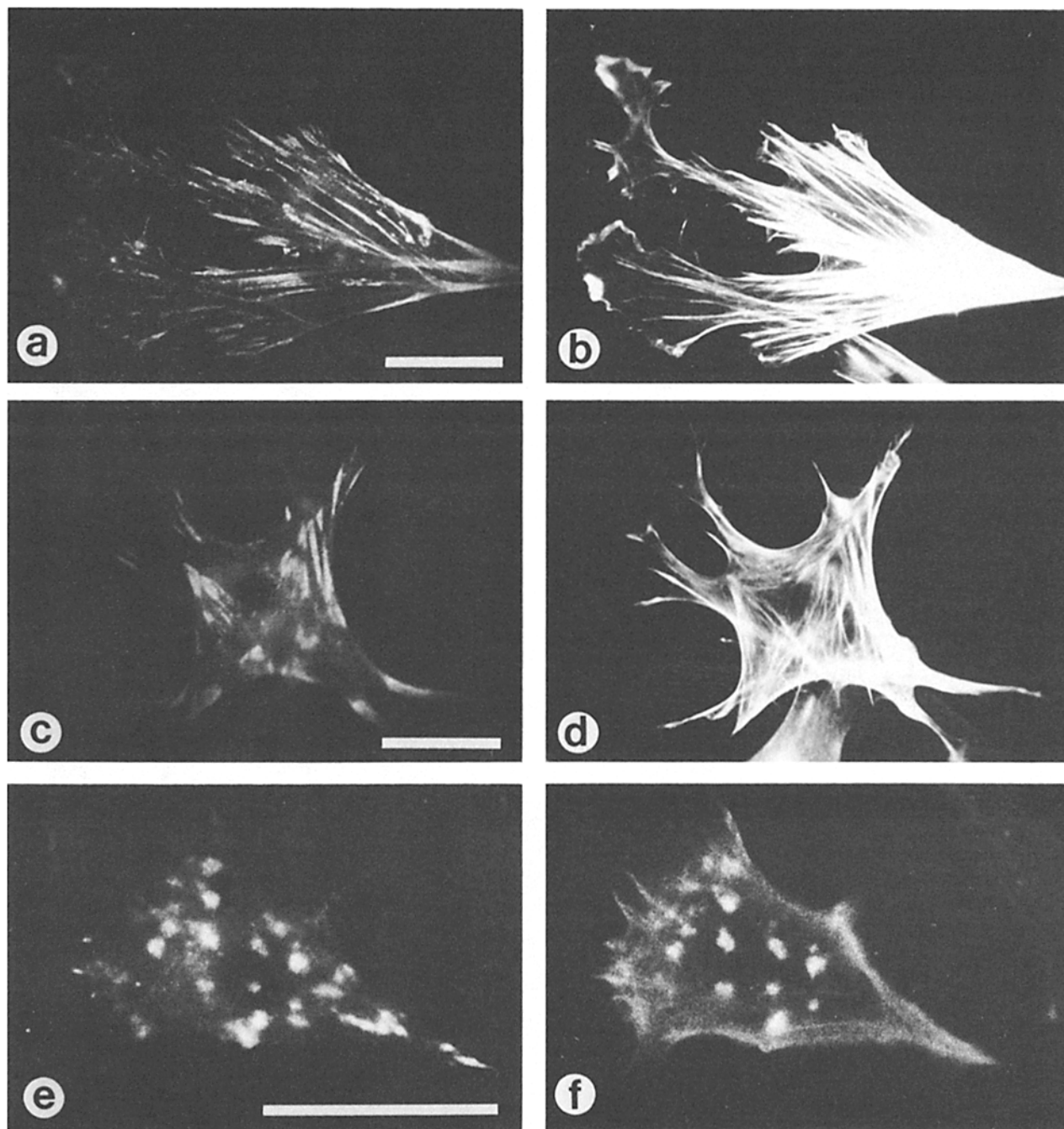We recently described a protein fraction derived from

Figure 2. Double-fluorescent staining of HA1-related molecules (*a*, *c*, and *e*) and F-actin (*b*, *d*, and *f*) in CEFs. In all photographs the plane of focus is at the level of the cell–substrate interface. In normal CEFs (*a–d*) the predominant indirect immunofluorescent staining by HA1 antibodies followed with fluorescein-labeled secondary antibody (*a* and *c*) is at the ends of actin filament bundles stained by rhodamine-labeled phalloidin (*b* and *d*). Additionally, the cells display a faint diffuse cytoplasmic staining and variable staining along stress fibers (*a*). In Rous sarcoma virus–transformed CEFs (*e* and *f*) antibody staining is localized at the actin containing rosettes, which are characteristic of transformed fibroblasts. Control samples in which the incubation with primary antibodies was omitted, in which the same concentration of affinity-purified nonimmune rabbit IgG was substituted for the primary antibodies, or in which the permeabilization step was omitted showed only a diffuse fluorescence too faint to be photographically recorded. Bars, 20 μm.

smooth muscle vinculin preparations made by a published method (12). This fraction was designated HA1 (26) because it eluted ahead of vinculin on a hydroxylapatite column and was shown to contain proteins immunologically distinct from vinculin (see below). HA1 has the ability to reduce the low-shear viscosity of F-actin and to inhibit monomer addition to the barbed end (morphologically defined by myosin S1 decoration) of actin filaments. In this study, we use immunological techniques to characterize polypeptides in HA1 as well as in an alternate preparation (HA1a) that provides a larger quantity of active material.

To initiate our investigation of the cellular function of the HA1 polypeptides, we raised antibodies in rabbits against the entire fraction. A crude IgG fraction was prepared from the antiserum, and was further purified by affinity chromatography on an HA1a-agarose column. In immmunoblotting experiments, the antibodies bound to a number of bands in HA1 and HA1a (Fig. 1) and in the 25–35% ammonium sulfate fraction from which the two preparations were derived. By comparison, the antibodies did not cross-react significantly with any of the proteins in the 35–40% ammonium sulfate fraction, which is enriched in talin (5). There is also no detectable cross-reactivity between the antibodies and the other well characterized components of these fractions: filamin, vinculin, and alpha actinin.

The HA1 antibodies produced a particularly interesting staining pattern in cultured chicken embryo fibroblasts examined by double-label indirect immunofluorescence microscopy (Fig. 2). Areas of strong fluorescence are seen at the ends of stress fibers on the ventral surface of the cells in a pattern reminiscent of that observed for vinculin and talin (e.g., 5, 6). In addition, there is a faint, diffuse staining of the cytoplasm, and staining that can sometimes be seen for variable distances along the stress fibers. Some samples were incubated with HA1 antibodies preabsorbed with vinculin purified by the method of Feramisco and Burridge (12) followed by hydroxylapatite chromatography (26). The staining pattern obtained was indistinguishable from that of samples incubated with antibodies that had not been preabsorbed, in-

dicating that the staining observed is not a result of contamination with anti–vinculin antibodies (data not shown).

In fibroblasts transformed with Rous sarcoma virus, strongly fluorescent spots are colocalized with F-actin in the form of rosettes characteristic of transformed fibroblasts. This type of staining pattern has also been described for vinculin and talin that are normally present at the focal adhesions of nontransformed cells (5, 9).

To confirm that proteins immunologically related to HA1 are localized at the adhesion plaques, we labeled chicken embryo fibroblasts with HA1 antibodies and with a monoclonal antibody directed against vinculin. The results of this experiment showed that HA1-related polypeptides are clearly colocalized with vinculin (Fig. 3).

The localization of molecules that are recognized by HA1 antibodies was also determined in several other cell types. Fig. 4 shows an immunofluorescence micrograph of a PtK2 cell stained with HA1 antibodies. Bright fluorescence can be seen at the ends of stress fibers, apparently in the focal adhesion areas, at areas of cell–cell contacts, and along stress fibers. This experiment also demonstrates that molecules immunologically related to those in HA1 from avian smooth muscle are present in mammalian epithelial cells.

We then performed a similar experiment to investigate whether molecules that are recognized by HA1 antibodies are also present in avian striated muscle. Interestingly, when myofibrils from chicken breast muscle were stained with the antibodies, staining was detected exclusively at the Z-lines, as can be seen by comparing fluorescent with phase-contrast images (Fig. 5). We also examined the staining pattern of HA1 antibodies in cultured embryonic chicken cardiac muscle cells. Fig. 6 shows a fluorescence pattern similar to that observed in skeletal muscle. In addition to the Z-line staining, fluorescence was also seen at regions at the ends of myofibrillar structures.

Because the HA1 antibodies used for the immunofluorescence studies described above labeled a number of bands on immunoblots of protein fractions derived from smooth muscle (Fig. 1), we wanted to see if any of these bands could be
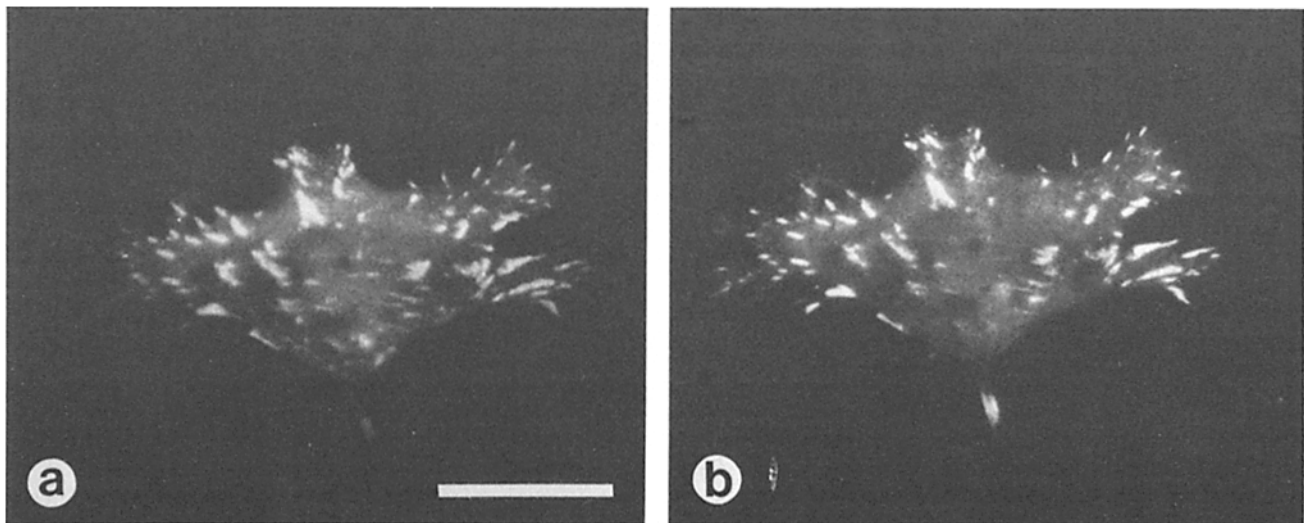

Figure 3. Double-fluorescent staining of HA1-related molecules and vinculin in CEFs. The plane of focus is at the level of the cell–substrate interface. HA1 antibodies detected by a fluorescein-labeled secondary antibody (a) stain CEFs in a manner virtually identical to that of the antibody directed against vinculin (an adhesion plaque protein) detected by a rhodamine-labeled secondary antibody (b). Bar, 20 μm.
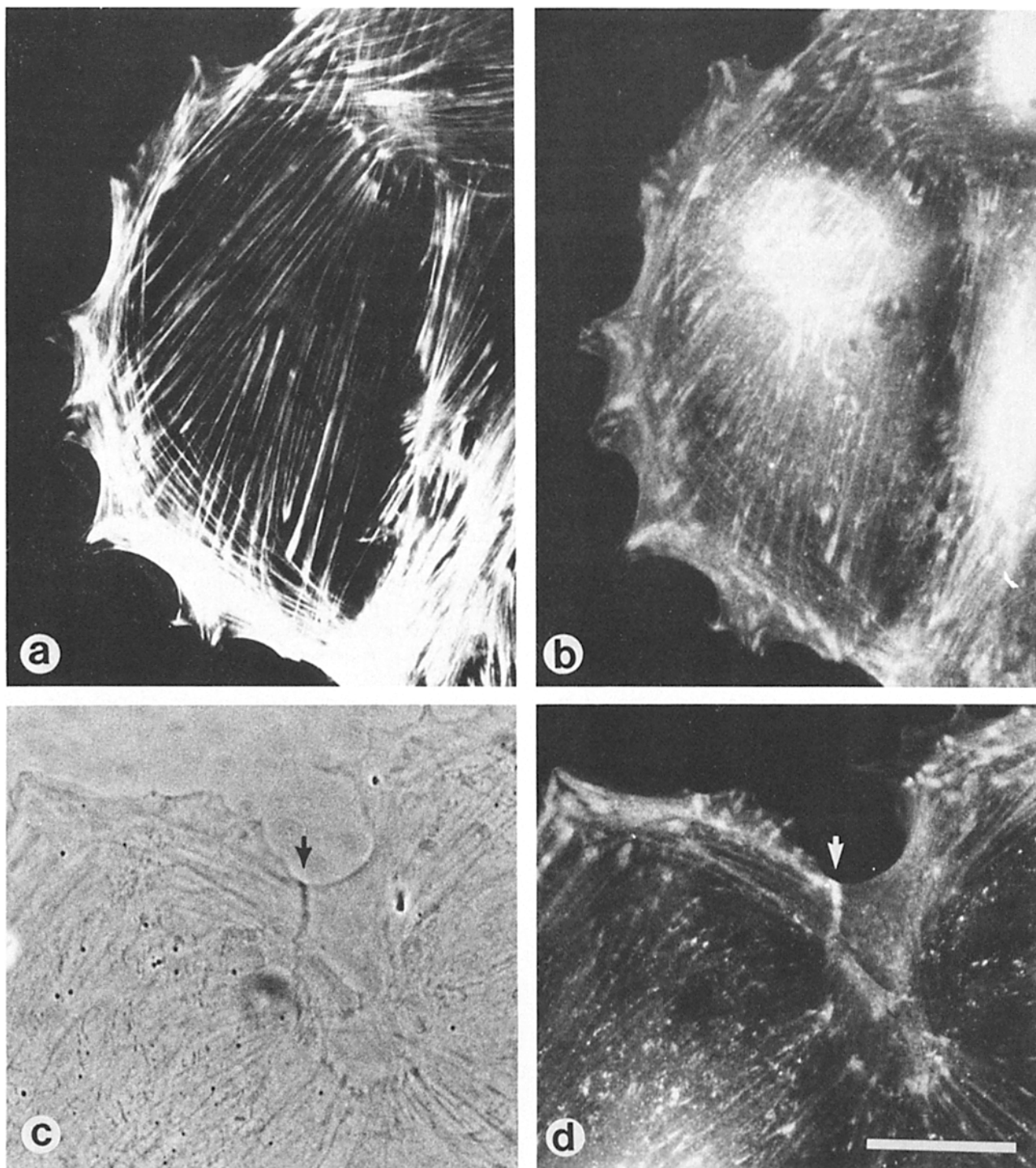
Figure 4. Double-fluorescent staining of HA1-related molecules and F-actin in PtK2 cells. Comparison of phalloidin staining of F-actin (*a*) and indirect immunofluorescent staining with HA1 antibodies (*b*) (using the reagents described in Fig. 2) in the same PtK2 cell reveals that the latter is localized predominantly at the ends of actin filament bundles and also along the bundles. In addition, intense staining with the antibodies is observed at cell–cell contacts, which can be resolved as phase-dense structures (*arrows* in *c* and *d*). The plane of focus is at the level of the cell–substrate interface in *a* and *b* and at the level of the cell–cell contact area in *c* and *d*. Control samples in which the incubation with primary antibodies was omitted or the same concentration of affinity-purified nonimmune rabbit IgG was substituted for the primary antibodies showed only a faint diffuse fluorescence. Bar, 20 μm.

detected in protein preparations from other cells and tissues. We found that the pattern of proteins recognized by the HA1 antibodies was critically dependent on the inclusion of protease inhibitors and EGTA (to inhibit Ca^{++}-dependent pro-

teases; reference 27) in the electrophoresis sample buffer to control proteolysis during sample preparation. Fig. 7, *A* and *B* shows that in samples treated to inhibit proteolysis the HA1 antibodies bound to polypeptides with M_r's substantially
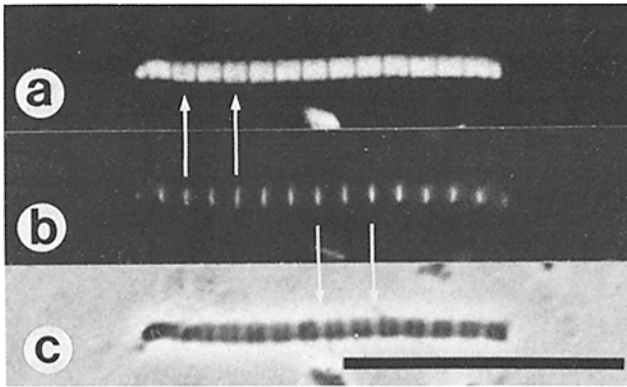
Figure 5. Double-fluorescent staining of HA1-related molecules and F-actin in chicken breast myofibrils. Myofibrils were fluorescently stained for F-actin (*a*) and HA1-related molecules (*b*) using fluorescein-phalloidin and rhodamine-labeled secondary antibody, respectively. The brightly stained structures in *b* (*arrows*) coincide with the Z-line (center of the I band) seen by phase-contrast optics (*c*) and with the region midway between the H-zones not stained by the phalloidin (*a*). Control samples in which the incubation with primary antibodies was omitted or the same concentration of affinity-purified nonimmune rabbit IgG was substituted for the primary antibodies did not show fluorescent staining. Bar, 20 μm.

higher than the 92,500-D marker in both gizzard and fibroblasts. Intensely stained bands of ∼200 and 60 kD in chicken cardiac muscle and 60 and 31 kD in chicken breast muscle were detected on immunoblots using the HA1 antibodies. Occasionally, a doublet appeared in the 60-kD region of the immunoblots of cardiac and breast muscle tissues. In contrast to samples prepared in the presence of protease inhibitors, the same samples prepared without protease inhibitors showed a complex pattern of staining of many bands, including a few with molecular masses similar to those in HA1 (Fig. 7 *D*).

To determine the molecular masses of the bands labeled by the HA1 antibodies in the gizzard and fibroblast samples more precisely, we analyzed these protein preparations on 7% SDS gels (Fig. 8). The immunoblots clearly show that the HA1 antibodies bound to bands with very similar molecular masses in the two samples. However, the staining intensities of the two bands were reversed. The apparent molecular masses of the two bands were estimated to be 200,000 and 150,000 D, based on their electrophoretic mobilities relative to those of standard proteins.

To find out whether the high molecular mass polypeptides that bound the HA1 antibodies on immunoblots were responsible for the staining observed in the immunofluorescence experiments, we repeated the cellular localization experiments with antibodies eluted from different areas of a preparative immunoblot. In this experiment, proteins from lyophilized chicken gizzard tissue were fractionated on preparative SDS-polyacrylamide gels with precautions taken to limit proteolysis, transferred to nitrocellulose sheets, and overlaid with HA1 antibodies. The pattern of antibody binding revealed by the use of alkaline phosphatase–labeled second antibody (see Materials and Methods) was similar to that obtained in Fig. 7 *B*, except that bands of lower molecular masses of 66,000 and 25,000 D were also detected. Antibodies eluted from the 200,000- and 150,000-D bands each stained both adhesion plaques in CEFs (Fig. 9, *a* and *b*) and

Z-lines of chicken skeletal muscle myofibrils (Fig. 9, *c* and *d*) in a manner comparable to that of the original HA1 antibody preparation (see e.g., Fig. 2 and 5). In addition, antibodies eluted from two bands with molecular masses of ∼25,000 D were found to stain focal adhesions (data not shown). In contrast, antibodies eluted from the 66,000-D band did not produce any specific staining pattern in the immunofluorescence experiments.

The immunofluorescence experiment described above strongly suggests that the 200,000- and 150,000-D polypeptides are immunologically related to the polypeptides in the HA1 preparation. This conjecture was supported by the finding that immunoblots of HA1a stained with antibodies eluted from the two bands were similar to each other and had characteristics similar to those stained with HA1 antibodies (Fig. 10 a). In a related experiment, the antibodies eluted from either the 200,000- or the 150,000-D band were used to probe strips cut from nitrocellulose blots of gizzard and cardiac muscle proteins. Antibodies eluted from either band stained both of the bands on strips from chicken gizzard (Fig. 10 *b*), indicating that these polypeptides are immunologically related. The eluted antibodies also recognized both the 200,000- and 60,000-D species on strips prepared from cardiac muscle (Fig. 10 *c*), although the staining of the 200,000-D species was stronger than that of the 60,000-D species. Although we were able to see staining of chicken breast myofibrils with both of the eluted antibody preparations in immunofluorescence experiments (see Fig. 9), the quantity of antibodies obtained was apparently too low in the case of the 200-kD eluate to get adequate staining of immunoblots of chicken breast skeletal muscle. The 150-kD eluate was seen to stain the 60 kD, but not the 31-kD band on immunoblots of breast muscle (data not shown).

A distinguishing characteristic of HA1 and HA1a is their ability to lower the low-shear viscosity of F-actin (Fig. 11 *A*), a property shared by many proteins which bind to the barbed ends of actin filaments (8). Because a protein(s) recognized by the HA1 antibodies was found at cellular attachment sites for F-actin, it was of interest to know whether the antibodies bound to actin binding proteins in the HA1 preparation. The immunoprecipitation experiment in Fig. 11 *B* shows that the activity of HA1a was removed in a dose-dependent manner by HA1 antibodies.

Discussion

Much attention has been focused in recent years on the molecular nature of the attachment of actin filaments to membranous structures. To date, such interactions are best understood in the case of the erythrocyte membrane (1). The end-on association of actin filaments with structures such as focal adhesions of nonmuscle cells and Z-lines of striated muscle remains unclear at the molecular level, although it appears that proteins immunologically related to those associated with erythrocyte membranes are not involved in these systems (e.g., 1).

Our studies on the interaction of vinculin with actin in vitro have led to the identification of a group of active contaminants that we refer to as HA1 (26). Attempts at further fractionation and characterization of this preparation with conventional biochemical techniques have not been fruitful because of its heterogeneity and the difficulty in obtaining
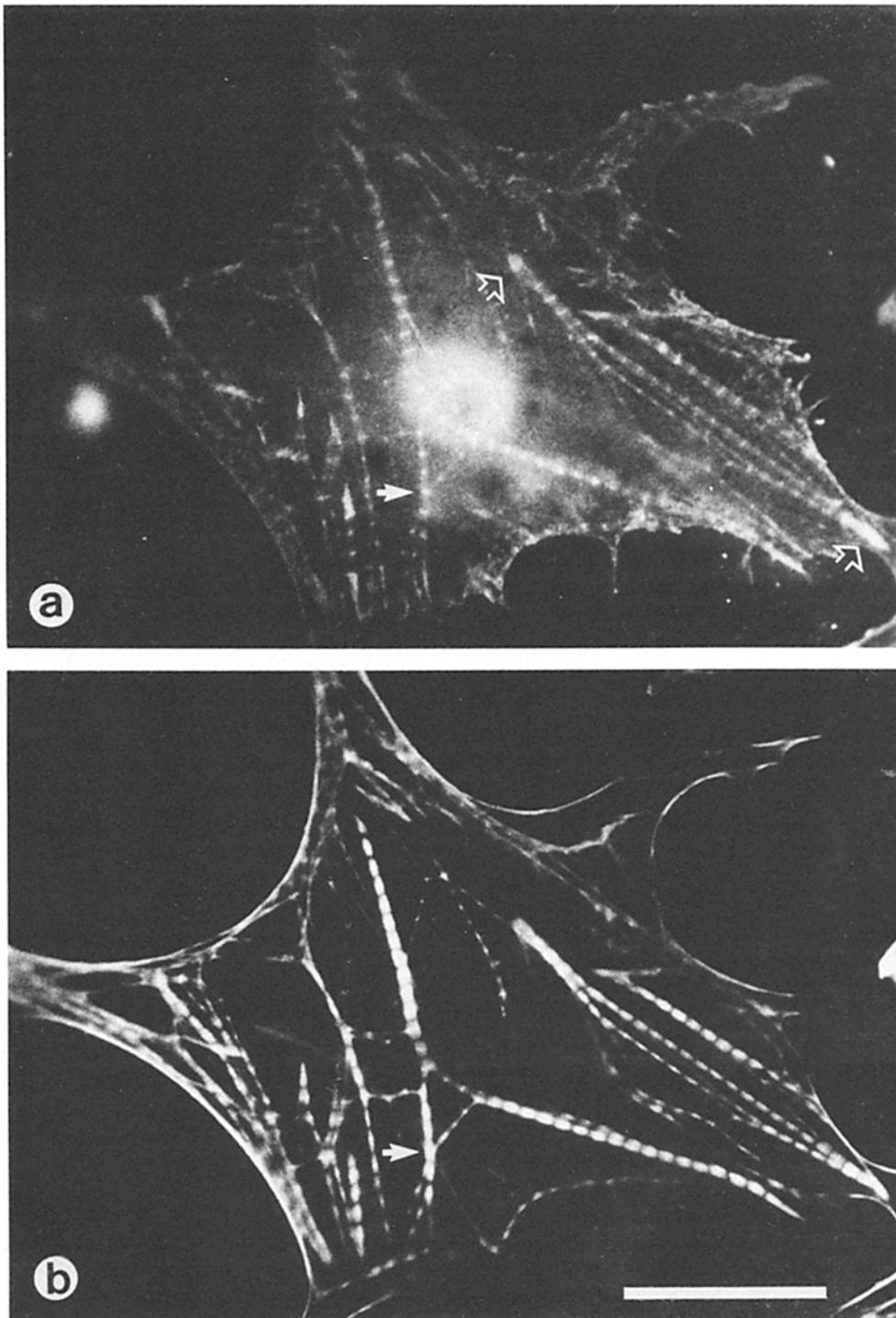
Figure 6. Double-fluorescent staining of HA1-related molecules (*a*) and F-actin (*b*) in a cultured embryonic chicken cardiac myocyte, with the reagents described in Fig. 2. Antibody staining is localized at Z-lines (closed arrows in *a* and *b*) as well as at the ends of myofibrillar structures (open arrows in *a*). Control samples in which the incubation with primary antibodies was omitted or the same concentration of affinity-purified nonimmune rabbit IgG was substituted for the primary antibodies showed only a faint diffuse fluorescence. Bar, 20 μm.

sufficient quantities of the material. In taking an alternative approach to this problem, we raised polyclonal antibodies to the entire HA1 preparation and used these as probes to investigate the localization of immunologically related molecules in a number of cellular systems. Although this approach has obvious limitations because of the heterogeneity of the antigen preparation, we were able to make a number of intriguing observations that could form the basis of future investigations into the molecular mechanism of actin–membrane interaction.

The finding that HA1 antibodies bind to specific areas of both nonmuscle cells (focal adhesions) and striated muscle (Z-lines) where the barbed ends of actin filaments insert into specialized structures represents quite an interesting convergence. Only alpha actinin has thus far been localized at both of these sites. The polypeptides in HA1 are not, however, immunologically related to alpha actinin (e.g., Fig. 1) and interact with actin in a distinct way. In vitro, alpha actinin binds to the sides of F-actin, causing the filaments to form a cross-linked network (19). The polypeptides in HA1 bundle F-actin
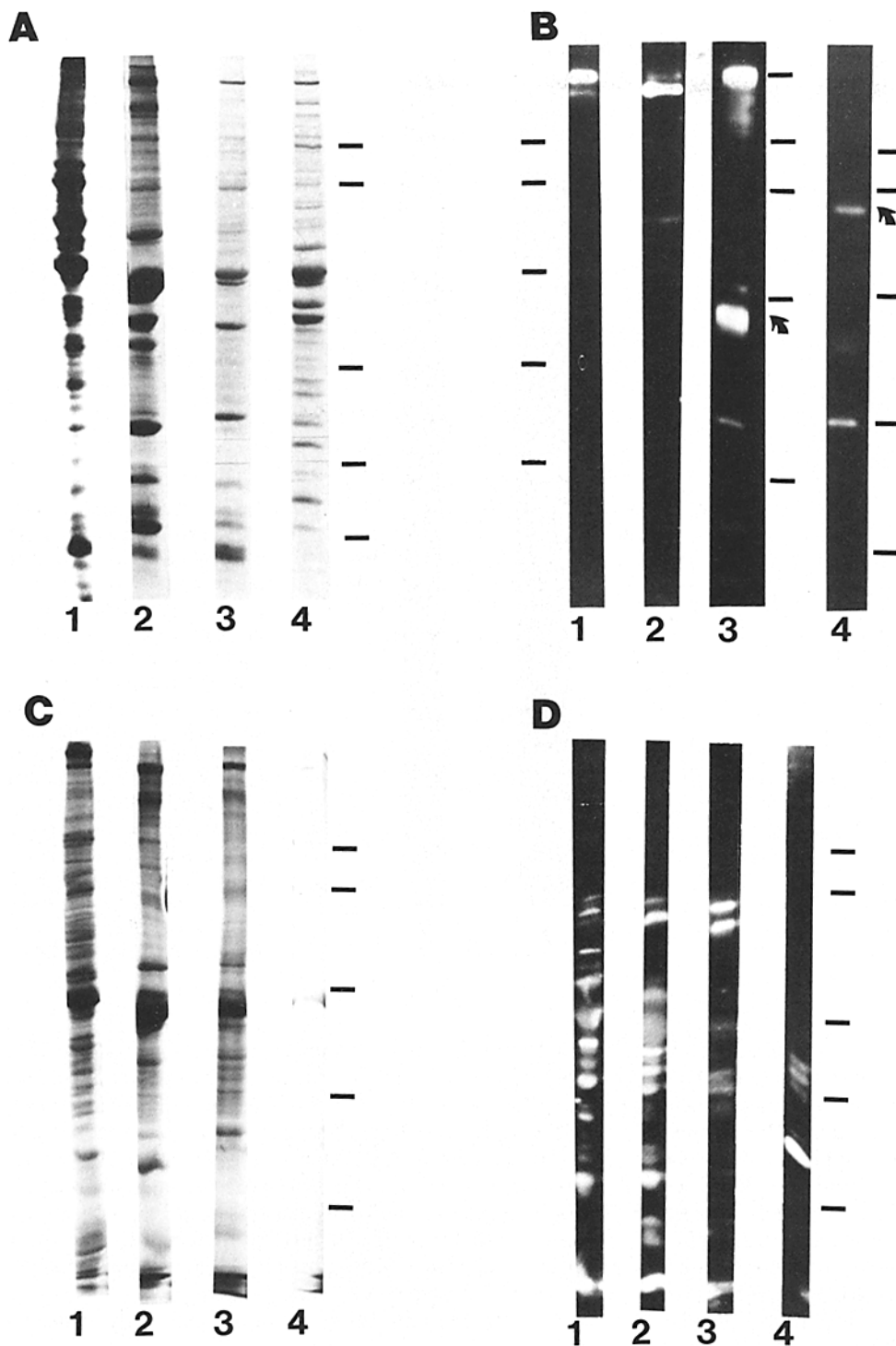
Figure 7. Immunoblots of proteins from adult chicken tissues prepared in the presence and absence of protease inhibitors, probed with HA1 antibodies. To control proteolysis (*A* and *B*), whole adult chicken tissues (cardiac, breast, and gizzard muscle) were prepared by immersion in liquid N$_2$ followed by lyophilization. Lyophilized tissue was then immersed in boiling electrophoresis sample buffer containing 5 mM diisopropylfluorophosphate and 5 mM EGTA; CEFs were prepared by immersion of cells directly into the above buffer. Samples prepared in the absence of protease inhibitors (*C* and *D*) were homogenized in TBS. The homogenates were mixed with an equal volume of SDS sample buffer without diisopropylfluorophosphate or EGTA. All samples were run on 10% SDS polyacrylamide gels as described in Materials and Methods. Gels were stained with Coomassie Blue or transferred to nitrocellulose and probed with HA1-antibodies, followed by incubation with radiolabeled goat anti–rabbit IgG or protein A. (*A* and *C*) Representative gels of samples, stained with Coomassie Blue. (*B* and *D*) Autoradiograms of immunoblots. Lanes *1*, CEFs; lanes *2*, chicken gizzard; lanes *3*, chicken cardiac muscle; lane *4*, *A* and *B*, chicken breast muscle; lane *4*, *C* and *D*, HA1, 1 µg. Blots in lanes *1* and *2*, *A* were probed with 4 µg/ml of HA1 antibodies and those in the other lanes were probed with 40 µg/ml of the antibodies. Positions of molecular mass standards are shown to the right of *A*, *C*, and *D*. Their values in *A* (from top to bottom) were: 92.5, 66.2, 45, 31, 21.5, and 14.4 kD. The 14.4-kD standard was deleted from the markers in *C* and *D*. In *B*, the molecular mass standards for lanes *1* and *2* are to the left of those lanes and are the same as those in *A*. The molecular mass standards for lanes *3* and *4*, *B* are shown to the right of those lanes and are the following. Lane *3*: 200, 116, 92.5, 66, and 45 kD; lane *4*: 92.5, 66, 45, 31, and 21.5 kD. Note that the positions of the 60-kD bands (marked by *arrows*) differs in lanes *3* and *4*, *B*.

at low concentrations (26), but more importantly in this context, interact with the barbed end of actin filaments (20, 26) in a manner similar to filament capping proteins that have been described in recent years (see e.g., 8, 22). The immunofluorescence data coupled with the finding that the HA1 antibodies are able to remove the ability of HA1a to interact with

F-actin raise the exciting possibility that HA1 polypeptides may participate in linking the barbed ends of actin filaments to structures with which they must interact to transmit contractile force during cellular contractility and motility.

The immunoblotting experiments also produced several interesting results. We found that a number of polypeptides
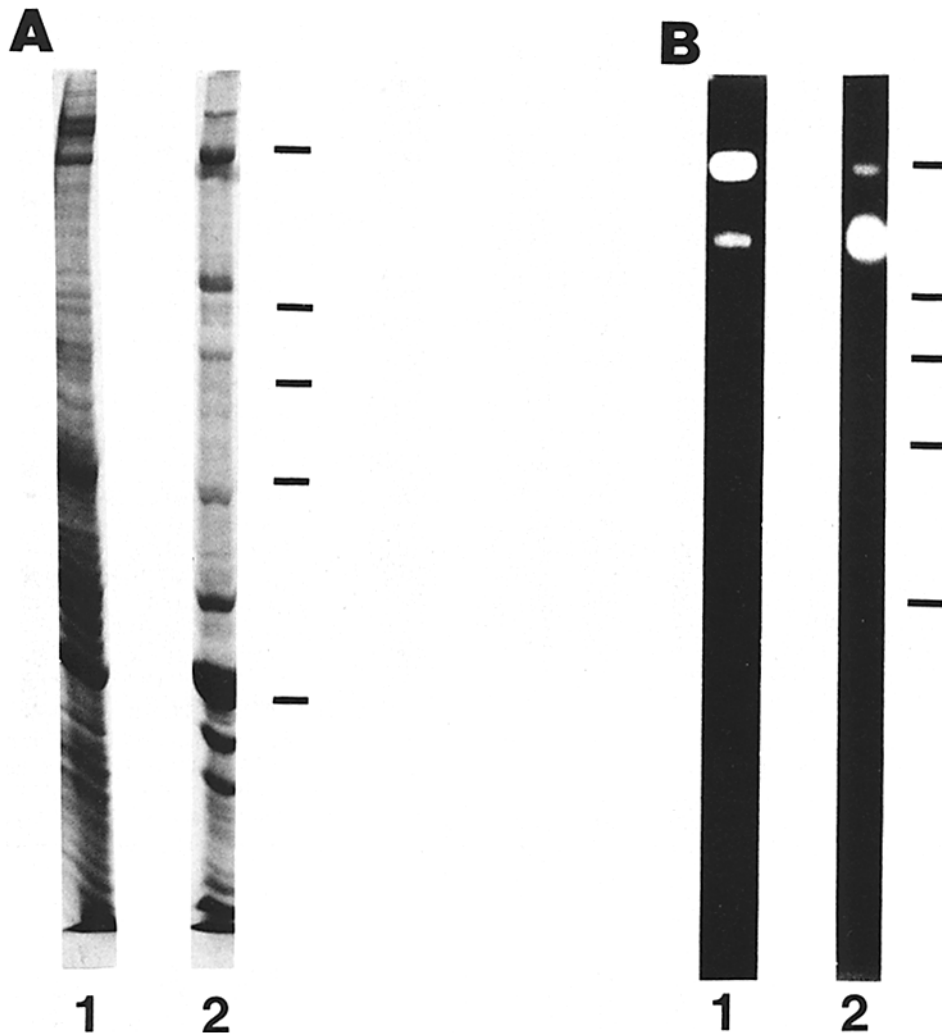
A

1   2

B

1   2

Figure 8. Immunoblots of CEFs and adult chicken gizzard probed with HA1 antibodies. CEFs and adult chicken gizzard tissue were prepared in the presence of protease inhibitors as described in Fig. 7 and electrophoresed on 7% SDS polyacrylamide gels. The gels were stained with Coomassie Blue (*A*) or transferred to nitrocellulose and probed with HA1 antibodies (4 μg/ml). Lanes *1*, CEFs; lanes *2*, adult chicken gizzard. Marks to the right of the panels show the positions of molecular mass standards: from top, 200, 116, 92.5, 66, and 45 kD.

in the HA1 preparation with molecular masses between 20,000 and 45,000 D were recognized by the antibodies. Many of these polypeptides were not seen in gels stained with Coomassie Blue. However, their molecular masses fell within the range of those with actin capping activity, as previously determined in gel elution experiments (26). The immunoblotting experiments also suggest that the molecules responsible for antibody staining of the focal adhesions and Z-lines have higher molecular masses than those present in the HA1 preparation. Our present working hypothesis is that the 200,000- and 150,000-D species seen in immunoblots of smooth muscle treated to minimize proteolysis are the precursor molecules from which HA1 polypeptides are generated by proteolysis during the prolonged extraction step at 37°C (12). This idea is supported by several lines of evidence. First, immunoblots of smooth muscle treated to minimize proteolysis showed little, if any, material cross-reacting with HA1 antibodies in the HA1 region. Second, the pattern of immunoreactive bands in HA1 and HA1a varies somewhat from preparation to preparation. Third, antibodies eluted from the 200,000- and 150,000-D regions of nitrocellulose blots of gizzard proteins bound to polypeptides in HA1. Finally, when vinculin was extracted from smooth muscle at

low temperature in the presence of protease inhibitors, HA1 polypeptides were not obtained as a contaminant during the initial stages of purification (Wilkins, J. A., M. A. Risinger, and S. Lin, unpublished results). We attempted to use antibodies eluted from the 200- and 150-kD regions of preparative immunoblots of chicken gizzard to do immunoprecipitation experiments similar to that shown in Fig. 11 using the column affinity purified anti-HA1. However these experiments were unsuccessful, possibly due to the small amounts of antibodies obtained by such methods.

Another question raised by the present study is the relationship between the immunoreactive polypeptides of ∿60,000 D found in striated muscle tissues and the 200,000- and 150,000-D polypeptides found in smooth muscle and nonmuscle cells. Although we know that antibodies eluted from the 200,000- and 150,000-D regions of nitrocellulose transfers of smooth muscle proteins bind to Z-lines of skeletal muscle myofibrils and that a 200,000-D species is present in cardiac muscle, further experiments must be performed to see whether the 60,000-D polypeptides detected in cardiac and skeletal muscles are derived from the high molecular mass species (e.g., by proteolysis), or whether they are HA1 analogs found only in striated muscle.
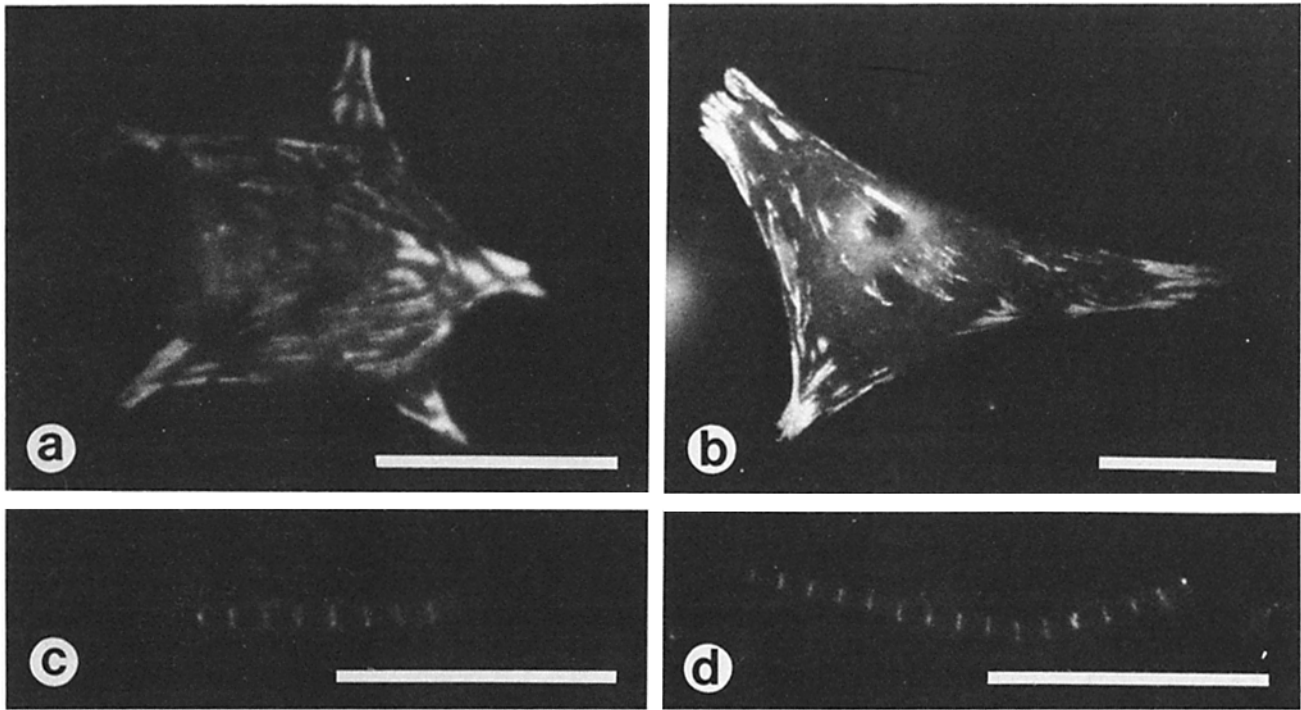
Figure 9. Immunofluorescent staining of CEFs and chicken breast myofibrils with HA1 antibodies eluted from immunoblots of chicken gizzard. HA1 antibodies were incubated with preparative immunoblots and those binding to the 150,000- and 200,000-D regions of the blots were eluted as described in Materials and Methods. These undiluted antibody preparations were then incubated with either CEFs (*a* and *b*) or chicken breast myofibrils (*c* and *d*). The antibodies were localized using fluorescein-labeled goat anti–rabbit IgG as described in Materials and Methods. (*a* and *c*) Staining performed with antibodies eluted from the 200-kD region. (*b* and *d*) Staining performed with antibodies eluted from the 150-kD region.
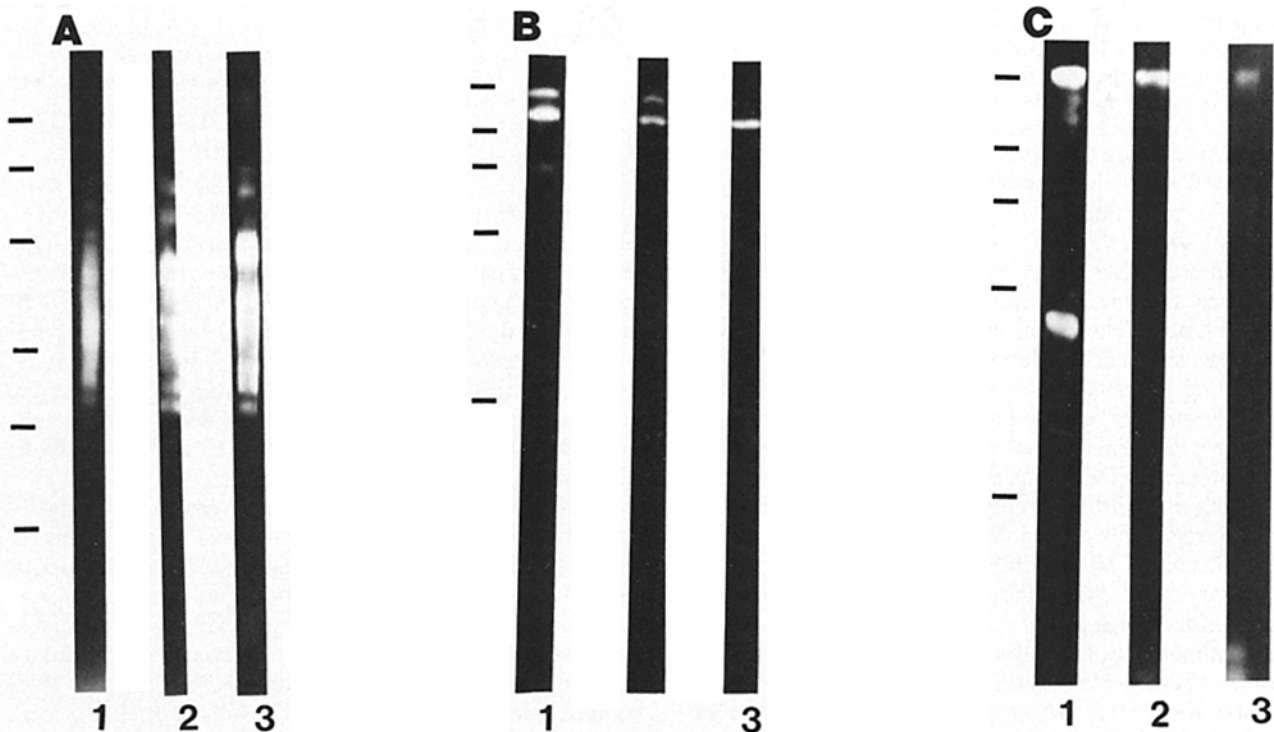

Figure 10. Immunoblots of HA1a polypeptides, chicken gizzard, and chicken cardiac muscle probed with different antibody fractions eluted from preparative immunoblots of chicken gizzard smooth muscle tissue. Strips were cut from preparative nitrocellulose blots of HA1a (*A*), chicken gizzard (*B*), or chicken cardiac muscle (*C*). Chicken gizzard and cardiac muscle tissues were prepared as described in the legend to Fig. 7 to avoid proteolysis during sample preparation and electrophoresis. The strips were immersed in the following solutions: HA1 antibodies (40 µg/ml; lanes *1*); a subfraction of antibodies eluted from the 200,000-D region (lanes *2*), or those eluted from the 150,000-D region (lanes *3*) of a preparative immunoblot of chicken gizzard proteins as described in Materials and Methods. After an overnight incubation at 4°C, bound antibodies were detected by incubation with [125I] protein A, followed by autoradiography. Marks to the left of the lanes indicate the positions of molecular mass standards: *A* (top to bottom), 92.5, 66, 45, 31, 21.5, and 14 kD; *B* and *C* (top to bottom), 200, 116, 92.5, 66, and 45 kD.
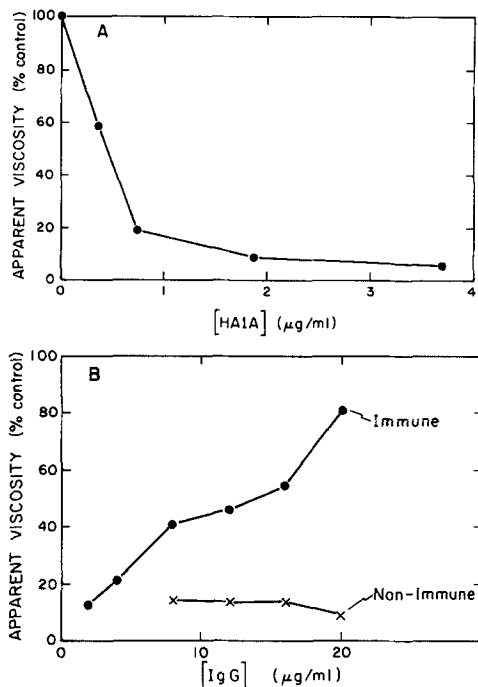
Figure 11. Effects of HA1 antibodies on the ability of HA1a to reduce the low-shear viscosity of F-actin. (*A*) Various amounts of HA1a were mixed with fixed amounts (0.5 mg/ml) of gel-filtered actin in buffer B. After actin polymerization, the viscosity of the samples was measured in falling ball viscometers as previously described (26). The apparent viscosity is expressed as a percentage of the control sample containing only actin. (*B*) Fixed amounts of HA1a (0.37 μg) were mixed with various amounts of HA1 antibodies (●) or nonimmune rabbit IgG (X) and the samples were treated as described in Materials and Methods. After removal of the antibodies, equal volumes (190 μl) of supernatants were added to gel-filtered actin (0.125 mg) and the final volumes of the samples were adjusted to 0.25 ml by adding IgG buffer. MgCl₂ was added to a final concentration of 2 mM and the low-shear viscosity of the samples (expressed as a percentage of the control sample containing only actin) was measured after a 1-h incubation at 30°C.

In summary, the present study has raised the possibility that proteins immunologically related to the HA1 polypeptides are involved in the attachment of the barbed end of actin filaments to specialized structures in muscle and nonmuscle cells. Our working hypothesis at present is that the polypeptides in the HA1 preparation are proteolytic fragments containing actin binding domains of larger molecules (i.e., the 150- and 200-kD species in smooth muscle). The final proof of this conjecture must await the eventual isolation of each of the high molecular mass species revealed by the use of the HA1 antibodies described here and the demonstration of their ability to bind to the barbed end of actin filaments.

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