Proteins of the human placental microvillar cytoskeleton

α -Actinin

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The human placental syncytiotrophoblast microvilli are supported by an underlying cytoskeleton consisting mainly of actin microfilaments. The major proteins associated with the actin have M_r values of 105 000, 80 000 and 68 000. The 105 000- M_r protein is recognized by an antibody preparation raised to purified chicken gizzard α -actinin. Electron microscopy has shown that the human placental protein has dimensions similar to those reported for muscle α -actinin. About half of the placental microvillar α -actinin is released from the cytoskeleton in the presence of Ca^{2+} . This effect occurs at concentrations of Ca^{2+} greater than $0.3 \mu\text{M}$ and has been used as the basis of a method for the purification of the placental α -actinin. This sensitivity to Ca^{2+} is not affected by trifluoperazine and is therefore likely to be a property of the α -actinin as such rather than being mediated via calmodulin.

INTRODUCTION

The microvillar plasma membrane of the human placental syncytiotrophoblast is in direct contact with maternal blood. As such, it is active in the selective materno-foetal transfer of physiologically important materials (for a review see Truman & Ford, 1984). The microvilli increase the surface area of the plasma membrane by a factor of about 10-fold. Their characteristic finger-like shape is maintained by an underlying cytoskeleton consisting of actin microfilaments, which are cross-linked to each other and which are directly or indirectly attached to the plasma membrane.

In a previous study, we reported that the microvillar cytoskeleton contained, in addition to actin, major proteins of M_r 105 000, 80 000 and 68 000 (a doublet) and calmodulin (Booth & Vanderpuye, 1983). The placental microvilli differ considerably from the well-studied intestinal microvilli. Intestinal microvilli contain villin, a Ca^{2+} -regulated actin-capping protein (Bretscher & Weber, 1980; Craig & Powell, 1980; Glenney *et al.*, 1981; Cribbs *et al.*, 1982), and fimbrin, an actin-cross-linking protein (Bretscher, 1981; Glenney *et al.*, 1981), but are devoid of α -actinin (Mooseker & Stephens, 1980). In contrast, the placental microvilli are devoid of villin and fimbrin and, as reported in the present paper, contain a Ca^{2+} -sensitive α -actinin. In this respect they may resemble the branched microvilli of ascites adenocarcinoma cells, which have been reported to contain significant quantities of α -actinin (Carraway *et al.*, 1980).

We now report the identification of the placental 105 000- M_r protein as α -actinin and report its purification and partial characterization.

MATERIALS AND METHODS

Normal term human placentae were obtained from the Delivery Suite, Leeds General Infirmary, Leeds, U.K., and were processed within 15 min of delivery. Frozen chicken gizzards were purchased locally. A goat

antiserum raised to rabbit IgG and horseradish peroxidase–rabbit anti-peroxidase antibody complex (PAP) were obtained from Miles Laboratories, Slough, Berks., U.K. Human IgG was obtained from Sigma Chemical Co., Poole, Dorset, U.K. Trifluoperazine was from May and Baker, Dagenham, Essex, U.K. Nitrocellulose sheets were obtained from Anderman and Co., Kingston-upon-Thames, Surrey, U.K. All other reagents were of the highest purity available from Sigma Chemical Co. or BDH Chemicals, Poole, Dorset, U.K.

Placental microvilli were prepared as previously described (Booth *et al.*, 1980) except that the initial wash with 0.05 M- CaCl_2 was omitted. Cytoskeletons were prepared from the microvilli by resuspending them to a protein concentration of 5 mg/ml in buffer A (75 mM- NaCl /5 mM- MgCl_2 /1 mM-EGTA/1 mM-dithiothreitol/1 mM-phenylmethanesulphonyl fluoride/10 mM-Hepes/ NaOH buffer, pH 7.4) containing 4% (v/v) Triton X-100 and incubating on ice for 15 min. After centrifugation at 20 000 g for 15 min in a Sorvall SS-34 rotor, the pelleted material was resuspended and washed twice in buffer A containing 0.1% (v/v) Triton X-100. Finally, the pelleted material was washed, and resuspended to a protein concentration of 3 mg/ml in detergent-free buffer A.

α -Actinin was purified from chicken gizzards by the method of Feramisco & Burridge (1980). Final purification was achieved by using preparative polyacrylamide-gel electrophoresis in the presence of SDS. The protein was recovered from the polyacrylamide gel and used for immunization of New Zealand White rabbits as described by Lazarides (1976). The antisera collected were recycled through columns containing chicken gizzard α -actinin immobilized on CNBr-activated Sepharose 4B. After the columns had been washed with 0.15 M- NaCl /10 mM-sodium phosphate buffer, pH 7.4, the bound antibodies were eluted with 0.2 M-glycine/ HCl buffer, pH 2.8, and were immediately neutralized with 1.0 M-Tris base and dialysed against 0.15 M- NaCl /10 mM-sodium phosphate buffer, pH 7.4. These antibodies were found to be specific

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for α -actinin when used to stain total gizzard proteins that had been transferred to nitrocellulose sheet after separation by SDS/polyacrylamide-gel electrophoresis.

Electrophoresis in 7–17% (w/v) polyacrylamide gradient gels in the presence of SDS was performed by the method of Laemmli (1970). Gels were stained and destained as described previously (Booth & Wilson, 1981). Gel strips were scanned at a wavelength of 620 nm by using a Gilford model 240 spectrophotometer equipped with a model 222 gel-scanning attachment. The slit width used was 0.1 mm. The strips were scanned at 5 cm/min with the chart recorder running at 60 cm/min. Full-scale deflexion corresponded to an absorbance of 1.2. The area under each peak was quantified by cutting it from the chart recording and comparing its weight with that of a known area of chart paper. Electrophoretic transfer of proteins from gels to nitrocellulose sheets was performed as described by Towbin *et al.* (1979), with the Bio-Rad TransBlot apparatus (Bio-Rad Laboratories, Watford, Herts., U.K.). Transferred antigens were localized by the following method. Excess protein-binding sites were blocked by incubation for 18 h at 4 °C in Tris-buffered saline (0.15 M-NaCl/10 mM-Tris/HCl/buffer, pH 7.4) containing 3% (w/v) bovine serum albumin. All further manipulations were carried out at room temperature. After each stage the strips were washed as follows: three times for 10 min in 1.0 M-NaCl/10 mM-Tris/HCl buffer, pH 7.4, containing 0.5% (v/v) Tween 20 and then twice for 10 min in Tris-buffered saline. The blocked nitrocellulose strips were incubated for 1 h in Tris-buffered saline containing 3% (w/v) bovine serum albumin and 20 μ g of the anti- α -actinin antibodies/ml. After being washed, strips were then incubated for 1 h in Tris-buffered saline containing a 1:100 dilution of a goat anti-(rabbit IgG) serum, 2.5 mg of human IgG/ml and 3% (w/v) bovine serum albumin. The strips were then washed and incubated for 1 h in Tris-buffered saline containing 3% (w/v) bovine serum albumin and a 1:100 dilution of PAP complex. After further washing, the bound PAP complex was detected by incubation with 10 mM-sodium acetate/acetic acid, pH 5.0, containing 2 mM-3-amino-9-ethylcarbazole and 4.5 mM-H₂O₂.

Samples of purified proteins were examined, after negative staining the samples with uranyl acetate on carbon/Formvar grids, in a Philips EM300 electron microscope operating at 80 kV.

RESULTS

Evidence that the placental 105000- M_r protein is a placental form of α -actinin

Samples of placental microvillar cytoskeleton preparations were subjected to SDS/polyacrylamide-gel electrophoresis. The gel was divided into two portions. One was stained immediately with Coomassie Blue. The resolved proteins in the other half were electrophoretically transferred to nitrocellulose sheets and the strips were stained by using the rabbit anti-(chicken gizzard α -actinin) antibody preparation as described in the Materials and methods section. The result is shown in Fig. 1. The antibody stained a single band whose mobility was identical with that of the 105000- M_r protein. Two-dimensional electrophoresis of cytoskeletal proteins (Booth & Vanderpuye, 1983) has shown that there is a single protein in this band whose isoelectric point is

Fig. 1. Identification of placental microvillar α -actinin

Human placental microvillar cytoskeletons were subjected to SDS/polyacrylamide-gel electrophoresis. The resolved proteins were either stained with Coomassie Blue or electrophoretically transferred to nitrocellulose sheet and stained by using an antibody preparation raised to chicken gizzard α -actinin as described in the Materials and methods section. The left-hand strip (a) shows the Coomassie Blue-stained gel, and the right-hand strip (b) shows the antibody-stained nitrocellulose sheet from a sample run on the same gel.

similar to that reported for non-muscle α -actinin. Thus the microvillar protein of M_r 105000 can be identified as a placental form of α -actinin.

Evidence that the interaction of the placental α -actinin with the cytoskeleton is sensitive to micromolar concentrations of Ca²⁺

We have already shown that the placental protein of M_r 105000 interacts with the cytoskeleton in an ATP-independent and Ca²⁺-sensitive manner (Booth & Vanderpuye, 1983). In the presence of Ca²⁺, α -actinin, actin and a protein of M_r 80000 were shown to be released from the cytoskeletons. However, those experiments were performed with 1 mM-Ca²⁺. This concentration is several orders of magnitude greater than that reported to have effects on other Ca²⁺-sensitive proteins. We therefore prepared modified versions of buffer A

Fig. 2. Effect of Ca^{2+} on the interactions of microvillar proteins with the cytoskeleton

Samples of microvillar cytoskeleton preparations were resuspended in buffers containing defined concentrations of Ca^{2+} as described in the Results section. After centrifugation at 20000 g for 15 min, the supernatants (s) and pelleted material (p) were examined by SDS/polyacrylamide-gel electrophoresis. The concentrations of Ca^{2+} used were: tracks 1, 0; tracks 2, 30 nM; tracks 3, 100 nM; tracks 4, 300 nM; tracks 5, 1 μM ; tracks 6, 3 μM ; tracks 7, 1 mM. Tracks marked M contained M_r -marker proteins. These were vinculin (M_r 130000), transferrin (M_r 80000), bovine serum albumin (M_r 68000), ovalbumin (M_r 45000), soya-bean trypsin inhibitor (M_r 21500) and haemoglobin (M_r 17000).

Fig. 3. Effect of the Ca^{2+} concentration on the interaction of the placental α -actinin with the cytoskeleton

Samples of microvillar cytoskeleton preparations were resuspended in buffers containing defined concentration of free Ca^{2+} as described in the Results section. After centrifugation at 20000 g for 15 min, the supernatants and

containing defined concentrations of free Ca^{2+} . These buffers were adjusted to pH 6.8, contained no Mg^{2+} and contained CaCl_2 and EGTA in the ratios appropriate to produce free Ca^{2+} concentrations of 0, 30 nM, 100 nM, 300 nM, 1 μM , 3 μM or 1 mM. The dissociation constant used to calculate these ratios was that given by Hellewell & Taylor (1979), i.e. $1.95 \times 10^6 \text{ M}^{-1}$ at pH 6.8. Seven samples of cytoskeleton preparations (3 mg of protein/ml in buffer A) were centrifuged at 20000 g for 15 min in a Sorvall SS-34 rotor. The pelleted material from each sample was resuspended in one of the modified buffers and incubated on ice for 15 min. After centrifugation at 20000 g , samples of each supernatant and pellet were examined by SDS/polyacrylamide-gel electrophoresis. A

pelleted materials were subjected to SDS/polyacrylamide-gel electrophoresis. The gels were stained and destained, then each line of the gel was scanned and the amount of α -actinin released into the supernatant was calculated by manual integration as described in the Materials and methods section. The results shown represent the means of four separate experiments. $K_{0.5}$ for the effect of Ca^{2+} on the interaction of the placental α -actinin with the cytoskeleton was 0.6 μM .

Fig. 4. Effect of trifluoperazine on the Ca^{2+} -mediated release of α -actinin from microvillar cytoskeletons

Samples of placental microvillar cytoskeleton preparations were dialysed against a buffer containing 1 mM-EGTA in the presence (tracks 1, 2, 5, 6, 9, 10, 13 and 14) or absence (tracks 3, 4, 7, 8, 11, 12, 15 and 16) of 1 mM-trifluoperazine. After centrifugation at 20000g for 15 min, the supernatants and pelleted material were subjected to SDS/polyacrylamide-gel electrophoresis. The samples were run in supernatant-pellet pairs, odd-numbered tracks contained the supernatants and even-numbered tracks contained the corresponding pelleted material. The track marked M contained the M_r -marker proteins described in the legend to Fig. 2. It can be seen that 1 mM-trifluoperazine had no effect after immediate or prolonged exposure to the cytoskeletons.

typical result is shown in Fig. 2. The placental α -actinin was maximally released from the cytoskeletons at concentrations of Ca^{2+} of $1 \mu\text{M}$ or greater. To obtain quantitative results, the percentage of α -actinin released was measured by scanning gel strips as described in the Materials and methods section. Fig. 3 shows the combined results of four such experiments. The α -actinin was released in a complex manner. At concentrations of Ca^{2+} less than $0.3 \mu\text{M}$, relatively little α -actinin was released. The most obvious effect of Ca^{2+} occurred between $0.3 \mu\text{M}$ and $1.0 \mu\text{M}$. The proportion of α -actinin released rose from approx. 6% to approx. 25%, with a $K_{0.5}$ of $0.6 \mu\text{M}$. Thereafter, as the concentration of Ca^{2+} increased, progressively more α -actinin was released, up to a maximum of 50%. Never more than 50% of the α -actinin was released, even at Ca^{2+} concentrations of 1 mM or greater.

As shown in Fig. 2, α -actinin was not the only protein whose interaction with the cytoskeleton was affected by Ca^{2+} . Several polypeptides showed the opposite characteristics to α -actinin, i.e. their binding to the cytoskeleton was enhanced in the presence of Ca^{2+} . Of these, the major polypeptides had M_r values of 25000, 36000, 69000, 72000, 180000 and 190000. The $K_{0.5}$ for the effect of Ca^{2+} on the interaction of the 36000- M_r protein with the cytoskeleton was identical with that for α -actinin,

possibly suggesting a connection between the functions of these two proteins.

Evidence that the sensitivity of the placental α -actinin to Ca^{2+} is not affected by trifluoperazine

We have previously demonstrated that the human placental microvillar cytoskeletons contain calmodulin (Booth & Vanderpuye, 1983). This raised the possibility that the placental α -actinin might exist as a complex with calmodulin. The latter protein might then be responsible for the apparent Ca^{2+} -sensitivity of the α -actinin. To test this possibility, the effect of Ca^{2+} on the release of α -actinin from the microvillar cytoskeleton was examined in the presence of trifluoperazine, a phenothiazine compound known to interfere with calmodulin function (Levin & Weiss, 1977, 1978).

Samples of cytoskeleton preparations were resuspended to a protein concentration of 3 mg/ml in either buffer A or buffer A containing 1 mM-trifluoperazine, and were dialysed against the same buffer for 18 h at 4°C . Both samples were then divided into four portions and centrifuged at 20000 g for 15 min. The pellets were resuspended to a protein concentration of 3 mg/ml in one of the following buffers: buffer A; buffer A containing 1 mM-trifluoperazine; buffer B (75 mM-NaCl/5 mM- MgCl_2 /1 mM-dithiotreitol/1 mM-phenylmethane

Fig. 5. Purification of human placental microvillar α -actinin

Cytoskeletons were dialysed for 18 h against 2 mM- CaCl_2 /2 mM- NaN_3 /10 mM-Hepes/NaOH buffer pH 7.1. After centrifugation at 90000 g for 30 min, the solubilized proteins were concentrated by applying the supernatant to a column of DEAE-cellulose equilibrated with the same buffer. The proteins were eluted with 0.6 M- NaCl /2 mM- CaCl_2 2 mM- NaN_3 /10 mM-Hepes/NaOH buffer, pH 7.1. After dialysis against the first buffer, $(\text{NH}_4)_2\text{SO}_4$ was added to 25% saturation. After standing on ice, the precipitated material was pelleted by centrifugation at 20000 g for 30 min. $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant to 40% saturation, and the precipitated material was collected as before. At each stage samples were examined by SDS/polyacrylamide-gel electrophoresis. The gel shown carried the following samples: track 1, placental microvillar cytoskeletons; track 2, proteins released by Ca^{2+} ; track 3, material eluted from the DEAE-cellulose column; track 4, material precipitated by between 25% and 40% with $(\text{NH}_4)_2\text{SO}_4$; track 5, α -actinin precipitated by between 25% and 40% saturation with $(\text{NH}_4)_2\text{SO}_4$; track 6, material not precipitated by 40% saturation with $(\text{NH}_4)_2\text{SO}_4$.

sulphonyl fluoride/1 mM-Hepes/NaOH buffer, pH 7.4); buffer B containing 1 mM-trifluoperazine. After incubation on ice for 15 min, each sample was centrifuged at 20000 g for 15 min. The material in each pellet and supernatant was examined by SDS/polyacrylamide-gel electrophoresis. The results are shown in Fig. 4. The presence of trifluoperazine in the incubation or preincubation buffers had no effect on the Ca^{2+} -mediated release of placental α -actinin from the cytoskeletons. There is therefore no evidence for the involvement of calmodulin in the Ca^{2+} -mediated release of α -actinin from the placental microvillar cytoskeletons, and it is likely that this is therefore a property of the α -actinin as such.

Purification of the human placental α -actinin

The Ca^{2+} -mediated release of α -actinin from the microvillar cytoskeletons has been used as the basis for its purification. Cytoskeletons (100 mg of protein) were resuspended in 30 ml of buffer C (2 mM- CaCl_2 /2 mM- NaN_3 /10 mM-Hepes/NaOH buffer, pH 7.1) and dialysed for 18 h against 4 litres of the same buffer. After centrifugation at 90000 g for 30 min in a Beckman AL 30 rotor, the solubilized proteins were concentrated by applying the supernatant to a column (5 ml) of DEAE-cellulose previously equilibrated with buffer C. The absorbed proteins were eluted with buffer C

containing 0.6 M- NaCl . The eluted material contained three major proteins. These were actin, α -actinin and a protein of M_r 80000. This last protein remains unidentified, although a similar protein is present in chicken intestinal microvilli (Bretscher, 1983). The placental protein is recognized by an antiserum, kindly provided by Dr. Bretscher, that has been raised to the intestinal protein (H. C. Edwards & A. G. Booth, unpublished work).

After dialysis for 18 h against buffer C, the preparation was kept on ice, and $(\text{NH}_4)_2\text{SO}_4$ was added to 25% saturation. After standing for 30 min, precipitated material was removed by centrifugation at 20000 g for 30 min. Further $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant to adjust it to 40% saturation. After standing on ice for 30 min, the precipitated material was sedimented at 20000 g for 30 min. Both pellets were resuspended in 3 ml of buffer C and, together with the final supernatant, were dialysed for 18 h against 4 litres of buffer C. All three samples were examined by SDS/polyacrylamide-gel electrophoresis. The result is shown in Fig. 5. The protein precipitated at between 0 and 25% saturation with $(\text{NH}_4)_2\text{SO}_4$ contained mainly actin. The α -actinin was precipitated at between 25% and 40% saturation, and the 80000- M_r protein remained soluble in 40%-saturated $(\text{NH}_4)_2\text{SO}_4$. Samples of the α -actinin preparation were

examined by electron microscopy after negative staining with uranyl acetate. The molecules appeared as short rods, similar in appearance to that reported for muscle α -actinin by Podlubnaya *et al.* (1975).

DISCUSSION

Human placental syncytiotrophoblast microvilli contain a protein whose M_r value and appearance in the electron microscope are very similar to those reported for muscle α -actinin. In addition, the human placental protein is recognized by an antibody preparation specific for chicken gizzard α -actinin. We are therefore confident that the microvillus preparations used in these studies contain a placental form of α -actinin.

The extensively studied microvilli obtained from chicken enterocytes do not contain α -actinin (Bretscher & Weber, 1979; Craig & Lancashire, 1980; Mooseker & Stephens, 1980). However, α -actinin has been identified in many plasma-membrane preparations, and is present in the microvilli of MAT-CI mammary adenocarcinoma cells (Carraway *et al.*, 1980). It is possible therefore that the irregular, often branched, microvilli found on the syncytiotrophoblast, on the adenocarcinoma cells and on many other cells may represent a different class of structure from the rigid arrays of microvilli found in the brush borders of the intestine and kidney.

The interaction of the placental α -actinin with the microvillar cytoskeleton is sensitive to Ca^{2+} . This has also been reported for the α -actinins isolated from HeLa cells (Burridge & Feramisco, 1981), platelets (Rosenberg *et al.*, 1981; Landon & Olomucki, 1983), macrophages (Bennett *et al.*, 1984), brain (Duhaiman & Bamburg, 1984) and *Dictyostelium discoideum* (Condeelis *et al.*, 1984). In the case of the placental α -actinin, the main effect of Ca^{2+} occurs at concentrations greater than $0.3 \mu\text{M}$. This is in line with the values reported for the α -actinins from the other sources, although the effect that we have observed seems to be more marked than that reported by other workers. Presumably this effect is important in modulating the structure of the microvillus cytoskeleton, since it occurs at concentrations of Ca^{2+} that are within the physiological cytoplasmic range. Furthermore, at least one other cytoskeletal protein shows exactly the reverse properties, binding to the cytoskeleton in the presence of Ca^{2+} with the same $K_{0.5}$ as α -actinin. It is difficult to avoid the implication that these two proteins might have reciprocal functions, rather like the Ca^{2+} -dependent calmodulin- or actin-binding 'flip-flop' proteins reported by Sobue *et al.* (1983). Indeed, one of their proteins has an M_r value identical with that of α -actinin. It is unlikely that calmodulin is a direct binding partner for the placental α -actinin, since its binding characteristics are unaffected by trifluoperazine.

It is likely that, at low cytoplasmic concentrations of Ca^{2+} , the microvillar α -actinin acts as an actin-cross-linking protein, reinforcing the cytoskeleton. However, if the cytoplasmic Ca^{2+} concentration were to rise locally, perhaps in response to ligand-receptor interactions at the plasma membrane, some of the α -actinin would be

released. Although the physiological significance of this is not clear, in view of the considerable endocytic activity occurring at this plasma membrane, it is likely that it has some role in allowing the microvillus to accommodate the events of endocytosis.

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