

Identification and characterization of tropomodulin and tropomyosin in the adult rat lens

Mary K. Woo and Velia M. Fowler*

Department of Cell Biology, The Scripps Research Institute, North Torrey Pines Road, La Jolla, CA 92037, USA

*Author for correspondence

SUMMARY

The lens fiber cells express all the major components of the erythrocyte membrane skeleton including spectrin, protein 4.1 and ankyrin. We have used immunoblot and immunoprecipitation analyses, as well as immunofluorescence localization to identify and characterize two additional components of the membrane skeleton in the rat lens: tropomyosin and the tropomyosin-binding protein tropomodulin. In the erythrocyte, tropomyosin and tropomodulin are proposed to stabilize and limit the lengths of the short actin filaments of the spectrin-actin network, thus influencing the organization and mechanical properties of the erythrocyte membrane skeleton.

Antibodies directed against erythrocyte tropomodulin specifically recognize a M_r 43,000 polypeptide from rat lens that comigrates with erythrocyte tropomodulin on SDS-gels. A non-muscle isoform of tropomyosin is also present in the lens. This tropomyosin isoform migrates on SDS-gels with a M_r of ~28,000 and is distinct from the two erythrocyte isoforms of tropomyosin (M_r 27,000 and 29,000). Indirect immunofluorescence staining of 5 μ m cryosections of adult rat lens reveals that both tropomodulin and tropomyosin colocalize with rhodamine phalloidin staining for actin filaments on fiber cell plasma membranes. Lens tropomodulin exhibits many characteristics that are similar to its erythrocyte counterpart. For example, lens tropomodulin binds tropomyosin in a solid-phase blot

binding assay, and extraction experiments with Triton X-100, urea and NaOH show that the membrane-bound tropomodulin in the lens is a tightly associated peripheral membrane protein that is a component of the Triton-insoluble cytoskeleton. However, unlike the erythrocyte, there are approximately 2000 actin monomers per tropomodulin in the lens. This differs greatly from the 16/1 ratio seen in the erythrocyte, where actin, tropomodulin and tropomyosin associate to form the short actin filaments in the membrane skeleton. In addition, we have identified a large soluble pool of actin and tropomodulin in the lens, which does not exist in erythrocytes. These differences imply the presence of distinct pools of cytoskeletal components in the lens. The membrane-bound portion of tropomodulin and tropomyosin may associate with that portion of lens actin that is associated with spectrin and protein 4.1 to form a membrane skeleton in the lens fiber cell, whereas the soluble pools of tropomodulin and actin may serve as precursors necessary for rapid assembly of the membrane skeleton during fiber cell elongation. The membrane skeleton may play a role in the maintenance of cell shape and deformability during visual accommodation as well as in the generation of plasma membrane domains during fiber cell differentiation.

Key words: lens, cytoskeleton, actin, tropomodulin, tropomyosin

INTRODUCTION

Underlying the plasma membrane of many eukaryotic cell types is a cytoskeletal meshwork referred to as the membrane skeleton (for recent reviews see Bennett, 1990; Bennett and Lambert, 1991; Luna and Hitt, 1993; Mangeat, 1988). The molecular organization and function of the membrane skeleton has been best defined in the human erythrocyte where it provides structural support to maintain the biconcave shape of these cells and confers upon them unique deformability properties that are essential for their survival in the exceedingly small apertures of the capillary bed as well as in the high shear environment of the aorta. The components of the membrane skeleton are arranged in a lattice-like hexagonal network where spectrin tetramers radiate outward like spokes from central

hubs composed of short actin filaments and actin-associated proteins (Bennett, 1989). Two tropomyosin (TM) molecules are associated with each short actin filament, one in each groove of the filament (Fowler and Bennett, 1984a). TM is proposed to mechanically stabilize the actin filaments and also to regulate the number of spectrin molecules bound to each actin filament, thus influencing the hexagonal organization of the spectrin-actin lattice (Fowler and Bennett, 1984b; Shen et al., 1986; for a recent review see Bennett, 1989).

A new 40.6 kDa, TM-binding protein, tropomodulin (Tmod) (Fowler, 1987; Sung et al., 1992) has been localized recently to the short actin filaments (Ursitti and Fowler, 1994). Tmod is proposed to limit the lengths of the actin filaments based on its ability to block elongation and depolymerization at the slow-growing (pointed) ends of actin filaments, together with its

ability to bind to one end of TM and block TM head-to-head association along actin filaments (Fowler, 1990; Fowler et al., 1993; A. Weber, G. Babcock, C. Pennise and V. Fowler, unpublished data). Stoichiometric analysis supports this hypothesis, since there are about 2.8 Tmod molecules and two TM molecules for every 12-16 actin monomers (the number of monomers making up the length of a short actin filament in the erythrocyte) (Fowler and Bennett, 1984a; Fowler, 1987; Fowler et al., 1993). Restriction of actin filament length is one mechanism by which the hexagonal organization of the spectrin-actin membrane skeleton could be regulated.

The spectrin-based membrane skeleton plays a diverse role in non-erythroid cells, reflecting the greater complexity of their plasma membrane domains as well as the functional diversity of the cell types in which these components have been identified (Bennett, 1990; Bennett and Lambert, 1991; Luna and Hitt, 1992; Morrow, 1989). The lens fiber cells are no exception and express a host of cytoskeletal components that are identical or related to those found in other tissue types. Isoforms of the erythrocyte membrane skeleton components: spectrin, protein 4.1, 4.9 (dematin) and adducin as well as proteins that link the membrane skeleton to the membrane: ankyrin and band 3, are expressed in the lens, suggesting that a membrane skeleton similar to that found in the erythrocyte may function to maintain membrane stability in the lens (Allen et al., 1987; Aster et al., 1984; Faquin et al., 1988; Granger and Lazarides, 1984; Green and Maisel, 1984; Kaiser et al., 1989; Lehto and Virtanen, 1983; Repasky et al., 1982).

To investigate further the molecular organization and function of the membrane skeleton in the lens, we have identified and characterized two additional components of the membrane skeleton in the lens: tropomodulin and tropomyosin. Their location at the plasma membrane of the lens fiber cells suggests they may play a role in regulating the organization of the membrane skeleton in this tissue. The identification of Tmod and TM in the lens adds to the number of membrane skeletal components identified in the lens, and supports the notion that the membrane skeleton of the lens may be similar structurally and functionally to that of the erythrocyte. However, in contrast to the erythrocyte, there is a large soluble pool of actin and Tmod in the lens and the ratio of actin to Tmod in the lens differs greatly from that of the erythrocyte. These observations suggest that there may be at least two distinct populations of actin and Tmod in the lens, which provide different cellular functions.

MATERIALS AND METHODS

Materials

Chemicals and reagents used in this study were obtained from Sigma (St Louis, MO) or Fisher (Pittsburgh, PA) unless specified.

Antibodies

Affinity-purified antibodies to human erythrocyte Tmod or TM were prepared as described previously (Fowler, 1990; Fowler et al., 1993; Ursitti and Fowler, 1994). Antiserum against γ actin and antiserum against β actin were generous gifts from J. C. Bulinski (Columbia University, New York, NY). Since the lens contains both β and γ actin, these individual antisera were mixed for use in our immunoblot experiments. Anti-bovine MP26 was a generous gift from Nalin Kumar (The Scripps Research Institute, La Jolla, CA).

Immunofluorescence staining

Adult rat lenses were frozen at -20°C in embedding compound (OCT, Tissue Tek, Miles, Elkhart, IN). Sections ($5\ \mu\text{m}$) were cut using a Leica-Reichert-Jung Cryocut 1800 at -15°C and processed for indirect immunofluorescence staining according to Gregorio et al. (1988). Briefly, sections were melted onto chromogel (1 mM chromium potassium sulphate dodecahydrate, 0.5% gelatin)-coated coverslips, fixed in 2.0% formaldehyde in PBS and stored at 4°C in PBS-azide. Prior to incubation with the primary antibodies, the sections were permeabilized for 5 minutes in 0.2% Triton X-100, followed by incubation for 20 minutes in 2% BSA/PBS-azide to block non-specific binding of antibodies. For immunolocalization of Tmod, the sections were incubated with $10\ \mu\text{g}/\text{ml}$ affinity-purified anti-erythrocyte Tmod antibodies or pre-immune IgG for 40 minutes in 0.05% Triton. After a 30 minute wash in PBS-azide, the sections were co-incubated for 20 minutes with fluorescein-conjugated goat anti-rabbit IgG (1:200) (Boehringer Mannheim, Indianapolis, IN) together with rhodamine-labelled phalloidin (1:200) (Molecular Probes, Eugene, Oregon) to detect filamentous actin. The coverslips were then washed for 45 minutes in PBS-azide before mounting on glass slides with mounting medium (40 mM Tris-HCl, pH 8.0, 75% glycerol, 1% *p*-phenylenediamine). Since the staining with affinity-purified anti-erythrocyte TM antibodies was much weaker than the staining with anti-Tmod antibodies, for immunolocalization of TM an additional antibody step was added to enhance the signal. Specifically, after incubation with the rabbit anti-erythrocyte TM antibody the sections were incubated with unconjugated goat anti-rabbit IgG (1:200) (Boehringer Mannheim, Indianapolis, IN) followed by co-incubation with fluorescein-conjugated donkey anti-goat antibodies (1:200) (Boehringer Mannheim, Indianapolis, IN), together with rhodamine-labelled phalloidin as described above. Immunofluorescence staining was viewed with a Zeiss Axiophot fluorescence microscope using a $63\times$ oil immersion PlanApo objective with the appropriate barrier filters and photographed using T-MAX ASA 400 film with an automatic camera.

Isolation and extraction of lens plasma membranes

Lenses were dissected from the eyes of adult (9 week) male Lewis rats. For most experiments, the lens nucleus was removed immediately and discarded, while the remaining capsule, epithelial and cortical fiber cells were kept on ice or quick frozen in liquid nitrogen before use. All procedures were performed at 0°C in the presence of the following protease inhibitors: PMSF ($100\ \mu\text{g}/\text{ml}$), aprotinin ($1\ \mu\text{g}/\text{ml}$), leupeptin and pepstatin A ($5\ \mu\text{g}/\text{ml}$ each) and tosyl-L-lysyl chloromethyl ketone ($100\ \mu\text{g}/\text{ml}$). Lenses were washed in PBS, then homogenized on ice using a Dounce homogenizer in lens buffer (100 mM NaCl, 20 mM Hepes, pH 7.4, 2 mM MgCl_2 , 1 mM EGTA, 1 mM DTT) at 4 lenses/ml ($\sim 30\ \text{mg}/\text{ml}$). The resulting homogenate was centrifuged at $30,000\ g$ for 20 minutes at 4°C . The supernatant was removed and the pellet was resuspended to the initial volume in lens buffer using a glass stir rod followed by vortexing. The centrifugation step was then repeated, followed by two more rounds of resuspension and centrifugation. The resulting pellet contained the washed plasma membranes. The first supernatant was subjected to a high-speed spin of $100,000\ g$ for 2 hours at 4°C to yield a $100,000\ g$ supernatant and pellet. Samples of the total extract, $30,000\ g$ supernatant and pellet, and $100,000\ g$ supernatant and pellet, each representing half a lens, were removed and combined with an equal volume of $2\times$ SDS sample buffer (Laemmli, 1970), sonicated extensively with a microprobe sonicator and boiled for 5 minutes. The gel samples were then stored at -20°C until use. In the case of the MP26 immunoblot analysis, the samples were not boiled prior to gel electrophoresis as this resulted in the aggregation of the MP26 into an insoluble complex (Wong et al., 1978). The proteins were separated by SDS-gel electrophoresis as described below.

Immunoprecipitation, electrophoresis and immunoblotting

Immunoprecipitation of Tmod and TM from whole lens extracts was

performed as described by Fowler et al. (1993). After immunoprecipitation, the proteins were solubilized by boiling in SDS sample buffer and electrophoresed on a 7.5% to 15% linear gradient acrylamide gel (Laemmli, 1970). The gel was stained with Coomassie Blue or transferred to nitrocellulose and then incubated with the appropriate antibody, followed by ^{125}I -Protein A as described (Fowler, 1990; Fowler et al., 1993).

Determination of the quantity of Tmod, TM and actin in the lens

Tmod

Tmod was quantitatively immunoprecipitated from duplicate samples of 40 adult rat lenses with 40 μg of affinity-purified anti-erythrocyte Tmod antibodies. The immunoprecipitated proteins were separated by SDS-PAGE and then stained with Coomassie Blue. The amount of Tmod in each lane was determined by scanning densitometry using an LKB Ultrascan densitometer, by comparison with known amounts of purified erythrocyte Tmod (Fowler, 1990) electrophoresed on the same gel.

TM

TM was immunoprecipitated from duplicate samples of 4 adult rat lenses with 30 μg of affinity-purified anti-erythrocyte TM antibodies. The proteins were separated by SDS-PAGE along with a standard curve of purified erythrocyte TM (Fowler and Bennett, 1984a) and transferred to nitrocellulose. The portion of the blot containing the TM was probed with 3 $\mu\text{g}/\text{ml}$ anti-erythrocyte TM antibodies followed by ^{125}I -Protein A. The amount of TM in each sample was determined by cutting out and counting the labelled band in a γ -counter (Beckman) and corrected for nonspecific binding as described (Fowler, 1987).

Actin

Duplicate samples representing 1/100th of a whole adult rat lens extract were separated by SDS-PAGE along with a standard curve of purified rabbit skeletal muscle actin as described above. The band representing actin was well separated and easily detected on the Coomassie Blue-stained gel and was confirmed to be actin by immunoblot analysis of a duplicate lane of total lens extract. The amount of actin in the total extract samples was determined in two ways: (1) the Coomassie-stained gel was scanned using a video scanning module (Sony) and saved as an Image (NIH version 1.47) file on a Macintosh computer (Apple Computers). The density of each band was then determined using this software. (2) The actin bands were excised from the Coomassie-stained gel followed by pyridine elution of the Coomassie stain (Fenner et al., 1975). The amount of eluted stain in each sample was determined by spectrophotometry at A_{605} .

RESULTS

Identification of immunoreactive isoforms of Tmod and TM in the lens

Fig. 1 demonstrates that anti-erythrocyte Tmod antibodies specifically recognize a single polypeptide on immunoblots of whole adult rat lens extracts, which comigrates with erythrocyte Tmod (Fig. 1, lanes b and c). Anti-erythrocyte TM antibodies also specifically recognize a single polypeptide on blots of material enriched for TM by immunoprecipitation from whole lens extracts with anti-TM antibodies (Fig. 1, lane e). Lens TM migrates on SDS-PAGE as a single band of $\sim M_r$ 28,000 (lane e) similar to some non-muscle isoforms of TM (Matsumura et al., 1983) but different from both of the erythrocyte TM isoforms, which migrate at M_r 27,000 and 29,000

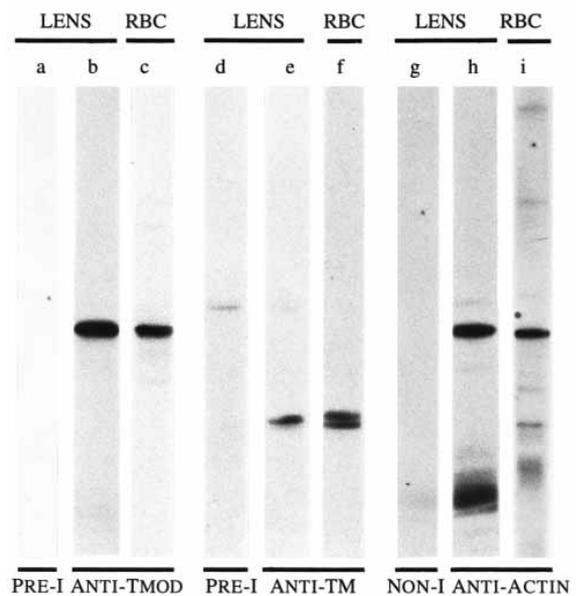
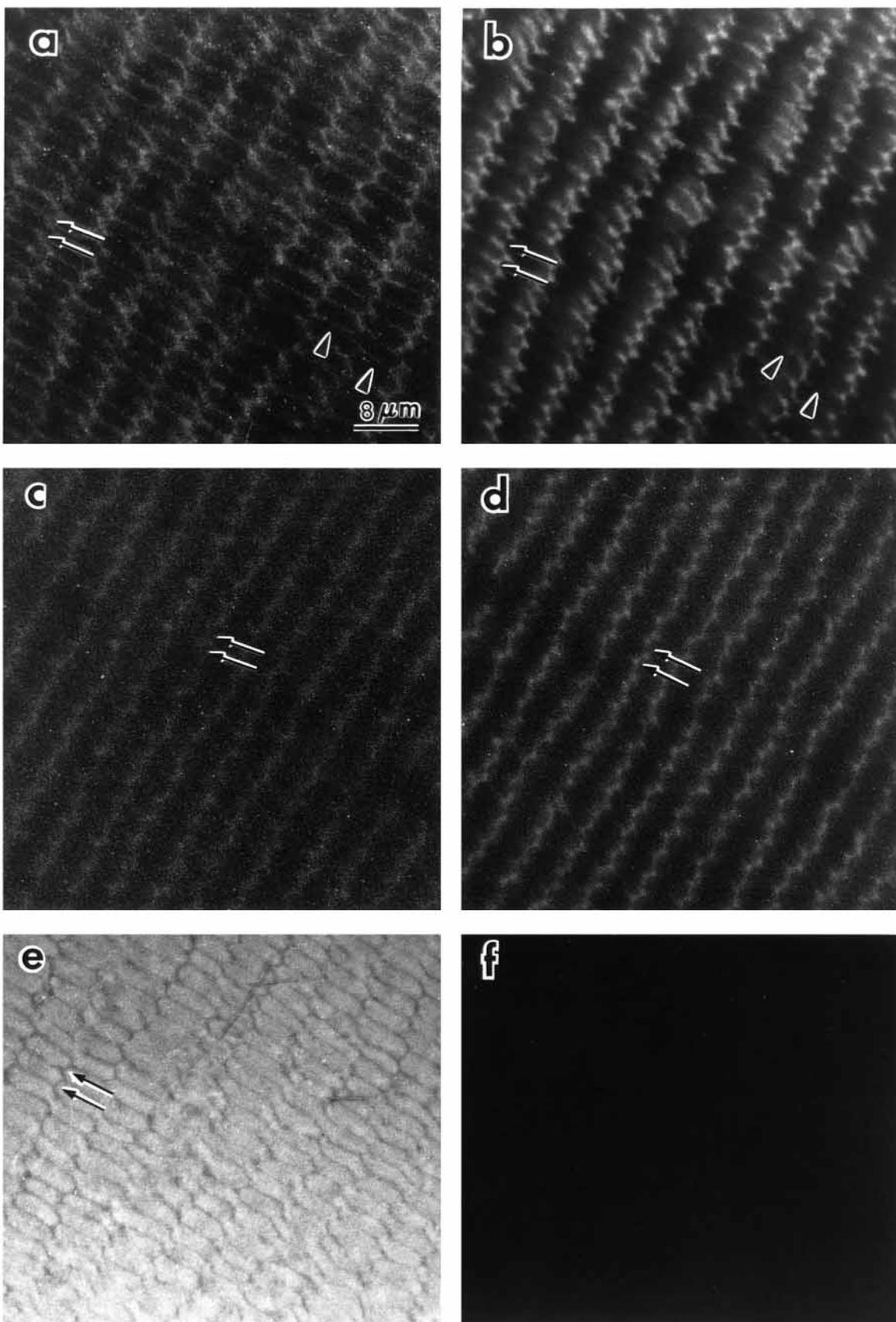


Fig. 1. Identification of Tmod, TM and actin in adult rat lens. Tmod and actin: nitrocellulose strips of whole lens extract (1/20th lens per lane) or erythrocyte membranes (20 μl per lane) were probed with pre-immune IgG (lane a), non-immune IgG (lane g), anti-erythrocyte Tmod antibodies (lanes b and c), or antisera against β and γ actin (lanes h and i) followed by ^{125}I -labelled Protein A. TM: nitrocellulose strips of immunoprecipitated TM from whole rat lens extracts were probed using pre-immune (lane d) or anti-erythrocyte TM antibodies (lane e) and a nitrocellulose strip of erythrocyte membrane proteins was probed with anti-erythrocyte TM antibodies (lane f), followed by ^{125}I -labelled Protein A.

(lane f) (Fowler and Bennett, 1984). We were able to detect lens TM only after immunoprecipitating the TM from whole lens extracts prior to immunoblot analysis. This was necessary because in our hands TM antibodies are less sensitive than either the Tmod antibodies or the actin antisera. This may be due to weak cross-reactivity of TM antibodies with lens TM or to a very low amount of TM in the lens. We also were unable to detect TM on immunoblots of whole lens extracts using anti-chicken gizzard TM antibodies or anti-rabbit muscle TM antibodies (data not shown). The mixture of anti- β - and γ -actin antisera used in our experiments also specifically recognizes a single polypeptide band on immunoblots of whole lens extracts (Fig. 1, lane h). It has previously been shown that both the β and γ isoforms of actin are present in the lens (Mousa and Trevithick, 1979). Pre-immune or non-immune IgG do not label any of these polypeptides (lanes a, d and g).

Immunofluorescence localization of Tmod, F-actin and TM in cryosections of rat lens

To characterize lens Tmod and TM further we used indirect immunofluorescence staining to compare the subcellular localization of these proteins with respect to filamentous actin (stained with rhodamine-phalloidin). In Fig. 2, 5 μm frozen sections of adult rat lenses were doubly stained for Tmod and F-actin or for TM and F-actin. Both Tmod and TM exhibit membrane-associated staining that colocalizes with the F-actin staining in lens fiber cells (Fig. 2, compare arrows in panels a



and c with panels b and d). As previously reported (Kibbelaar et al., 1980), the intensity of F-actin staining appears to be greatly enhanced at the angles and short sides of the hexagonal fiber cells (arrows). A slightly higher intensity of staining for Tmod and TM was also observed in this region (Fig. 2a and c; arrows). In contrast to the actin staining, however, the Tmod and TM staining appears to be somewhat more evenly distributed about the whole plasma membrane as revealed by the staining on the longer sides of the hexagons (Fig. 2, compare arrowheads a and b).

Subcellular distribution of Tmod and actin

In order to examine the subcellular distribution of Tmod and actin in the lens, we prepared lens membranes by three successive rounds of homogenization in physiological salt buffer followed by centrifugation at 30,000 g. This buffer contains 2 mM MgCl₂, which is sufficient to retain the tropomyosin on the membrane (Fowler and Bennet, 1984a). The proteins in each fraction were separated by SDS-PAGE, transferred to nitrocellulose, and then probed with anti-erythrocyte Tmod antibodies or with a mixture of antisera directed against the γ and β isoforms of non-muscle actin followed by ¹²⁵I-Protein A. It was not feasible to examine the subcellular distribution of TM by this method due to the negligible TM signal detected on immunoblots of whole lens extracts (see above). A representative Coomassie Blue-stained gel of these fractions and the corresponding immunoblots are presented in Fig. 3A and B. A quantitative measure of the level of Tmod or actin associated with each fraction was obtained by excising the radiolabelled bands from the nitrocellulose filters and counting them in a γ counter. The results of this experiment show that 38±8% of lens Tmod and 32±9% of lens actin is associated with the pellet fraction under these extraction conditions, while the remainder of these components is found in the first supernatant. The second and third washes with physiological salt buffer contained no detectable Tmod or actin (data not shown).

The Tmod and actin found in the 30,000 g supernatant fraction are not associated with small membrane vesicles, since all of the fiber cell-specific, integral membrane protein, MP26, is found in the 30,000 g pellet fraction (Fig. 3B, MP26 panel). Furthermore, the Tmod in the 30,000 g supernatant is not associated with short actin filaments or with large aggregates, since Tmod and actin are not found in the pellet fraction even after centrifugation at 100,000 g for 2 hours (Fig. 3B). When low ionic strength buffer was used in the initial homogenization instead of physiological salt, the proportion of Tmod and actin

found associated with the membranes in the pellet fraction remained unchanged at 30-40% (data not shown).

Extraction of Tmod and actin from lens membranes

To examine the nature of the interaction of actin and Tmod with lens plasma membranes, the 30,000 g pellet fraction obtained in the previous experiment was extracted with a variety of salt, detergent and pH conditions followed by recentrifugation at 30,000 g. The resulting fractions were probed by

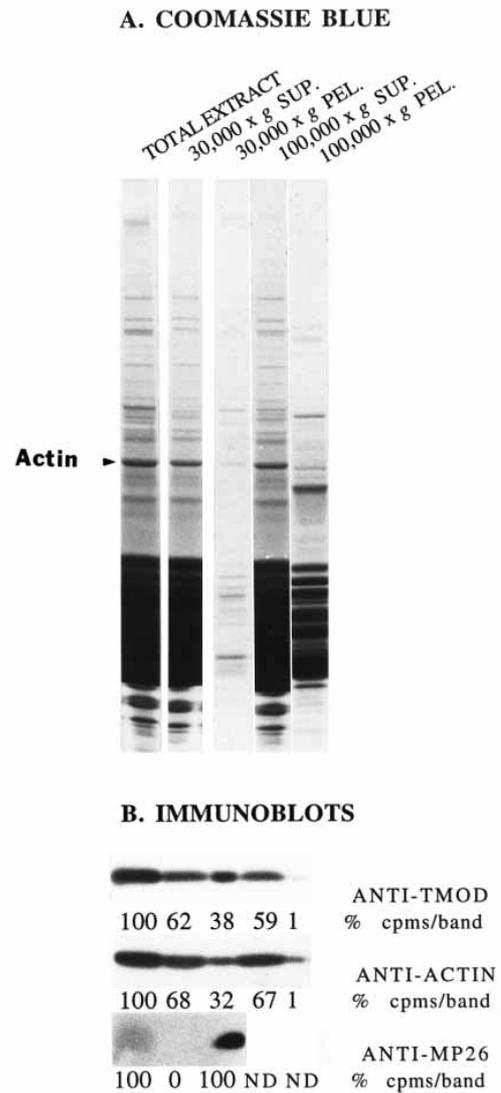


Fig. 3. Subcellular distribution of Tmod and actin in the lens. Subcellular fractions of the capsule and cortex layers of 10 adult rat lenses were examined for the presence of Tmod, actin and MP26. (A) Coomassie Blue-stained SDS-polyacrylamide gel of the subcellular fractions (1/30th lens per lane). (B) Immunoblot analysis of the fractions probed with either anti-erythrocyte Tmod antibodies, anti- β - and - γ -actin antisera, or anti-bovine MP26 antibodies followed by ¹²⁵I-Protein A. The percentage of Tmod, actin or MP26 found in each fraction is indicated below the labelled bands. Except for the 100,000 g supernatant and pellet fractions, the percentages for Tmod are the average of 8 experiments, the percentages for actin are the average of 6 experiments and the percentages for MP26 are the average of 3 experiments. The values for the 100,000 g supernatant and pellet fractions are an average of 3 experiments.

Fig. 2. Tmod and TM colocalize with F-actin on the fiber cell membranes of adult rat lenses. Immunofluorescence microscopy of frozen sections of adult rat lenses using antibodies to erythrocyte Tmod (a), erythrocyte TM (c), or using rhodamine-phalloidin to visualize F-actin (b and d). The phase-contrast micrograph in e corresponds to the labelled section in a and b. Note the hexagonal pattern of the fiber cell membranes, which is characteristic of a section through the cortical region of the lens that is perpendicular to the anterior/posterior axis. The non-immune control is shown in f. Note that the intensity of staining on the angles and short sides of the hexagonal fiber cells (thin arrows) is greater than the staining on the long sides (arrowheads). TM staining was also present on the long sides of the hexagons but was too dim to reproduce on the micrograph.

Table 1. Extractability properties of Tmod and actin from lens membranes

Condition	Tmod (%)		Actin (%)	
	Pellet	Sup	Pellet	Sup
9.2 M urea	20	80	20	80
1% Triton	85	15	93	7
1 M NaCl	70	30	80	20
0.1 M NaOH (pH 12)	60	40	62	38
2 mM NaCarb (pH 10)	43	57	93	7

The membrane pellet from 40 rat lenses (capsules and cortexes) was divided into five tubes and extracted by several strokes with a Dounce homogenizer under the indicated conditions at 4°C, except for the urea extraction, which was performed at room temperature. The extracts were incubated on ice less than 10 minutes before centrifugation at 30,000 *g*. The resulting supernatant and pellet fractions were analyzed by immunoblot analysis as described in Materials and Methods. The results are the average of data obtained from three separate experiments, except the NaOH results, which are the average of data obtained from two experiments. NaCarb, sodium carbonate. Extraction conditions: 9.2 M urea, in double-distilled water (ddH₂O); 1% Triton X-100, in lens buffer; 1 M NaCl, in lens buffer at final NaCl of 1 M; 0.1 M NaOH, in ddH₂O; 2 mM NaCarb, in ddH₂O.

immunoblot analysis for the presence of Tmod and actin and the results were quantified as described above. Treatment with 9.2 M urea solubilized greater than 80% of both the Tmod and actin, while essentially all of the Tmod and actin were found in the membrane pellet after extraction with 1% Triton X-100 (Table 1). These results are similar to previous findings in the erythrocyte and confirm that the membrane-associated actin and Tmod are peripheral membrane proteins that are associated with the Triton-insoluble cytoskeleton (Fowler, 1987).

To investigate further the extractability properties of Tmod and actin from the lens plasma membranes, the lens membranes were also extracted with either 1 M NaCl, 2 mM sodium carbonate (pH 10) or 0.1 M NaOH (pH 12), followed by centrifugation at 30,000 *g*. MP26 is found only in the pellet fractions after these treatments (data not shown), confirming that all of the membranes were pelleted at 30,000 *g*. Only 30% of the membrane-associated Tmod, and 20% of the membrane-associated actin was solubilized from the membranes by extraction with 1 M NaCl, while extraction with 0.1 M NaOH at pH 12 released about 40% of both the membrane-associated Tmod and actin from the lens membranes (Table 1). Thus, these results suggest that both Tmod and actin are tightly associated with the plasma membrane. Interestingly, sodium carbonate at pH 10 solubilized 60% of the membrane-associated Tmod but only 7% of the membrane-associated actin from the pellet (Table 1). This implies that the actin is more tightly associated with the membranes than is Tmod and is consistent with the notion that actin filaments could be attached to a greater number of different membrane binding sites than Tmod. We cannot rule out, however, the possibility that the residual Tmod and/or actin associated with the pellet fractions after some or all of these treatments could be due to the presence of inaccessible membrane vesicle compartments (i.e. inside-out vesicles) (Allen et al., 1987; Hubbard and Ma, 1983).

¹²⁵I-TM binding to lens Tmod

To determine whether rat lens Tmod binds TM, we performed a ¹²⁵I-TM blot overlay experiment on the immunoprecipitated lens Tmod. This method was used previously to characterize

TM-Tmod interactions and is both sensitive and specific (Fowler, 1987; Fowler et al., 1993; Sussman and Fowler, 1992). Fig. 4 shows that ¹²⁵I-erythrocyte TM binds to lens Tmod. In lanes 1 and 2, duplicate Coomassie Blue-stained lanes show the results of immunoprecipitations of Tmod from 40 rat lenses demonstrating the specificity of the anti-erythrocyte Tmod antibodies. Lane 3 shows the results of a control immunoprecipitation using pre-immune IgG. In lane 4, the immunoprecipitated Tmod was blotted to nitrocellulose and probed with ¹²⁵I-labelled TM. The specificity of TM binding to lens Tmod is demonstrated by competition with a 40-fold molar excess of unlabelled TM, which inhibits the TM binding to lens Tmod by 80% (Fig. 4, lane 5). When pre-immune IgG is substituted for anti-Tmod antibodies, no radiolabelled bands are detected (lane 6).

Determination of the quantity of Tmod, TM and actin in the lens

To determine the amount of Tmod and TM per adult rat lens, we analyzed quantitative immunoprecipitations from whole lens extracts either by densitometry of Coomassie Blue-stained gels (Tmod) or by immunoblot analysis followed by quantitation using a γ -counter (TM). The immunoprecipitates were co-electrophoresed on the same gel as a standard curve of purified erythrocyte Tmod or TM. To ensure that all of the proteins were completely immunoprecipitated from the samples, the extracts were subjected to a second round of immunoprecipitation using a fresh sample of antibodies. No additional Tmod or TM was detected in this second round of immunoprecipitations either by Coomassie Blue stain or by immunoblot analysis with the appropriate antibodies (data not shown). From the standard curves, the amount of Tmod per rat lens was determined to be 20 ng and the amount of TM per lens was determined to be 13 ng (Table 2). However, the value for the amount of TM in the lens may be underestimated if the erythrocyte TM antibodies do not cross-react as well with the rat lens TM as with the erythrocyte TM. The amount of Tmod per lens is calculated directly from Coomassie Blue staining and is not subject to this uncertainty.

We also determined the ratio of actin to Tmod in the lens. The amount of actin per lens was determined by densitometry of Coomassie Blue-stained gels of total lens extract and comparison to an actin standard curve (data not shown). The actin band was easily detected on the Coomassie Blue-stained gels of whole lens extracts (see Fig. 3, total extract). These results show that there are 45 μ g of actin per lens, which is roughly 1% of the total protein as has been previously determined by others (Mousa and Trevithick, 1979).

DISCUSSION

We report here the identification of Tmod and TM in the adult rat lens and show by immunofluorescence staining that they colocalize with actin filaments on the plasma membrane of fiber cells. This staining pattern is as expected for a membrane-skeleton-associated protein in the lens and is similar to previous results obtained for lens spectrin, band 4.1, adducin and band 4.9 (dematin) (Faquin et al., 1988; Granger and Lazarides, 1984; Green and Maisel, 1984; Kaiser et al., 1989). Interestingly, in cross-sections of the rat lens, we observed an

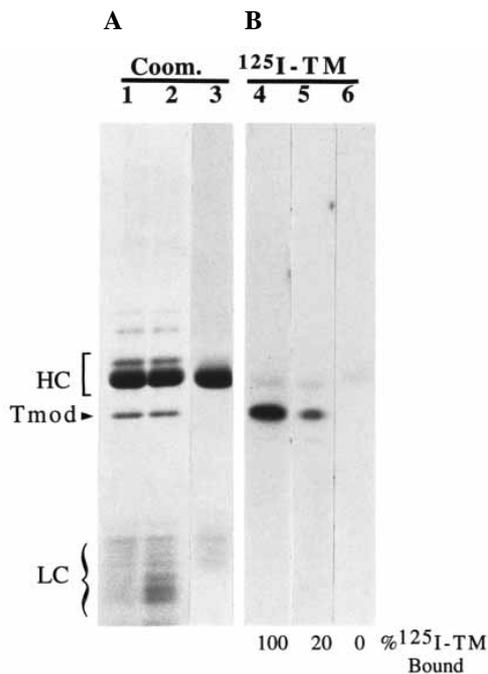


Fig. 4. Lens Tmod is a TM-binding protein. (A) Coomassie Blue-stained gel of proteins immunoprecipitated from 40 adult rat lenses per lane. Lanes 1 and 2, duplicate immunoprecipitations using anti-erythrocyte Tmod antibodies (0.8 µg Tmod/lane). Lane 3, Control immunoprecipitation using pre-immune IgG. (B) Autoradiograph of ^{125}I -erythrocyte TM blot binding to Tmod immunoprecipitated from extracts adult rat lenses (~50 ng Tmod/2.5 lenses per lane). Lane 4, incubation with 0.5 µg/ml ^{125}I -erythrocyte TM. Lane 5, incubation with 0.5 µg/ml ^{125}I -erythrocyte TM in the presence of 20 µg/ml unlabelled erythrocyte TM. Lane 6, control immunoprecipitation using pre-immune IgG, incubated with ^{125}I -erythrocyte TM. ^{125}I -TM blot binding was performed as described (Fowler, 1987). The numbers below the strips indicate the relative amount of ^{125}I -erythrocyte TM bound to each strip, determined by excising and counting the labelled bands in a γ -counter. These results are from a representative experiment, which has been repeated twice with similar results.

enrichment in the staining of F-actin, Tmod and TM at the angles and short sides of the hexagonally shaped fiber cells. In previous studies, actin has been shown to be concentrated at the angles and short sides of the fiber cells in rat lens (Kibbelaar et al., 1979, 1980). Re-examination of published immunofluorescence micrographs reveals that both band 4.1 and spectrin also appear to exhibit increased staining in these regions (Granger and Lazarides, 1984; Green and Maisel, 1984; Repasky et al., 1982). Enrichment of these proteins at the angles and short sides of the fiber cells is significant because ultrastructural analysis of lens fiber cells has revealed the angles and short sides to be the location of adherens junctions (Benedetti et al., 1976; Kistler and Bullivant, 1989). Furthermore, the lens cadherin, A-CAM (a marker for adherens junctions), appears to colocalize with F-actin in these regions of the fiber cell membrane (Geiger et al., 1985). Alternatively, the increased staining intensity of these proteins at the angles and the short sides of the hexagons could be due to an increase in membrane surface area due to the ball-and-socket interdigitations present in this region (Kistler and Bullivant, 1989).

The portion of Tmod and actin that is found associated with

Table 2. Amount of Tmod, TM and actin per adult rat lens

	No. lenses	ng§	ng/lens
Tmod*	40	800	20
TM†	4	52	13
Actin‡	1/100	450	4500

*The amount of Tmod immunoprecipitated from extracts of 40 lenses was determined by Coomassie Blue staining and comparison to a standard curve of purified erythrocyte Tmod.

†The amount of TM immunoprecipitated from extracts of four lenses and was determined by immunoblot analysis and comparison to a standard curve of purified erythrocyte TM.

‡The amount of actin in 1/100 of a whole lens extract was determined by Coomassie Blue staining and comparison to a standard curve of purified rabbit muscle actin.

§Values are the average of duplicate determinations.

the lens plasma membranes is largely Triton X-100-insoluble, indicating that the membrane Tmod and actin are stably associated with the cytoskeleton. Furthermore, these components can be solubilized from the membrane pellet by extraction with urea, which identifies them as peripheral membrane components. Previous studies have also shown similar extractability properties for lens actin, band 4.1, spectrin and ankyrin (Allen et al., 1987; Granger and Lazarides, 1985; Kibbelaar et al., 1979; Nelson and Veshnock, 1986; Repasky et al., 1982). Tmod and actin appear to interact tightly with the plasma membranes as demonstrated by the results of our extraction experiments with high salt, low ionic strength buffers and high pH (Table 1). The extractability of Tmod from the lens plasma membranes is similar to the results obtained by others for spectrin and 4.1, except that, overall, a greater percentage of the Tmod remains associated with the membranes in our study. This difference could be explained by the fact that our extractions were performed by brief Dounce homogenization on ice rather than by incubation overnight or at 37°C, as was the case for many of the previous studies. We were concerned about the stability of Tmod and therefore chose a more rapid method of extraction. In general, the results of our experiments and those of others indicate that Tmod and the spectrin-based membrane skeleton in the lens are tightly associated with the plasma membrane of the lens fiber cells.

This study supports the hypothesis that a membrane skeleton similar in nature to that of the erythrocyte exists in the lens. To date, all of the components identified in the membrane skeleton of the erythrocyte have been identified in the lens. Furthermore, their localization on the lens fiber cell plasma membrane supports the hypothesis that they may form a membrane skeleton similar to that found in the erythrocyte. Indeed, a membrane skeleton in the lens may provide a similar function to that in the erythrocyte, providing mechanical stability to the plasma membrane and contributing to its elastic properties. These functions could be important in the lens fiber cells as the lens deforms during visual accommodation.

Assuming that the erythrocyte TM antibodies cross-react equally well with lens TM we calculate the stoichiometry of lens Tmod to lens TM to be 2.3:1, which would be similar to that found in the erythrocyte where 1.4 Tmod molecules are present for each TM (Fowler, 1987; Fowler et al., 1993). However, comparison of the amount of Tmod to that of actin in the lens gives a stoichiometry of ~2000 actin monomers to 1 Tmod. On the basis of the extraction results presented in Fig.

3, the stoichiometry of membrane-associated actin to Tmod is 1700:1; similar to that calculated for the total lens, suggesting that the majority of the actin is assembled into long filaments that are not associated with Tmod. This is considerably different from the situation found in the erythrocyte where the stoichiometry of 12-16 actin monomers to 1-2 Tmods is consistent with the association of 1 or 2 Tmod molecules with the end of each short actin filament (Fowler, 1987; see footnote 1, Fowler et al., 1993). This result suggests that not all of the actin filaments are associated with Tmod, and therefore that most of the actin is not organized into short actin filaments as it is in the erythrocyte. Ultrastructural analysis confirms that there are relatively long actin filaments extending into the cytoplasm of lens fiber cells (Ireland and Maisel, 1983; Mousa and Trevithick, 1979; Rafferty et al., 1979), as well as filaments organized into polygonal arrays that closely appose the membranes at the epithelio-fiber cell junction (Rafferty and Scholz, 1984). The actin found in these structures may account for the high ratio of actin to Tmod found in the lens. Furthermore, lens fiber cell membranes are less uniform in their protein distribution than erythrocyte membranes, having specialized plasma membrane domains, such as adherens junctions and gap junctions, which are more similar to other polarized epithelial cells. Localization of specific integral membrane proteins in these domains could be generated by cytoskeletal elements that attach to and cross-link them into large arrays. For example, immunolocalization studies have revealed a colocalization of F-actin with A-CAM in adherens junctions (Geiger et al., 1985). Therefore, the membrane-associated cytoskeletal components may serve a dual role in the lens fiber cell, one of providing mechanical stability and another of establishing and maintaining polarized domains of integral membrane proteins.

The presence of a large soluble pool of Tmod (60%) and actin (70% of the total) in the lens is similar to previous reports of actin solubility in the lens (Ramaekers et al., 1981) and differs from the erythrocyte where all of the Tmod and essentially all of the actin are found associated with the membrane (Fowler, 1987; Pinder and Gratzner, 1983). Similarly, preliminary subcellular fractionation studies have revealed a soluble pool of spectrin in the lens (M. Woo and V. Fowler, unpublished observations). A soluble pool of some membrane skeletal components has also been observed in other cell types. For example, a soluble pool of spectrin has been identified in non-polarized kidney epithelial cells that have been maintained at a low culture density so as to be 'cell-contact naive'. The soluble pool of spectrin is associated with ankyrin, Na⁺,K⁺-ATPase and other components in a high molecular mass complex. Upon polarization of these cells due to increased cell-cell contact the soluble spectrin, ankyrin and Na⁺,K⁺-ATPase rapidly become Triton-insoluble and are localized to the basolateral membranes (Nelson and Hammerton, 1989; Nelson and Veshnock, 1986, 1987). It is tempting to speculate that some of the soluble Tmod, spectrin and actin in the lens might exist in a high molecular mass complex similar to that described in the kidney epithelial cells. In addition, much of the soluble actin in the lens is in monomeric form (Ramaekers et al., 1981) and may be sequestered by such proteins as profilin or thymosin β 4 (proteins that prevent actin from polymerizing) as has been found to be the case in many cells and tissues

(Carlsson et al., 1977; Safer et al., 1991; and for reviews see Korn, 1982; Fechheimer and Zigmond, 1993).

Lens fiber cells differentiate from a polarized epithelial cell by a process of dramatic cell elongation and a huge increase in the plasma membrane surface area with respect to the volume of the cell. This elongation is inhibited in cell culture by the addition of cytochalasins to block actin polymerization, suggesting a role for actin filaments in the elongation process (Mousa and Trevithick, 1977; Ramaekers et al., 1982/1983). Rapid assembly of a spectrin-based membrane skeleton may accompany fiber cell elongation in which case a pool of pre-assembled complexes containing spectrin, actin, Tmod and TM as well as other membrane skeleton components might be required to facilitate the assembly process. It would be interesting to determine whether the soluble pool of Tmod, spectrin and actin exists in all fiber cells, or predominantly in those at the equatorial junction between epithelial cells and fiber cells where rapid elongation takes place. Future studies using bovine lenses will be useful in examining this question, since epithelial cell and superficial fiber cell layers can be easily dissected and separated from the more highly differentiated cortical and nuclear fiber cells. Furthermore, lens epithelial cells can be induced to differentiate and elongate in culture, and associations of cytoskeletal or membrane skeleton proteins can be examined by in vitro labelling and cell fractionation experiments.

We thank C. Gregorio for providing assistance and advice during the immunofluorescence labelling experiments, for critical reading of the manuscript, and for many helpful scientific discussions. We also thank N. Kumar and G. Babcock for their useful comments regarding our work. This research was supported by a grant from the NIH (GM34225). V.M.F. is a recipient of an Established Investigator Award from the AHA and M.W. is the recipient of a NRSA post-doctoral fellowship award from the NEI: EY06386.

REFERENCES

- Allen, D. P., Low, P. S., Dola, A. and Maisel, H. (1987). Band 3 and ankyrin homologues are present in eye lens: evidence for all major erythrocyte membrane components in the same non-erythroid cell. *Biochem. Biophys. Res. Commun.* **149**, 266-275.
- Aster, J. C., Welsh, M. J., Brewer, G. J. and Maisel, H. (1984). Identification of spectrin and protein 4.1-like proteins in mammalian lens. *Biochem. Biophys. Res. Commun.* **119**, 726-734.
- Benedetti, E. L., Dunia, I., Bentzel, C. J., Vermorken, A. J. M., Kibbelaar, M. and Bloemendal, H. (1976). A portrait of plasma membrane specializations in eye lens epithelium and fibers. *Biochim. Biophys. Acta* **457**, 353-384.
- Bennett, V. (1989). The spectrin-actin junction of erythrocyte membrane skeletons. *Biochim. Biophys. Acta* **988**, 107-121.
- Bennett, V. (1990). Spectrin-based membrane skeleton: a multipotential adaptor between plasma membrane and cytoplasm. *Physiol. Rev.* **70**, 1029-1065.
- Bennett, V. and Lambert, S. (1991). The spectrin skeleton: from red cells to brain. *J. Clin. Invest.* **87**, 1483-1489.
- Carlsson, L., Nystrom, L. E., Sundkvist, I., Markey, F. and Lindberg, U. (1977). Actin polymerizability is influenced by profilin, a low molecular weight protein in nonmuscle cells. *J. Mol. Biol.* **115**, 465-483.
- Faquin, W. C., Husain, A., Hung, J. and Branton, D. (1988). An immunoreactive form of erythrocyte protein 4.9 is present in non-erythroid cells. *Eur. J. Cell Biol.* **46**, 168-175.
- Fechheimer, M. and Zigmond, S. H. (1993). Focusing on unpolymerized actin. *J. Cell Biol.* **123**, 1-5.
- Fenner, C., Traut, R. R., Mason, D. T. and Wikman-Coffelt, J. (1975). Quantification of Coomassie blue stained proteins in polyacrylamide gels based on analyses of eluted dye. *Anal. Biochem.* **63**, 595-602.

- Fowler, V. M. and Bennett, V.** (1984a). Erythrocyte membrane tropomyosin. *J. Biol. Chem.* **259**, 5978-5989.
- Fowler, V. M. and Bennett, V.** (1984b). Tropomyosin: a new component of the erythrocyte membrane skeleton. In *Erythrocyte Membranes 3: Recent Clinical and Experimental Advances*, pp. 57-71. New York, Alan R. Liss, Inc.
- Fowler, V. M.** (1987). Identification and purification of a novel M_r 43,000 tropomyosin binding protein from human erythrocyte membranes. *J. Biol. Chem.* **262**, 12792-12800.
- Fowler, V. M.** (1990). Tropomodulin: a cytoskeletal protein that binds to the end of erythrocyte tropomyosin and inhibits tropomyosin binding to actin. *J. Cell Biol.* **111**, 471-482.
- Fowler, V. M., Sussman, M. A., Miller, P. G., Flucher, B. E. and Daniels, M. P.** (1993). Tropomodulin is associated with the free (pointed) ends of the thin filaments in rat skeletal muscle. *J. Cell Biol.* **120**, 411-420.
- Geiger, B., Volk, T. and Volberg, T.** (1985). Molecular heterogeneity of adherens junctions. *J. Cell Biol.* **101**, 1523-1531.
- Granger, B. and Lazarides, E.** (1984). Membrane skeletal protein 4.1 of avian erythrocytes is composed of multiple variants that exhibit tissue-specific expression. *Cell* **37**, 595-607.
- Granger, B. L. and Lazarides, E.** (1985). Appearance of new variants of membrane skeletal protein 4.1 during terminal differentiation of avian erythroid and lenticular cells. *Nature* **313**, 238-241.
- Green, J. and Maisel, H.** (1984). Lens fodrin binds actin and calmodulin. *Curr. Eye Res.* **3**, 1433-1440.
- Gregorio, C. C., Hudecki, M. S., Pollina, C. M. and Repasky, E. A.** (1988). Effects of denervation on the concentration of spectrin in avian muscle. *Muscle Nerve* **11**, 372-378.
- Hubbard, A. L. and Ma, A.** (1983). Isolation of rat hepatocyte plasma membranes. II. Identification of membrane-associated cytoskeletal proteins. *J. Cell Biol.* **96**, 230-239.
- Ireland, M. and Maisel, H.** (1983). Identification of native actin filaments in chick lens fiber cells. *Exp. Eye Res.* **36**, 531-536.
- Kaiser, H. W., O'Keefe, E. and Bennett, V.** (1989). Adducin: Ca^{2+} -dependent association with sites of cell-cell contact. *J. Cell Biol.* **109**, 557-569.
- Kibbelaar, M., Selten-Versteegen, E., Dunia, I., Benedetti, E. L. and Bloemendal, H.** (1979). Actin in mammalian lens. *Eur. J. Biochem.* **95**, 543-549.
- Kibbelaar, M. A., Ramaekers, F. C. S., Ringens, P. J., Selten-Versteegen, A. M. E., Poels, L. G., Jap, P. H. K., van Rossum, A. L., Feltkamp, T. E. W. and Bloemendal, H.** (1980). Is actin in eye lens a possible factor in visual accommodation? *Nature* **285**, 506-508.
- Kistler, J. and Bullivant, S.** (1989). Structural and molecular biology of the eye lens membranes. *Crit. Rev. Biochem. Mol. Biol.* **24**, 151-181.
- Korn, E.** (1982). Actin polymerization and its regulation by proteins from nonmuscle cells. *Physiol. Rev.* **62**, 672-737.
- Laemmli, U. K.** (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.
- Lehto, V.-P. and Virtanen, I.** (1983). Immunolocalization of a novel, cytoskeleton-associated polypeptide of M_r 230,000 daltons (p230). *J. Cell Biol.* **96**, 703-716.
- Luna, E. J. and Hitt, A. L.** (1992). Cytoskeleton-plasma membrane interactions. *Science* **258**, 955-964.
- Mangeat, P.-H.** (1988). Interaction of biological membranes with the cytoskeletal framework of living cells. *Biol. Cell* **64**, 261-281.
- Matsumura, F., Yamashiro-Matsumura, S. and Lin, J. J.-C.** (1983). Isolation and characterization of tropomyosin-containing microfilaments from cultured cells. *J. Biol. Chem.* **258**, 6636-6644.
- Morrow, J. S.** (1989). The spectrin membrane skeleton: emerging concepts. *Curr. Opin. Cell Biol.* **1**, 23-29.
- Mousa, G. Y. and Trevithick, J. R.** (1977). Differentiation of rat lens epithelial cells in tissue culture II. Effects of cytochalasins B and D on actin organization and differentiation. *Dev. Biol.* **60**, 14-25.
- Mousa, G. and Trevithick, J.** (1979). Actin in the lens: changes in actin during differentiation of lens epithelial cells in vivo. *Exp. Eye Res.* **29**, 71-81.
- Nelson, W. J. and Hammerton, R. W.** (1989). A membrane-cytoskeletal complex containing Na^+ , K^+ -ATPase, ankyrin, fodrin in madin-darby canine kidney (MDCK) cells: implications for the biogenesis of epithelial cell polarity. *J. Cell Biol.* **108**, 893-902.
- Nelson, W. J. and Veshnock, P. J.** (1986). Dynamics of membrane-skeleton (fodrin) organization during development of polarity in madin-darby canine kidney epithelial cells. *J. Cell Biol.* **103**, 1751-1765.
- Nelson, W. J. and Veshnock, P. J.** (1987). Modulation of fodrin (membrane skeleton) stability by cell-cell contact in madin-darby canine kidney epithelial cells. *J. Cell Biol.* **104**, 1527-1537.
- Pinder, J. C. and Gratzer, W.** (1983). Structural and dynamic states of actin in the erythrocyte. *J. Cell Biol.* **96**, 768-775.
- Rafferty, N., Goosens, W. and Roth, A.** (1979). Cytoplasmic filaments in aging lens of rodents. *Ophthalmol. Res.* **11**, 276-282.
- Rafferty, N. S. and Scholz, D. L.** (1984). Polygonal arrays of microfilaments in epithelial cells of the intact lens. *Curr. Eye Res.* **3**, 1141-1149.
- Raemaekers, F., Boomkens, T. and Bloemendal, H.** (1981). Cytoskeletal and contractile structures in bovine lens cell differentiation. *Exp. Eye Res. Prelim. Notes* **135**, 454-461.
- Ramaekers, F., Jap, P., Mungyer, G. and Bloemendal, H.** (1982/1983). Microfilament assembly during lens cell elongation in vitro. *Curr. Eye Res.* **2**, 169-181.
- Repasky, E. A., Granger, B. L. and Lazarides, E.** (1982). Widespread occurrence of avian spectrin in nonerythroid cells. *Cell* **29**, 821-833.
- Safer, D., Elzinga, M. and Nachmias, V. T.** (1991). Thymosin β_4 and Fx, an actin sequestering peptide, are indistinguishable. *J. Biol. Chem.* **266**, 4029-4032.
- Shen, B. W., Josephs, R. and Steck, T. L.** (1986). Ultrastructure of the intact skeleton of the human erythrocyte membrane. *J. Cell Biol.* **102**, 997-1006.
- Sung, L. A., Fowler, V. M., Lambert, K., Sussman, M. A., Karr, D. and Chien, S.** (1992). Molecular cloning and characterization of human fetal liver tropomodulin: a tropomyosin-binding protein. *J. Biol. Chem.* **267**, 2616-2621.
- Sussman, M. A. and Fowler, V. M.** (1992). Tropomodulin binding to tropomyosins. Isoform-specific differences in affinity and stoichiometry. *Eur. J. Biochem.* **205**, 355-362.
- Ursitti, J. A. and Fowler, V. M.** (1994). Immunolocalization of tropomodulin, tropomyosin and actin in spread human erythrocyte skeletons. *J. Cell Sci.* (in press).
- Wong, M. M., Robertson, N. P. and Horwitz, J.** (1978). Heat induced aggregation of the sodium dodecyl sulfate solubilized main intrinsic polypeptide isolated from bovine lens plasma membrane. *Biochem. Biophys. Res. Commun.* **84**, 158.

(Received 6 December 1993 - Accepted 28 January 1994)