Gelsolin Is Expressed in Early Erythroid Progenitor Cells and Negatively Regulated during Erythropoiesis

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Abstract. We have identified an ~85-kD protein in chicken erythrocytes which is immunologically, structurally, and functionally related to the gelsolin found in many muscle and nonmuscle cell types. Cell fractionation reveals a Ca^{2+}-dependent partitioning of gelsolin into the soluble cytoplasm and the membrane-associated cytoskeleton of differentiating or mature erythrocytes. Depending on either the presence of Ca^{2+} during cell lysis or on the preincubation of the intact cells with the Ca^{2+}-ionophore A23187, up to 40% of the total cellular gelsolin is found associated with the membrane skeleton. Expression of gelsolin shows a strong negative regulation during erythroid differentiation. From quantitations of its steady-state molar ratio to actin, gelsolin is abundant in early progenitor cells as revealed from avian erythroblastosis virus- and S13 virus-transformed cells which are arrested at the colony forming unit erythroid (CFU-e) stage of erythroid development. In these cells, which have a rudimentary and unstable membrane skeleton, gelsolin remains quantitatively cytoplasmic, irrespective of the Ca^{2+} concentration. During chicken embryo development and maturation, the expression of gelsolin decreases by a factor of $\sim 10^3$ in erythroid cells. This down regulation is independent from that of actin, which is considerably less, and is observed also when S13-transformed erythroid progenitor cells are induced to differentiate under conditions where the actin content of these cells does not change. In mature erythrocytes of the adult the amount of gelsolin is low, and significantly less than required for potentially capping of all membrane-associated actin filaments. We suggest that the gelsolin in erythroid cells is involved in the assembly of the actin filaments present in the membrane skeleton, and that it may provide for a mechanism, by means of its severing action on actin filaments, to extend the meshwork of the spectrin-actin-based membrane skeleton in erythroid cells during erythropoiesis.

ERYTHROID development involves the formation of a network of structural proteins underlining the cell membrane, the membrane skeleton, whose function is to maintain the shape and stability of the red blood cell. The membrane skeleton of mammalian and avian erythrocytes has been the subject of extensive investigations with regard both to the identification and biochemical characterization of the proteins involved, and the mechanisms governing the assembly of such a complex structure in the living cell during development (for recent reviews see 4, 28, 31). The major network-forming element is a tetramer of the filamentous protein spectrin. These building units are cross-linked by short actin filaments (15), thus forming the vertices of a more or less hexagonal lattice (7, 38). Several other proteins are involved, such as 4.1 which serves to stabilize the spectrin–actin complex (36, 41) and possibly to cap the slow polymerizing end of the actin filaments; ankyrin which attaches the spectrin–actin network to the membrane (4, 31); and tropomyosin which possibly stabilizes the actin filaments (13). Other actin-associated proteins like myosin (14) and protein 4.9 (39) have shown to be present but their physiological role remains to be established.

The restricted length of the actin filaments in the mature erythrocyte membrane skeleton is evident from both biochemical studies and electron microscopic observations (1, 7, 38), indicating that one unit may consist of about 15 to 20 monomers (34, 38). During development, how is the cell able to regulate the polymer state of actin towards short fragments of relatively constant length distribution when, in vitro, actin has the tendency to form spontaneously long filaments under physiological conditions?

A relatively large number of actin-binding proteins have been found in cells other than erythrocytes that are capable of inducing the formation of short actin filaments—at least in vitro (for review see references 37, 40). Restriction of filament length may be achieved by capping of a filament end and preventing further addition of monomers, or by an active severing of actin filaments. Proteins of the latter function type have been termed actin modulators (16); in vertebrate cells they usually resemble gelsolin from macrophages (45, 46, 48) with a molecular mass of ~85–90 kD. Characteristic of all actin modulators is their complex pattern of interaction

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with actin. In the presence of micromolar concentrations of Ca^{2+}, they are activated not only to sever actin filaments, but also to promote nucleation of actin polymerization and to cap the fast polymerizing end of actin filaments (8, 17).

Here we describe the identification of gelsolin in chicken erythrocytes, its Ca^{2+}- dependent association with the membrane skeleton, and its differential expression with respect to actin during erythroid development and maturation.

Materials and Methods

Culture and Preparation of Cells

Yolk sac-derived avian erythroblastosis virus-transformed erythroblasts (AEV cells)[1] (27) and erythroblasts transformed with the S13 virus (kindly provided by Dr. Peter K. Vogt) (2) were grown as described previously (44). For one experiment, $\sim 10^8$ cells (AEV) and 10^7 cells (S13), respectively, were collected by centrifugation and washed extensively at least four times with 200 vol DME supplemented with 3.5×10^{-5} M thioglycerol, to remove all contaminating plasma gelsolin from the chicken serum in the culture medium, as determined by immunoblotting (see Results).

Chicken blood from various stages of embryogenesis (5) was collected from fertilized eggs either by opening the heart of the embryo (4–6-d-old embryos) or by puncturing one of the main yolk veins with a needle (7–19-d embryos). Newly hatched chickens and adult chickens were bled by cardiac puncture. Erythrocytes were purified by diluting the blood into 155 mM choline chloride, 5 mM Hepes, pH 7.2, 0.02% Heparin, and 1 mM EGTA, and collecting the red blood cells by centrifugation. All buffy coat was removed and the cells were washed at least five times in 200 vol of 155 mM ice cold choline chloride, 5 mM Hepes, pH 7.4. The number of cells was determined by counting appropriately diluted samples with a conventional Haemacytometer.

Preparation of Antigens and Immunization

Chicken gizzard actin modulator (gelsolin) (ChGAM) was purified from chicken gizzard smooth muscle as described for a corresponding protein from pig stomach smooth muscle (19, 20). Aliquots of 1 mg of the purified protein were subjected to preparative SDS-PAGE and the protein was electrophoretically transferred onto nitrocellulose (see below). The actin modulator band was excised from the Ponceau S-stained nitrocellulose, destained by repeated washing in Tris-buffered saline, and cut into fine pieces with a razor blade to obtain an injectable suspension. Polyclonal antibodies against this protein were raised in rabbits by injecting 6-mo-old animals subcutaneously with this suspension in doses of 1 mg for the first injection and 0.5 mg for booster injections at days 28, 35, 42, and 49. 0.3 mg/ml of adjuvant peptide (Sigma Chemical Co., St. Louis, MO) was used in the first two injections. Blood was collected at 3-d intervals after the second injection. Actin antibody was prepared against actin from calf thymus tissue as described (29). The amino acid sequence of the NH_2-terminal end of chicken gizzard gelsolin was determined by conventional protein sequence analysis using an automated sequenator.

Cell Fractionation and Preparation of Membranes

Erythrocytes were fractionated into Triton-soluble and Triton-insoluble components by extraction with 5 vol of Triton lysis buffer (0.5% Triton X-100, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 0.2 mM leupeptin, 0.05 mM aprotinin, 10 mM Tris-HCl, pH 7.4, and 2 mM EGTA or 0.2 mM $CaCl_2$, respectively). The lysate was kept on ice for 5 min and then centrifuged in an Eppendorf centrifuge for 15 min. The pellet was washed once with Triton lysis buffer. The soluble and insoluble fractions were operationally defined as cytoplasmic and cytoskeletal fraction. Alternatively cells were lysed under hypotonic conditions in 10 vol of 5 mM $MgCl_2$, 5 mM Tris-HCl, pH 7.4, 1 mM DTT, 1 mM PMSF, 0.2 mM leupeptin, and 0.05 mM aprotinin, 2 mM EGTA, or 0.2 mM $CaCl_2$, respectively. After 5 min, lysed cells were centrifuged for 15 min at 10,000 *g*. The hypotonic pellets were repeatedly washed until the pellets were slightly pink. Cell membranes were purified from the hypotonic pellets after thorough homogenization with a tight-fitting Dounce homogenizer, which quantitatively separated nuclei and membranes. Nuclei were removed by centrifugation for 5 min at 300 *g*, and the membranes collected by centrifugation for 60 min at 50,000 *g*. The membranes were extracted with 0.5% Triton X-100 in hypotonic lysis buffer to obtain the membrane skeleton.

PAGE

SDS-PAGE was performed on 12.5% polyacrylamide gels using the Laemmli buffer system (26). The gels (12 × 14 cm) were run at 20 mA constant until the hemoglobin band had entered the lower buffer reservoir. Two-dimensional electrophoresis was performed as described by O'Farrell (32).

Immunoblot Analysis

Separated proteins were transferred electrophoretically onto nitrocellulose, using a semi-dry blot apparatus (25) with additional cooling. Transfer was quantitative after 2 h at 250 mA constant current. The nitrocellulose sheets were transiently stained with Ponceau S for evaluation of the transfer and trimming. After destaining in TBS nonspecific binding sites were blocked with 3% gelatin in TBS for 1 h, and then incubated with the first antibody for 4 h at room temperature. For detection of antigen, the alkaline phosphatase-conjugated goat anti-rabbit antibody was used with the corresponding development system (Bio-Rad Laboratories, Richmond, CA).

To obtain quantitative data from the immunoblot experiments, the colored bands on the nitrocellulose were scanned with an E-C densitometer linked to an HP 3390A integrator. Various factors that could affect the linearity of the relationship between amount of antigen and the stain intensity of the band to be quantified were taken into consideration. Using multiple loadings and repeated runs, bands of equal density and in the linear range for the samples to be compared were obtained. Additionally, only samples from the same piece of nitrocellulose were compared to eliminate the influence of different incubation conditions.

Results

An Antibody against ChGAM Detects a Gelsolin-like Protein in Chicken Erythrocytes and Blood Plasma

Immunoblot analysis shows that an antibody to ChGAM detects an antigenically related protein in several types of chicken cells (Fig. 1, *A* and *B*). The reactivity with fibroblasts is somewhat weaker than with muscles and myogenic cells. This antibody reacts significantly less with the corresponding mammalian (mouse) cells (data not shown). A very weak reaction is observed with adult chicken erythrocytes (Fig. 1, *A* and *B*; lanes *6*), but the reaction is relatively stronger with embryonic erythroid cells (Fig. 2 *A*). To establish the identity of the cross-reacting protein, we used the characteristic isoelectric variant pattern and mobility of the actin modulator in two-dimensional IEF gels (20). A two-dimensional IEF immunoblot of the purified erythrocyte membranes shows a configuration of four variants with different isoelectric points (Fig. 1 *C*). The migration pattern, isoelectric points, and relative position to actin of these variants is indistinguishable from that of the purified antigen and that of the chicken gizzard modulator in silver-stained gels and immunoblots of whole muscle samples (not shown). Further indication for the identity of the protein as gelsolin is the strong reaction of the antibodies with chicken blood plasma (Fig. 1, lane *8*). The presence of extracellular gelsolin, also called brevin, has been shown for mammalian blood plasma (47). However, unlike in mammals where the extracellular isoform has a higher molecular mass than the cytoplasmic protein, chicken plasma gelsolin and the intracellular protein have indistinguishable apparent molecular masses of 85 kD. This apparent molecular mass of the

1. *Abbreviations used in this paper*: AEV cells, avian erythroblastosis virus-transformed erythroblasts; ChGAM, chicken gizzard actin modulator.

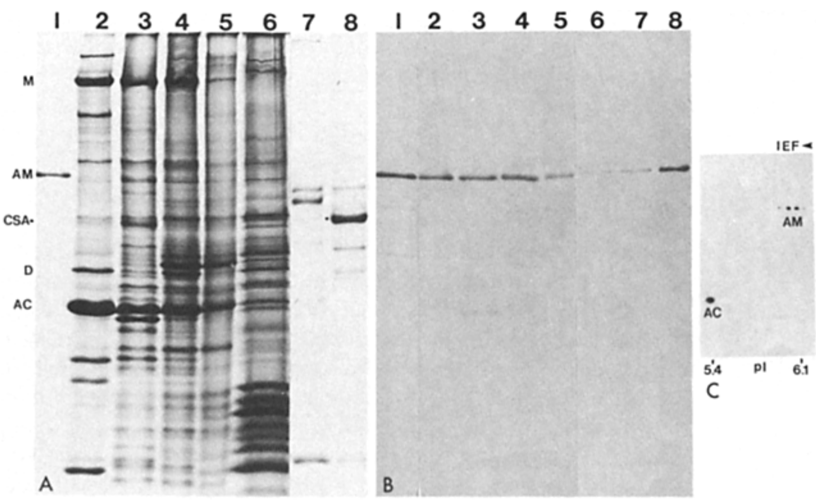

Figure 1. Specificity of ChGAM antibody. (*A*) Coomassie-stained SDS–polyacrylamide gel containing the following samples: purified antigen (lane *1*); chicken gizzard from 12-d-old chicken, whole muscle (lane *2*); breast muscle from 12-d-old chicken (lane *3*); cultured chicken myotubes, 2 d after the onset of fusion (lane *4*); chicken fibroblasts (lane *5*); erythrocytes from adult chicken (lane *6*); amniotic fluid from a fertilized egg with a 7-d-chicken embryo (lane *7*); blood plasma from adult chicken, diluted 1:50 (lane *8*). (*B*) Electrophoretic transfer of a gel identical to *A*, probed with the ChGAM antibody. (*C*) Part of an electrophoretic transfer from a two-dimensional electrophoresis gel of erythroid membranes from a 15-d chicken embryo probed simultaneously with anti-ChGAM and anti-actin. *M*, myosin; *AM*, actin modulator; *CSA*, chicken serum albumin (in lane *8*); *D*, desmin; *AC*, actin.

chicken protein appears to be ~2 kD less than the apparent mass of mammalian gelsolin (not shown). Finally, direct comparison of the sequence of the NH_2-terminal 26 amino acid residues of ChGAM with that of human plasma gelsolin (24) reveals an 81% homology between these two proteins over that segment (Fig. 3). The NH_2-terminal valine of ChGAM corresponds to amino acid 27 from the NH_2 terminus of human plasma gelsolin and to the second amino acid from the NH_2 terminus of rabbit macrophage cytoplasmic gelsolin.

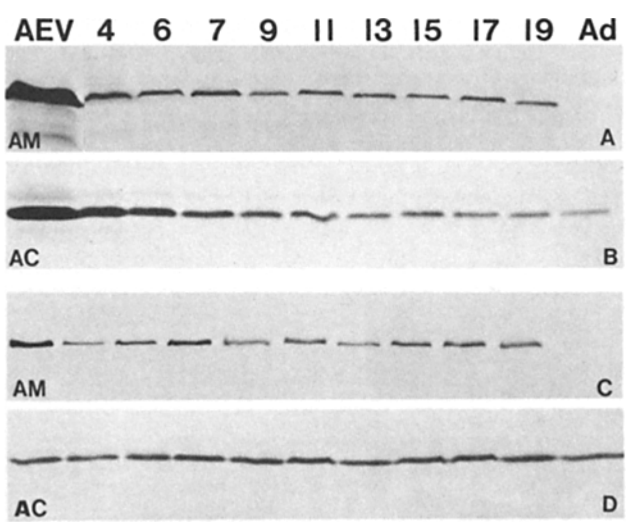

Figure 2. Decreased expression independent of gelsolin and actin in erythroid cells during differentiation and maturation. Immunoblot analysis of AEV cells, and erythroid cells from various embryonic stages and adult chicken. Total cells with ages indicated at the top margin were separated by SDS-PAGE, transferred onto nitrocellulose, and probed with anti-ChGAM (*A* and *C*) and anti-actin (*B* and *D*). Samples corresponding to 10^7 cells were loaded on each lane in *A* and *B*. Identical amounts of protein were loaded on each lane in *C* and *D*, hemoglobin excluded. All samples were run repeatedly and loadings adjusted until the densitometer scans varied by less than 10%. Only the relevant portions of the blots are displayed here.

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                  1                                                   26
ChGAM          NH2- V N H A M F A K A G K E P G L Q I W R I E K F D L V
Human plasma        •   •   •   •                               •
gelsolin            V E H P E F L K A G K E P G L Q I W R V E K F D L V
                    27                                                52
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Figure 3. Comparison of the amino acid sequence of ChGAM and human plasma gelsolin. The NH_2-terminal 26 amino acids of chicken gizzard actin modulator were determined by automated peptide sequence analysis and are displayed in the upper line. In the lower line the corresponding homologous amino acid sequence of human plasma gelsolin deduced from the nucleic acid sequence of full-length cDNAs (24) is displayed. The ChGAM sequence corresponds to amino acids 27–52 of human plasma gelsolin and to amino acids 2–27 of rabbit macrophage gelsolin exhibiting a homology of 81%. Nonidentical amino acids are indicated by a dot.

The strong reaction of the antibody with chicken blood plasma created a serious problem for the reliability of detection of the protein in erythrocytes, especially adult erythrocytes with a low abundance of the actin modulator. Excessive washing of the cells was necessary to ensure absence of trace contaminants of the protein from blood plasma. We routinely checked a sample of the last wash solution of the cell preparation by immunoblotting for possible plasma gelsolin content. Similar precautions were necessary for the virus-transformed cells, which are grown in media containing chicken serum. Finally, indirect immunofluorescence with the ChGAM antibody on circulating chicken embryo erythroid cells showed that the antigen could be detected only after permeabilization of the cells with Triton X-100 (data not shown), indicating that the antigen was indeed cytoplasmic. These results and additionally the Ca^{2+}-dependence of the erythrocyte protein, which will be shown in detail later, indicate that chicken erythrocytes contain a gelsolin-like protein. Similar results to those with chicken erythrocytes have been obtained also with mammalian (mouse) erythrocytes with an antibody to the actin modulator from pig stomach smooth muscle, where an antigen with an apparent molecular mass similar to that of mammalian gelsolin (87 kD) is detected (not shown).

Table I. Decrease of Relative Amount of Cytoplasmic Protein, Gelsolin, and Actin per Cell during Erythroid Development

	Protein (-Hb)	Actin	Actin modulator
AEV	100	100	100
4-d embryo	24.3	32.7	3.8
7-d embryo	12.6	18.1	2.9
9-d embryo	11.8	14.2	1.8
11-d embryo	11.0	14.8	2.1
15-d embryo	10.4	13.1	1.4
19-d embryo	7.2	9.1	1.2
1 wk old	7.0	9.4	1.0
4 wk old	6.3	7.6	0.5
1 mo old	5.6	6.7	0.15
S13	100	75	78
S13 diff	100	83	17

Samples of total erythrocytes from various stages of chicken development and maturation were resolved by SDS-PAGE. The relative amounts of protein were obtained by densitomeric quantification of Coomassie-stained gels. A sample equivalent to 10^7 cells was loaded in each lane. Amounts of actin and gelsolin were calculated from immunoblots with the respective antibody. Multiple loadings and several reruns were necessary to match the bands closely enough to enable a reliable densitomeric evaluation. All data were normalized to the amount determined for AEV cells as the earliest developmental stage.

Expression of Erythrocyte Gelsolin Decreases during Development and Maturation Independent of Actin

Yolk sac–derived AEV cells are arrested at an early stage of erythroid development (CFU-E stage; 27). For the experiments described below they will, therefore, be used as an earlier time point of erythroid differentiation (44). Immunoblots of electrophoretically resolved erythroid cell proteins at different stages of development and maturation show a dramatic reduction in the amount of gelsolin on a per cell basis (Fig. 2 *A*; Table I) with a precipitous decrease by a factor of almost 30 from AEV cells to the 4-d embryonic stage (circulating erythroblasts from 3-d embryos shown intermediate levels of gelsolin; not shown). There is again a fivefold difference between the last embryonic stage and the adult chicken, but the decrease is gradual and continues for several weeks after hatching. Between days 4 and 19 of embryogenesis, a significant fluctuation in the concentration of gelsolin is observed superimposed on the general decrease. As these fluctuations are not reflected in corresponding blots probed with anti-actin, they are not likely to be caused by experimental error. The amount of actin per cell decreases also during development but less dramatically and continuously (Fig. 2 *B*; Table I). Considering the fact that with the increase in hemoglobin content during erythroid differentiation the amount of the cytoplasmic proteins decreases (with the exception of some of the membrane skeleton proteins, which increase during development), the extent of decrease in the concentration of actin reflects this decrease in general protein content, as is also evident from Fig. 2 *D*. In contrast, gelsolin behaves characteristically different. Even with the gel loadings normalized to identical amounts of cytoplasmic protein (without hemoglobin), the AEV cells have a significantly higher amount of gelsolin, whereas, in adult cells the protein is practically undetectable under these conditions (Fig. 2 *C*). To eliminate the possibility that the high content of gelsolin in AEV cells is virus related, we have additionally investigated a cell line transformed by the S13 virus which is able to undergo spontaneous differentiation, as revealed by the onset of hemoglobin synthesis. Fig. 4 shows that while the amount of actin does not decrease in differentiating S13 cells, the amount of modulator is decreased by a factor of 4 (Table I). As only ~25% of the cell population actually turned hemoglobin-positive under the experimental conditions used here, the actual decrease of gelsolin in the differentiating S13-transformed cell population must be considerably higher.

The immunoblots do not allow any conclusion to be drawn about the ratio of actin and gelsolin in the cell. We have attempted a quantification of the actual molar ratio of both proteins from two-dimensional electrophoresis gels of AEV cell proteins labeled to steady state with [^{35}S]methionine (24 h) because the high gelsolin content in these cells enabled identification of the characteristic isoelectric variant configuration directly on the autoradiograph. By excising the spots of both proteins from the gel and measuring the radioactivity incorporated we obtained a ratio of 1:9.0 which would correspond to a molar ratio of ~1:18 gelsolin to actin, assuming that the relative incorporation of methionine is similar for both proteins.

Gelsolin Binds to the Erythrocyte Membrane Skeleton in a Calcium-dependent Manner

The interaction of gelsolin-like actin modulators with actin is Ca^{2+} dependent (12, 18, 21). We have investigated the amount of gelsolin associated in the presence and absence of Ca^{2+} with a Triton-insoluble cytoskeleton from erythroid cells at various stages of embryonic development (Table II). In AEV-transformed cells, almost all of gelsolin is soluble (Fig. 5, *AM*; Table II), independent of the Ca^{2+} concentration. Similarly, the vast majority of the actin appears to be soluble and only a small fraction of the actin appears to be associated with cytoskeletal components (Fig. 5, *AC*; Table II). This situation is changed considerably in all of the other developmental stages investigated (Table II). Despite the differences in the absolute amount of gelsolin, the ratio of soluble and insoluble actin is relatively constant for 9- and 17-d embryonic cells and for adult erythrocytes. When cells are lysed in EGTA, the amount of gelsolin found insoluble is ~10% of the total and increases by a factor of 3 upon lysis in the presence of Ca^{2+}. When the soluble fraction is cen-

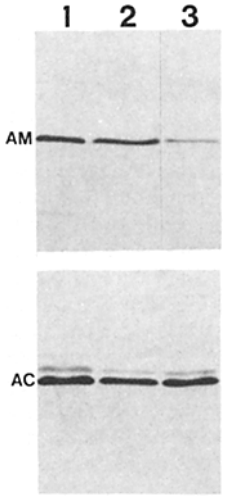

Figure 4. Induction of differentiation of S13-transformed cells causes reduced expression of gelsolin. Immunoblot analysis with anti-ChGAM and anti-actin. AEV cells serving as a reference (lane *1*), uninduced S13 cells (lane *2*), and S13 cells undergoing spontaneous differentiation as measured by hemoglobin synthesis (benzidine positive; 44) were adjusted to equal protein content, separated by SDS-PAGE, and transferred. The blots were probed with anti-ChGAM (*AM*) and anti-actin (*AC*). The cell line in lane *3* contained ~25% strongly benzidine-positive cells.

Table II. Partitioning of Actin and Gelsolin into Cytoplasmic and Cytoskeletal Fractions in Erythroid Cells of Various Stages of Development

	Actin modulator				Actin			
	EGTA		Ca^{2+}		EGTA		Ca^{2+}	
	Soluble	Insoluble	Soluble	Insoluble	Soluble	Insoluble	Soluble	Insoluble
	%	%	%	%	%	%	%	%
AEV	97	~2	96	~2	85	16	85	12
9-d embryo	84	13	60	36	52	44	50	49
17-d embryo	90	15	57	33	50	52	55	45
Adult	87	10	67	28	52	50	55	52

Erythroid cells were Triton-extracted in the presence and absence of Ca^{2+}, and the relative amounts of actin and gelsolin determined by quantitative evaluation of immunoblots of the respective samples resolved by SDS-PAGE. Data were normalized to the respective values obtained for a sample of the whole cells of this stage. Because of the limited precision of the method of quantitation, soluble and insoluble fraction may add up to slightly more or less than 100%.

trifuged at higher *g* force (3 h at 150,000 *g*) no additional gelsolin was found in the sediment, indicating that soluble gelsolin is not bound to filamentous actin (data not shown). The significance of the minor differences between the various stages cannot be evaluated here. The partitioning of actin into soluble and insoluble fractions is also constant, with ~50% of the actin soluble for the three erythroid stages investigated (Table II). The reason for the strikingly different situation in AEV cells is the apparent lack of a stably assembled membrane skeleton (44), which is most likely the only major site of structurally bound actin in erythroid cells.

The various fractions of membrane preparations prepared in the presence of either Ca^{2+} or EGTA show independent partitioning of gelsolin and actin (Fig. 6). Whereas the gelsolin content of the hypotonic supernatant and pellet is directly dependent on the Ca^{2+} concentration during lysis of the cell, actin partitions approximately equally into each supernatant and pellet fraction in a Ca^{2+}-independent manner. As the nuclei contain little actin and no detectable gelsolin, it has to be assumed that practically all of the insoluble fraction of the two proteins is associated with the cell membrane. This is in fact the case: if membranes are purified from the hypotonic pellet, the majority of the two proteins is recovered in the membrane fraction. Even after Triton extraction of the intrinsic membrane proteins, practically all of the actin and gelsolin are retained in the membrane skeleton, with the ratios unchanged as far as the Ca^{2+} concentration is concerned (Fig. 6, lanes *8* and *9*).

From quantitative evaluation of the immunoblots, we can therefore conclude that between 10 and 15% of the total amount of gelsolin is associated with the membrane skeleton when the cells are lysed in EGTA, and that upon cell lysis in the presence of Ca^{2+} this changes to ~30–35%. The amount of actin is practically unaffected by the Ca^{2+} concentration during extraction and purification of the membranes. A binding of gelsolin-like actin modulators to proteins other than actin has not been reported so far. We may, therefore, assume that the binding site of erythroid gelsolin in the membrane skeleton is the fast polymerizing end of actin filaments. Further evidence for actin as the binding site of gelsolin in the membrane skeleton comes from the relative irreversibility of the gelsolin membrane association in vitro. When a large amount of gelsolin is induced to bind to the membranes by cell lysis in the presence of Ca^{2+}, it cannot be removed subsequently just by lowering the Ca^{2+} concentration with EGTA. This is comparable to the behavior that has been described for the mammalian gelsolin–actin interaction in vitro (12, 21). On the other hand, erythrocyte gelsolin is removed from the membrane when actin is extracted. For example, dialysis of isolated plasma membranes against very low ionic strength solubilizes part of the actin and also releases considerable amounts of gelsolin from the membrane (data not shown).

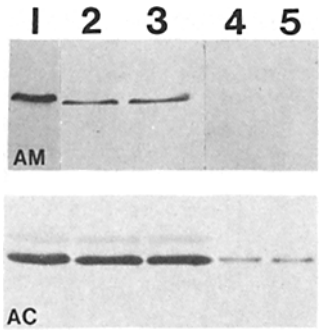

Figure 5. In AEV cells, the vast majority of gelsolin and actin partition into the Triton-soluble fraction regardless of Ca^{2+} concentration. AEV cells (lane *1*) were extracted with Triton X-100 in the presence of 1 mM EGTA (lanes *2* and *4*) or 0.2 mM $CaCl_2$ (lanes *3* and *5*); soluble fractions (lanes *2* and *3*) and insoluble fractions (lanes *4* and *5*) were separated by SDS-PAGE and the transfers probed with anti-ChGAM (*AM*) and anti-actin (*AC*).

Ionophore-induced Increase of Intracellular Ca^{2+} Concentration Leads to Reversible Binding of Gelsolin to the Membrane Skeleton In Vivo

Binding of gelsolin to membrane-associated actin may be regarded as an artifact induced by cell lysis which has little significance for the situation in vivo. To exclude this possibility, the translocation of cytoplasmic gelsolin to the plasma membrane was examined in vivo by incubation of intact cells with the calcium ionophore A23187. As shown in Fig. 7, when cells are lysed in EGTA after incubation with the ionophore, the partitioning of gelsolin into hypotonic supernatant and membranes resembles that of a preparation of the fractions after cell lysis in the presence of Ca^{2+}. This indicates that gelsolin must have been bound to the membrane fraction before cell lysis, otherwise EGTA would have prevented its interaction with actin. Subsequent incubation of the ionophore-treated cells in EGTA-containing medium apparently restores the normal partitioning of gelsolin because membranes prepared from these cells show the same amount of

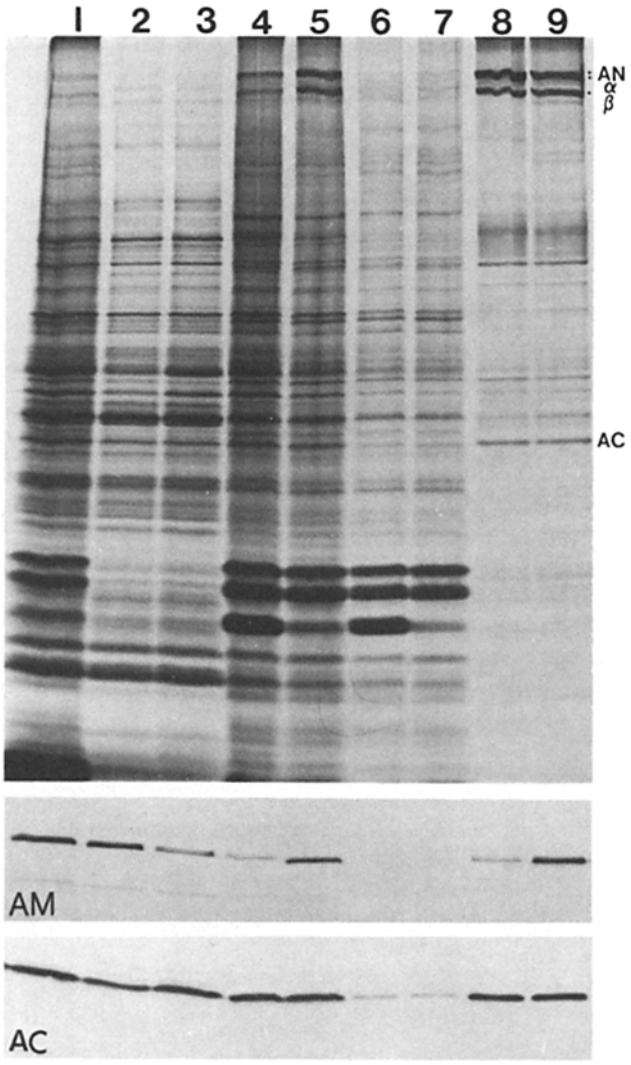

Figure 6. Different quantities of gelsolin and actin are incorporated into erythrocyte membrane skeletons in a Ca^{2+}-dependent manner. Purification of membrane skeletons from 16-d embryonic erythrocytes and SDS-PAGE of various fractions from the preparation: total erythrocytes (lane *1*); supernatant after hypotonic lysis in the presence of EGTA (lane *2*) and $CaCl_2$ (lane *3*); pellets after hypotonic lysis in the presence of EGTA (lane *4*) and $CaCl_2$ (lane *5*); purified nuclei from cells lysed in EGTA (lane *6*) and $CaCl_2$ (lane *7*); purified membrane skeletons from cells lysed in EGTA (lane *8*) and $CaCl_2$ (lane *9*). (*Upper panel*) Coomassie stain. (*AM* and *AC*) Immunoblots of corresponding gels probed with anti-ChGAM (*AM*) and anti-actin (*AC*). To enable quantitative comparison of lanes, all samples in *AM* and *AC* were normalized to the initial volume of the lysates.

gelsolin bound to the membrane as the control preparation (Fig. 7; cf. lanes *2* and *6*). It is not yet clear whether the cell is capable of separating actin and gelsolin, or if the actin-bound gelsolin is turned over and degraded during the relatively long incubation time of the cells under the experimental conditions used here. Collectively these results are consistent with the interpretation that free actin-binding sites are still available for gelsolin in the membrane skeleton, assuming that actin is the only binding site. Furthermore, they indicate that at least a fraction of the cytoplasmic gelsolin is capable of binding to actin, which implies that it is not capping any cytoplasmic actin filaments.

Discussion

In this paper we have presented evidence on the identification of a gelsolin-like protein in chicken erythrocytes, its Ca^{2+}-dependent association with the membrane skeleton, and its negatively regulated expression during differentiation and maturation of the erythroid cells. From in vitro studies on platelet (6, 23), plasma (12), and macrophage gelsolins (21, 45, 46, 48), and from direct comparative studies on various muscle and nonmuscle actin modulators (17, 18), which included the chicken gizzard protein used to generate the antibody of the present experiments, it is evident that this type of protein is conserved as far as its interaction with actin is concerned. Even modulator proteins from such different origins as the pig stomach smooth muscle and the acellular slime mold *Physarum polycephalum* reveal no more than small differences in Ca^{2+} dependence and severing of actin filaments (17). This conclusion is strengthened by the demonstration that the amino-terminal sequences of ChGAM, used here as an antigen, and rabbit macrophage gelsolin are highly homologous. Therefore, we have no reason to assume that the erythroid protein is not a Ca^{2+}-activated, actin filament–severing protein, also capable of capping the fast polymerizing filament end.

It is characteristic of gelsolin-like proteins that, though their association with actin is fully Ca^{2+} dependent, only one of the two actins bound dissociates in the presence of EGTA, and the remaining 1:1-complex still caps actin filaments (12, 18, 21). The increased binding of erythroid gelsolin to the membrane skeleton in the presence of Ca^{2+} may reflect a physiological situation as is suggested from the ionophore experiment where this effect was observed in vivo, but in a reversible manner. Apparently intracellular mechanisms exist that are capable of dissociating gelsolin from the membrane skeleton and restore the original situation. It has been reported that activation of platelets by the ionophore A23187

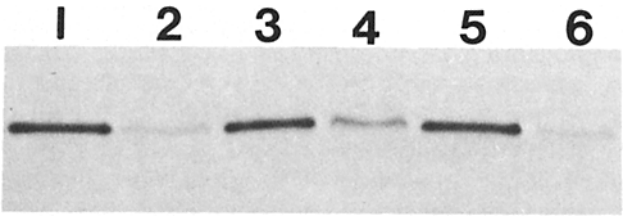

Figure 7. Ca^{2+}-dependent binding of gelsolin to cell membranes is induced in vivo by ionophore A23187 and is reversible. Erythrocytes from 16-d-old chicken embryos were incubated for 3 h in Ca-free MEM supplemented with 0.2 mM $CaCl_2$, and 5×10^{-6} M ionophore A23187. Half of the cells were hypotonically lysed after this time and cell membranes were prepared in hypotonic lysis buffer containing 2 mM EGTA. The other half was further incubated in Ca-free MEM supplemented with 1 mM EGTA for 3 h, after which the cells were lysed in hypotonic lysis buffer with 2 mM EGTA as before, and membranes were prepared. (Lanes *1* and *2*) Control preparation of normal erythrocytes lysed in the presence of EGTA. (Lanes *1*, *3*, and *5*) Soluble fractions; (lanes *2*, *4*, and *6*) purified membranes. (Lanes *3* and *4*) Ionophore-treated cells. (Lanes *5* and *6*) Ionophore-treated and EGTA-chased cells.

stimulated the formation of a gelsolin–actin complex (6). On the other hand, no effects were observed when gelsolin was microinjected into macrophages and fibroblasts and the intracellular Ca^{2+} concentration was raised by incubation with A23187 (11). These authors concluded that the intracellular Ca^{2+} concentration never reached a value sufficient to activate the gelsolin, and that the gelsolin does not express in vivo the properties found in vitro. The apparent discrepancies to our results remain to be resolved and may reflect differences in cytoplasmic Ca^{2+} levels between developing or adult erythrocytes and other cell types. None of the previously known actin-binding proteins of the erythrocyte membrane skeleton has the properties of a capping factor for the fast polymerizing end of the actin filament. The spectrin–4.1 complex was found to cap the (−) end of actin in vitro (33, 35), and there is also evidence from monomer addition experiments with erythrocyte ghost preparations that the (−) ends of the actin filaments are blocked in situ (34, 36). The same experiments indicate that the (+) ends are available for addition of further actin monomers and must therefore be uncapped (36). However, this does not contradict our results, as we have shown that the gelsolin-to-actin ratio in erythrocytes of the adult is very low, and there is less gelsolin than required to cap all actin filaments in the membrane skeleton, as we will discuss later on. When only a fraction of filament ends is capped, it may not be detected by monomer addition experiments.

Under all the lysis conditions we have investigated, the majority of the gelsolin was found soluble in the cytoplasmic fraction, so it can be concluded that at least part of gelsolin is present in a free form as indicated by the Ca^{2+} dependence of its association with the membrane skeleton. Likewise, gelsolin from macrophages and the actin modulator from pig stomach smooth muscle are extractable from these cell types mainly as free protein and not associated with actin (19–21). The apparent saturation of the binding to the membrane skeleton that we have observed for embryonic and adult erythrocytes limited the amount of gelsolin incorporated to a maximum of 40% in the presence of Ca^{2+}. It is not clear from these experiments whether the restriction is imposed by the limited number of binding sites or by the fact that gelsolin in the soluble phase is already bound to oligomeric or polymeric actin, or to another cytoplasmic protein.

In embryonic cells the membrane skeleton is a more dynamic structure than may be inferred from current models which depict the static and invariable arrangement of the fully differentiated cell. Proliferative erythroblasts and postmitotic cells continuously incorporate newly synthesized membrane skeleton proteins which probably induces structural rearrangements. Synthesis and assembly of membrane skeleton proteins have been the object of extensive studies (3, 9, 10, 30, 42–44; for review see reference 28). These investigations have led to the conclusion that formation of the membrane skeleton is a self-assembly process controlled posttranslationally by the sequential availability of high-affinity binding sites on the membrane. So far no experimental data are available that reveal at which point in the sequence of events actin, tropomyosin, and actin-binding proteins other than spectrin and protein 4.1 are assembled. We have shown that from an initially high steady-state concentration in early progenitor cells the amount of actin per cell decreases continuously during erythroid differentiation. In contrast to this, the steady-state concentration of spectrin, ankyrin, and 4.1 is very low in AEV cells, and the anion transporter (band 3) is not expressed at all (44). Only after the onset of band 3 synthesis does the steady-state concentration of the other three proteins increase and this correlates with increasing steady-state levels of these peripheral membrane skeleton proteins on the membrane. However, whereas these proteins are found to be quantitatively associated with the membrane (3, 42), characteristically no more than 50% of the steady-state amounts of actin are assembled onto the membrane skeleton. Unlike the other components, actin does not assemble spontaneously in a definite stoichiometric amount. How and when is the polymer size of actin determined? It has been speculated that tropomyosin serves as a length-determining element because of an apparent coincidence of its molecular length with the observed size of the actin oligomer (13). On the other hand, gelsolin-like actin modulators are in vitro potent factors for restricting actin filament length, and the differential expression of this protein in erythroid cells suggests a corresponding function in vivo. This possibility is strengthened by the molar ratio of the two proteins observed at various stages of differentiation. Our determined value of 1:15–1:20 for AEV cells is quite high in comparison to estimates of 1:100 for macrophages (21) and 1:200 for smooth muscle (Hinssen, H., unpublished observations). Thus, in AEV cells, the amount of gelsolin present is sufficient to generate very short actin filaments from the total amount of actin. From the determined ratio for AEV cells, we calculate an average gelsolin-to-actin ratio for embryonic erythroblasts and postmitotic erythrocytes of 1:100–1:150 and for erythrocytes of the adult of less than 1:500. The latter number indicates that at least the stabilization and maintenance of the short actin filaments in the fully differentiated membrane skeleton cannot be crucially dependent on the presence of gelsolin. Though the possibility exists that gelsolin initially generates a pool of short actin filaments in early progenitor cells which are assembled as soon as high affinity binding sites at the membrane in the form of spectrin–4.1 complexes become available, other mechanisms are also conceivable and consistent with the observed data. The actin may initially be associated with the membrane skeleton in the form of long filaments and subsequently fragmented by gelsolin until the terminal size is attained. This view is in accordance with the fact that, because of a low steady-state concentration of the involved proteins ankyrin, spectrin, and 4.1 (44), the meshwork density of the membrane skeleton in early erythroblasts and postmitotic embryonic cells is most likely lower than in the fully differentiated erythrocyte. As the spectrin-ankyrin-4.1 complexes are distributed more sparsely over the membrane, cross-linking of this system may require longer actin filaments and more actin. In fact, it is evident from our data that the amount of actin associated with the membrane skeleton is approximately threefold higher for a 9-d embryonic erythroid cell than for an adult erythrocyte. Therefore, as the spectrin–actin ratio is initially low, only few actin-binding sites are occupied. As soon as more spectrin-4.1 is assembled, an effective cross-linking is possible by shorter actin filaments and less actin. This could be achieved by fragmentation of existing filaments by gelsolin. Repeated fragmentation of actin and continuing incorporation of spectrin-4.1 would lead to a structural rearrangement of the membrane skeleton and to an increased meshwork

density. In the terminal situation most of the binding sites on the actin filament would be occupied by the various associated proteins and further fragmentation would be inhibited automatically. In the course of this process, gelsolin which remained at the (+) ends of the generated actin fragments could be turned over proteolytically, thus explaining the low amount of this protein in adult erythrocytes. Because of the lack of experimental data on the assembly kinetics of actin and some of its associated proteins, the ideas about a possible role of gelsolin must remain preliminary and speculative. Other mechanisms are conceivable as well (e.g., the assembly of actin at sites other than the membrane skeleton, such as the marginal band of microtubules [22]) and any experimental verification requires more information about the synthesis and the temporal sequence of the assembly of actin. However, as the physiological function of actin modulators has not been clarified yet for any other cell type, the erythroid cell may be a suitable model for further work in this direction, as it provides a system where the expression of the modulator is developmentally controlled.

We are grateful to Dr. Peter K. Vogt, Department of Microbiology, University of Southern California School of Medicine, for providing us with the AEV cells and S13 virus-transformed cells. We are also grateful to Chantal Champagne for determining the site of homology between this protein and human plasma gelsolin.

This work was supported by grants from the National Institutes of Health (AG 06078A and HL 35801A) and Muscular Dystrophy Association. H. Hinssen was supported by a Heisenberg Fellowship from the Deutsche Forschungsgemeinschaft.

Received for publication 25 March 1987, and in revised form 5 May 1987.

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