Posttranslational Control of Membrane-Skeleton (Ankyrin and $\alpha\beta$-Spectrin) Assembly in Early Myogenesis

W. JAMES NELSON and ELIAS LAZARIDES

Division of Biology, California Institute of Technology, Pasadena, California 91125. Dr. Nelson's present address is Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111.

ABSTRACT Adult chicken skeletal muscle cells express polypeptides that are antigenically related to α-spectrin (M_r 240,000) and β-spectrin (M_r 220,000–225,000), the major components of the erythrocyte membrane-skeleton, and to ankyrin (M_r 237,000; also termed goblin in chicken erythrocytes), which binds spectrin to the transmembrane anion transporter in erythrocytes. Comparative immunoblotting of SDS-solubilized extracts of presumptive myoblasts and fully differentiated myotubes cultured in vitro demonstrated that there is a dramatic accumulation of ankyrin and α- and β-spectrin during myogenesis and a concomitant switch in the subunit composition of spectrin from $\alpha\gamma$ to $\alpha\beta$. Analysis of early time points in myogenesis (12–96 h) revealed that these changes occur shortly after the main burst of cell fusion. To determine the temporal relationship between cell fusion and the accumulation of ankyrin and α- and β-spectrin, we treated presumptive myoblasts with 2 mM EGTA, which resulted in the complete inhibition of cell fusion. The incorporation of [^{35}S]methionine into total protein and, specifically, into α-, γ-, and β-spectrin remained the same in EGTA-treated and control cells. Analysis by immunoblotting of the amounts of ankyrin and α- and β-spectrin in fusion-blocked cells revealed that there was no effect on accumulation for the first 19 h. However, there was then a dramatic cessation in their accumulation, and thereafter, the amount of each protein at steady state remained constant. Upon release from the EGTA block, the cells fused rapidly (< 11 h), and the accumulation of ankyrin and α- and β-spectrin was reinitiated after a lag period of 3–5 h at a rate similar to that in control cells. The inhibition in the accumulation of newly synthesized ankyrin, α-spectrin, and β-spectrin in EGTA-treated myoblasts was not characteristic of all structural proteins, since the accumulation of the muscle-specific intermediate filament protein desmin was the same in control and fusion-blocked cells. These results show that in myogenesis, the synthesis of ankyrin and α- and β-spectrin and their accumulation as a complex, although concurrent, are not coupled events. We hypothesize that the extent of assembly of these components of the membrane-skeleton in muscle cells is determined by a control mechanism(s) operative at the posttranslational level that is triggered near the time of cell fusion and the onset of terminal differentiation.

Adult chicken skeletal muscle cells express a class of proteins that are antigenically and structurally related to the major components of the erythrocyte membrane-skeleton. These proteins are α-spectrin (M_r 240,000; references 43 and 56), β-spectrin (M_r 220,000–225,000; reference 43), and ankyrin (M_r 235,000; reference 46). In erythrocytes, α- and β-spectrin form an $(\alpha\beta)_2$ tetramer that binds actin oligomers (reviewed in references 2 and 10). Ankyrin (termed goblin in chicken erythrocytes; see references 38, 46, and 56) binds spectrin (4, 5; specifically the β-spectrin subunit [33, 40, 65]) to the anion transporter embedded in the plasma membrane (3, 6, 24). Indirect immunofluorescence of adult muscle cells has revealed that the muscle isoforms of ankyrin and $\alpha\beta$-spectrin are co-distributed on the sarcolemma in the form of rings, transverse to the long axis of the muscle fiber, which surround the myofibrils at the level of the Z-lines (43, 46, 56). By analogy to the role of the membrane-skeleton in erythrocytes (reviewed in references 2 and 10), it has been suggested that this structure in muscle cells may act as the anchorage site for myofibrils on the sarcolemma (46; reviewed in reference 47).

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Previous studies have demonstrated that the synthesis and accumulation of α- and β-spectrin in muscle cells is developmentally regulated and is characterized by a switch in spectrin subunit expression from αγ- to αβ-spectrin (44). Initially, replicating presumptive myoblasts in vitro synthesize predominantly α-spectrin, which is constitutively expressed throughout myogenesis, together with equimolar amounts of γ-spectrin (44), a spectrin subunit that is antigenically and structurally distinct from α- and β-spectrin (21, 43, 45, 56) and that is also expressed in brain and lens (32, 45). Upon terminal differentiation, the cells begin to synthesize β-spectrin, whereas the steady state amounts of γ-spectrin gradually decline (44). Analysis with specific cDNA clones has shown that during this period, there is a dramatic accumulation of mRNA coding for ankyrin and α- and β-spectrin (39). Together, these results suggest that transcriptional (or posttranscriptional) control of gene expression plays an important role in regulating the expression of these components of the muscle membrane-skeleton in early myogenesis, as has been shown also for other muscle-specific proteins such as myosin heavy chain (1, 13, 15, 17, 53, 57, 63), α-actin (15, 59, 63), tropomyosin (41), and the acetylcholine receptor (16, 35, 55, 62).

However, detailed studies on the switching of the subunit composition of muscle spectrin during myogenesis showed clearly that the gradual accumulation of β-spectrin and the concomitant decline in γ-spectrin is characterized by the differential stabilization of these two spectrin subunits (44); in mature myotubes, α- and β-spectrin had a half life of ~4–7 d, whereas that of γ-spectrin was < 30 h (44). We hypothesized that the differential accumulation of β- and γ-spectrin was determined at the posttranslational level by the availability of membrane binding sites specific for each of these subunits (presumably ankyrin), which when expressed resulted in the stable assembly of the subunits onto the membrane; in the absence of such a binding site, unassembled subunits would be susceptible to intracellular catabolism and thus, as in the case of γ-spectrin in the mature myotube, would be degraded relatively rapidly (44; reviewed in reference 47). This hypothesis predicts that synthesis of each of these three polypeptides and their accumulation as a complex during myogenesis are not coupled events. Experimental support for this hypothesis has, thus far, come from the analysis in vivo and in vitro of the assembly of these subunits of the membrane-skeleton in erythrocytes (reviewed in reference 31). These studies showed that synthesis of ankyrin and α- and β-spectrin and their assembly onto the membrane are not coupled events since these polypeptides, although synthesized simultaneously, are synthesized in excess of the amount assembled onto the membrane (9, 37, 38; reviewed in reference 31).

In the present study, we sought to determine whether control mechanisms exist also in muscle cells that regulate at the posttranslational level the accumulation of ankyrin and α- and β-spectrin. We have found that the period of myogenic cell fusion and the onset of terminal differentiation is a critical stage in the induction of the accumulation of these proteins. By inhibiting cell fusion with EGTA, we showed that the accumulation of these proteins was blocked, although at the same time their levels of synthesis remained constant. These results demonstrate that synthesis of these components of the muscle membrane-skeleton and their accumulation during myogenesis although concurrent, are not coupled events. We hypothesize that the accumulation of these proteins is determined by a control mechanism(s) operative at the posttranslational level that is triggered near the time of cell fusion. That this mechanism is specific for components of the membrane-skeleton and not for all structural proteins is supported by the observation that inhibition of myogenic cell fusion has little or no effect on either the synthesis or accumulation of the muscle-specific intermediate filament protein desmin.

MATERIALS AND METHODS

Antibodies: The antibodies used in this study were raised against chicken smooth muscle desmin (22), chicken erythrocyte α-spectrin (56), β-spectrin (43), and ankyrin (goblin) (46). Their specificities have been described in detail in these references.

Cell Culture: Primary cultures of embryonic chicken thigh muscle were prepared as described previously (19, 27). Cells were preplated twice to remove fibroblasts (19), which resulted in cultures that were 95% free of contaminating fibroblasts. Cells were plated at a density of either 2 or 6×10^6 cells per 100-mm collagenized petri dish in growth medium, minimum essential medium, supplemented with 15% horse serum, 5% 10-d chick embryo extract, 1% glutamine, and 1% nonessential amino acids. The cells were incubated at 37°C in air that contained 5% CO_2 in a humidified atmosphere. 11 h after the culture was initiated, the medium was replaced with fresh growth medium (control) or fresh growth medium that contained 2 mM EGTA, pH 7.2. At this time, < 4% of the nuclei were present in multinucleated cells (see Fig. 5), and few presumptive myoblasts exhibited alignment. 14 h later, the medium was removed from both the control and EGTA-treated cultures. The cells were gently rinsed with one change of fresh growth medium, and then incubated in normal growth medium for the remainder of the experiment. The kinetics of cell fusion were determined at regular time intervals (see Fig. 5). The culture dish was viewed under a Leitz inverted phase-contrast microscope (E. Leitz, Inc., Rockleigh, NJ) equipped with a 25× objective; the number of nuclei in single cells (unfused) and multinucleated (fused) cells that contained > 2 nuclei was scored in three random fields in the dish. Fusion kinetics are expressed as the percentage of nuclei in multinucleate cells compared to the total number of nuclei scored (see Fig. 5).

In Vitro Labeling of Cells with [^{35}S]Methionine: Cultures of myogenic cells were prepared and treated with EGTA as described above. Cells were labeled in vitro with L-[^{35}S]methionine (~1,100 Ci/mmol; New England Nuclear, Boston, MA) in duplicate as follows. Each petri dish was washed twice with 5 ml minimum essential medium without methionine and supplemented with 1% heat-inactivated dialyzed horse serum. The cells of each petri dish were then incubated in 4 ml minimum essential medium with 50 μCi [^{35}S] methionine at 37°C for 2 h. All subsequent operations were performed at 0–4°C unless otherwise stated. Each petri dish was washed in Ca^{++}- and Mg^{++}-free Earle's balanced salt solution, and the cells were scraped off the petri dish with a rubber policeman into 5 ml Ca^{++}- and Mg^{++}-free Earle's balanced salt solution. The cell suspension was centrifuged at 1,000 *g* in a clinical centrifuge, and the resulting pellet resuspended in 1 ml 20 mM Tris-HCl, pH 7.4, 120 mM NaCl (Tris-saline). 20 μl of the cell suspension was removed and precipitated with 10% trichloroacetic acid to determine total [^{35}S]methionine incorporation, and 150 μl was removed for DNA determination (25) as described previously (44). The remainder of the cell suspension was recentrifuged at 12,000 *g* in an Eppendorf microcentrifuge. The cell pellet was dissolved in 100 μl of 1% (wt/vol) SDS, 5 mM EDTA, 10 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride by boiling for 2 min. The sample was diluted 10-fold in 0.1% (wt/vol) SDS, 1% (wt/vol) Nonidet-P40, 1% Na-deoxycholate, 20 mM Tris-HCl, pH 7.4, 130 mM NaCl, 5 mM EDTA. 1 μl α-spectrin and 1 μl β-spectrin antibodies were added, and the sample was incubated at 4°C for 12 h. Immune complexes were isolated using protein A- bearing *Staphylococcus aureus*, according to method B of Blikstad et al. (9). Samples were finally boiled in SDS-sample buffer and the staphylococci were pelleted in an Eppendorf microcentrifuge. The supernatants were applied to a 10% SDS-polyacrylamide gel (see below). The gels were processed for fluorography with Enhance (New England Nuclear), dried, and exposed to Kodak XAR-5 X-ray film that had been preflashed to an absorbance of ~0.15 at 540 nm for 1–2 d at −80°C.

Immunoblotting: Cultures of myogenic cells were prepared and treated with EGTA as described above. Cells were harvested and washed as described above. Each cell pellet was resuspended in exactly 1 ml of Tris-saline. 150 μl of the suspension was removed for DNA determination as described above. The remainder of the cells was pelleted in an Eppendorf microcentrifuge and dissolved in 400–600 μl sample buffer by being boiled for 2 min. For SDS PAGE, the volumes of different samples loaded were normalized to the same equivalent amount of DNA. After SDS PAGE, the proteins were electrophoretically transferred to nitrocellulose filters according to the method described

by Towbin et al. (64) as modified and described previously (23). The filters were incubated with specific antisera diluted 1:1,000 and then with ^{125}I-protein A (for details see reference 23). Nitrocellulose filters were exposed to Kodak XAR-5 X-ray film with an intensifying screen for 0.25–36 h at −80°C. For quantitation, the areas on the nitrocellulose filters that corresponded to the bands on the autoradiograms were carefully excised and counted in a gamma counter. In all cases, half the amount of material loaded on the SDS-polyacrylamide gels yielded approximately half the total counts of ^{125}I.

Gel Electrophoresis: Proteins were solubilized in SDS sample buffer (26) by being boiled, and then separated by one-dimensional SDS 12.5% or 10% PAGE based on the system of Laemmli (28) as modified and described previously (26).

Indirect Immunofluorescence: Cultures of myogenic cells were grown on collagen-coated coverslips in 60-mm-diam petri dishes (five coverslips per dish) and treated with EGTA as described above. At intervals, coverslips were removed and the cells were permeabilized in phosphate-buffered saline (PBS) that contained 5 mM $MgCl_2$ and 0.5% (wt/vol) Triton X-100 for 15 s at room temperature; the cells were then fixed in PBS, 1.75% (vol/vol) formaldehyde at 37°C for 10 min. The coverslips were rinsed in PBS, 0.5% (wt/vol) Triton X-100 for 10 min at room temperature. Triplicate coverslips were incubated for 30 min at 37°C in a humidified atmosphere with either α- or β-spectrin–specific antibodies, or ankyrin antibodies (not shown), diluted 1:10 in PBS, 0.5% (wt/vol) Triton X-100. The coverslips were rinsed briefly and then incubated with fluorescein-conjugated goat anti-rabbit IgG (Miles-Yeda, Rehovot, Israel) diluted 1:150 in PBS, 0.5% (wt/vol) Triton X-100 for 30 min at 37°C. Coverslips were rinsed briefly as above, mounted in Elvanol (58), and photographed with Kodak Tri-X film with a phase/epifluorescence microscope (E. Leitz, Inc.).

RESULTS

Steady State Protein Levels of Ankyrin and α- and β-Spectrin Increase between Presumptive Myoblasts and Fully Differentiated Myotubes

Recent studies on the expression of ankyrin and α- and β-spectrin during myogenesis in vitro have demonstrated significant increases in the amounts of their respective mRNAs (39) and levels of protein synthesis (44) between rapidly dividing presumptive myoblasts and fully differentiated postmitotic myotubes. To determine whether these increases are also reflected in the accumulation of these proteins at steady state, we compared the amount of each protein in cells from a 12-h and an 8-d myogenic culture by immunoblotting; the former comprised ~95% rapidly dividing single cells, whereas the latter comprised ~80% multinucleated myotubes which were fully striated and were undergoing spontaneous contraction. Small amounts of ankyrin (Fig. 1 *a*; M_r 237,000) and α-spectrin (Fig. 1 *c*; M_r 240,000) were detected in cultures of 12-h presumptive myoblasts, but little or no β-spectrin was detected at steady state (Fig. 1 *e*; M_r 220,000–225,000). However, a similar analysis of 8-d myotubes revealed that these cells accumulated significantly greater amounts of ankyrin (Fig. 1 *b*), α-spectrin (Fig. 1 *d*), and β-spectrin (Fig. 1 *f*) than did the 12-h myoblasts. Quantitation of the relative amounts of each protein indicated a difference of ~10–20-fold between myoblasts and mature myotubes. It should be noted that since different titre antisera were used, and that the efficiency of transfer from the polyacrylamide gel to nitrocellulose varies between different proteins (see reference 23), the relative amounts of different proteins should not be compared directly.

Concomitant with the increase in amounts of ankyrin and α- and β-spectrin, there was a decrease in the amount of γ-spectrin during myogenesis; this is demonstrated by immunoprecipitation (Fig. 1, *i* and *j*). Although γ-spectrin did not react with either the α-spectrin or β-spectrin antibodies used here (see also reference 43), it could be detected as a result of the quantitative reassociation of SDS-solubilized γ-spectrin with α-spectrin during incubation with α-spectrin antibodies. The results show that γ-spectrin was present in equimolar amounts with α-spectrin in cultures of 12-h myoblasts but was undetectable in a similar immunoprecipitate from a culture of 8-d myotubes.

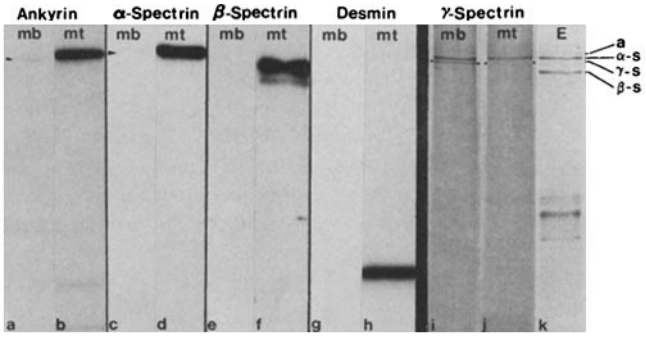

FIGURE 1 Comparison of protein levels of ankyrin, α-, β-, and γ-spectrin, and desmin between 12-h myoblasts and 8-d myotubes grown in vitro. Chick myogenic cell cultures were prepared as described in Materials and Methods. Cells were solubilized in SDS-sample buffer and processed for immunoblotting (*a–h*) with either ankyrin (*a* and *b*), α-spectrin (*c* and *d*), β-spectrin (e and *f*) or desmin-specific antibodies (*g* and *h*). The resulting autoradiograms are presented; exposures were for 0.25–36 h. The amount of total protein loaded from myoblasts (*mb*) and myotubes (*mt*) was normalized to DNA. Note that small amounts of ankyrin (a) and α-spectrin (c) are detected in myoblasts (arrowheads). Lanes *i* and *j* are Coomassie Blue–stained SDS gels of immunoprecipitates of SDS-solubilized extracts of myoblasts (*mb*, lane *i*) and 8-d myotubes (*mt*, lane *j*) with α-spectrin antibodies under conditions in which γ-spectrin (area between dots) is quantitatively co-immunoprecipitated (see Results). Note that the amount of α- (and γ-) spectrin immunoprecipitated was limited by the amount of antibodies used, which indicates erroneously that there are similar amounts of α-spectrin in myoblasts and myotubes (see, however, lanes c and *d*). Lane *k* is a Coomassie Blue–stained SDS gel of chicken erythrocyte membrane proteins: *a*, ankyrin; *α-S*, α-spectrin; *γ-S*, γ-spectrin (not present in erythrocytes); and *β-S*, β-spectrin.

Rapid Accumulation of Ankyrin and α- and β-Spectrin Is An Early Event in Myogenesis

To determine the stage of muscle differentiation at which ankyrin and α- and β-spectrin accumulate, we analyzed cultures of myogenic cells between 12 and 96 h by SDS PAGE, then by immunoblotting with antibodies specific for each of these proteins. The autoradiograms are presented in Fig. 2, and the quantitation in Fig. 3. With an initial cell inoculum of 6×10^6/100-mm petri dish, presumptive myogenic cells began to fuse with one another after 14 h in culture; 50% of the cells fused after ~36 h, and a maximum of 70–80% fused by 48 h (Fig. 3). Analysis of the relative amounts of ankyrin and α-spectrin reveals that the accumulation of these proteins was initiated soon after the onset of cell fusion (Figs. 2 and 3). The apparent rates of accumulation of these proteins appeared to be highest between 30 and 60 h, and then declined by 96 h (Fig. 3).

Rapid Decline in the Steady State Amount of γ-Spectrin Is an Early Event in Myogenesis

To determine whether the decline in the amount of γ-spectrin (Fig. 1) occurs during the early stages of myogenesis,

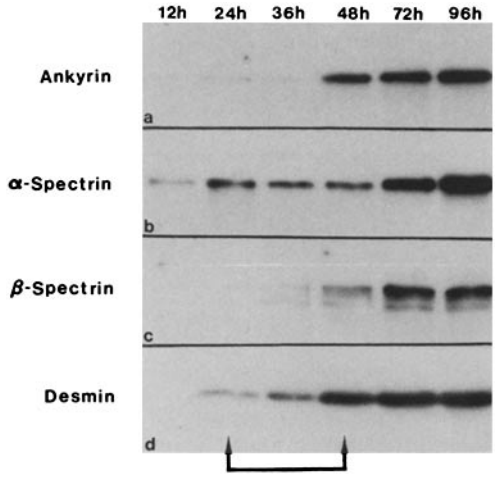

FIGURE 2 Induction in accumulation of ankyrin, α- and β-spectrin, and desmin in early myogenesis. Chick myogenic cell cultures were prepared as described in Materials and Methods. 12, 24, 36, 48, 72, and 96 h after initiation of the culture, cells were solubilized in SDS-sample buffer and processed for SDS PAGE and subsequent immunoblotting with ankyrin (a), α-spectrin (*b*), β-spectrin (c), and desmin-specific antibodies (*d*). The amount of protein loaded was normalized to DNA. The resulting autoradiograms are presented; exposure times were for 0.25–36 h. The arrows denote the time period of the main burst of cell fusion (see Fig. 3).

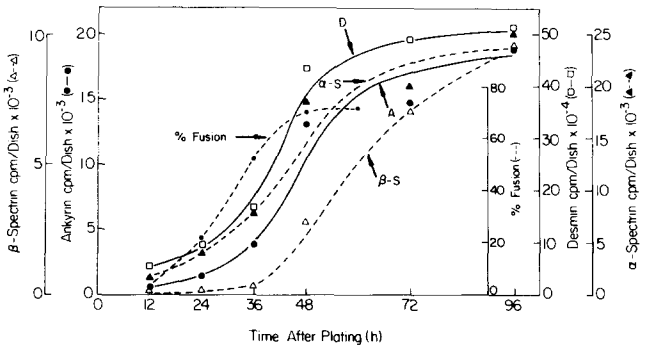

FIGURE 3 Graphic representation of the accumulation of ankyrin (*A*), α-spectrin (*α-S*), β-spectrin (*β-S*), and desmin (*D*) during early myogenesis. After immunoblotting with specific antisera was done, the areas of the nitrocellulose sheets that corresponded to the bands on the autoradiograms shown in Fig. 2 were carefully excised and counted in a gamma counter. The data are presented as counts per minute per petri dish; the variation in cell inoculum between petri dishes was <15%. It should be noted that using half the amount of sample for SDS-gel electrophoresis resulted in ~50% fewer counts after immunoblotting was done under identical conditions.

we examined 12–48-h cultures of myogenic cells for the presence of γ-spectrin by co-immunoprecipitation with α-spectrin by use of α-spectrin–specific antibodies (Fig. 4). Since it is difficult to quantitate accurately the relative amounts of γ-spectrin by Coomassie Blue staining, each time point is represented by immunoprecipitates from duplicate petri dishes to show qualitatively the reproducibility of the staining patterns of the spectrin subunits. 12 and 18-h cultures of presumptive myogenic cells accumulated at steady state equimolar amounts of α- and γ-spectrin; note that little or no β-spectrin was detected at this stage (see also Fig. 2). However, 24-h cultures of myogenic cells exhibited slightly lower amounts of γ-spectrin than α-spectrin. The decline in the amount of γ-spectrin relative to the amount of α-spectrin was more evident in 36 and 48 h cultures, by which time small amounts of β-spectrin were detected (see also Fig. 2).

Levels of Synthesis of α-, γ-, and β-Spectrin Are Not Affected by Blocking Cell Fusion

The results described above indicate that the time of myogenic cell fusion and the onset of terminal differentiation is critical in initiating the accumulation of ankyrin and α- and β-spectrin. To determine whether there are control mechanisms that regulate the synthesis or accumulation of these proteins during cell fusion, we sought to analyze the effect of inhibiting fusion of myogenic cells with EGTA. Previous studies showed that low concentrations of EGTA (< 2 mM) completely inhibit cell fusion (14, 53, 54, 60, 61, 62) but do not affect the synthesis of certain muscle-specific proteins, including creatine phosphokinase (14), myosin heavy chain (53), and the acetylcholine receptor (54, 62).

Fig. 5 *A* shows the increase in DNA per petri dish during early myogenesis. The difference observed between the levels in the control (−) and EGTA-treated (+) cells reflects a small loss of cells that rounded up in the presence of EGTA and were removed during changes of media. Fig. 5 *B* shows the fusion kinetics of the cells in the presence (+) and absence (−) of EGTA. EGTA was added to the culture before any significant cell fusion. There was little or no fusion during the course of the EGTA treatment. Furthermore, few cells exhibited any degree of alignment (see Fig. 8), which normally occurs before cell–cell fusion. The control cells exhibited a normal pattern of fusion that was maximal between 20 and 40 h after initiation of the culture. Cell fusion in the EGTA-treated culture occurred 2 h after removal of the EGTA from the medium, and within 5 h, ~50% of the cells had fused. The degree of cell fusion reached a maximum by 11 h (Fig. 5 *B*).

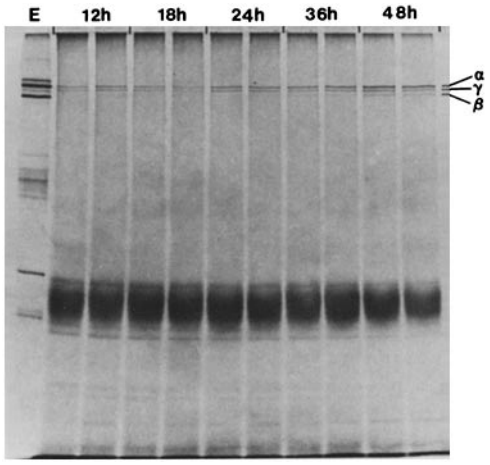

FIGURE 4 Analysis of protein levels of γ-spectrin during early myogenesis. Cultures of chick myogenic cells were prepared and, at given times (12, 18, 24, 36 and 48 h), processed for immunoprecipitation with a mixture of α- and β-spectrin specific antibodies as described in Materials and Methods. Under the conditions of immunoprecipitation used, γ-spectrin is quantitatively coimmunoprecipitated as a complex with α-spectrin (see Results). (*E*). Coomassie Blue–stained SDS gel of chicken erythrocyte membrane proteins. (α), α-spectrin, (γ), γ-spectrin, and (β) β-spectrin.

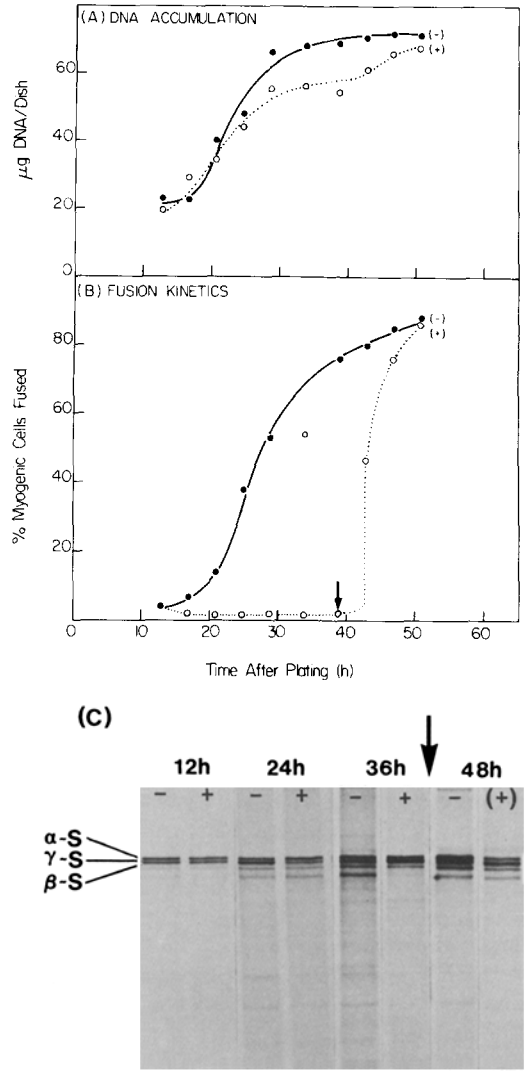

FIGURE 5 Effect of EGTA on the levels of synthesis of α-, β-, and γ-spectrin. Cultures of chick myogenic cells were prepared as described in Materials and Methods. EGTA (2 mM) was added to one-half of the culture (+); the rest were the control (−). During the experiment, the kinetics of cell fusion (*B*) were calculated as described in Materials and Methods. At given intervals, cells were labeled metabolically with [^{35}S]methionine and then processed for immunoprecipitation of spectrin subunits (*α-S*, α-spectrin; *β-S*, β-spectrin; and *γ-S*, γ-spectrin) and fluorography (*C*) as described in Materials and Methods. At the same time, aliquots of cell suspension were removed to determine the DNA concentration (*A*). The arrows mark the time of removal of EGTA from the culture medium. Note that a small amount of [^{35}S]methionine-labeled myosin which runs below β-spectrin is precipitated (nonspecifically; not shown) due to the low ionic strength of the immunoprecipitation buffer.

Cells were labeled metabolically with [^{35}S]methionine, and the level of synthesis of different spectrin subunits was determined by immunoprecipitation and fluorography (Fig. 5 *C*). At 12 h, the principal spectrin isoforms synthesized were α- and γ-spectrin; in addition, a small amount of [^{35}S]methionine incorporation could be detected in β-spectrin, as shown previously (44). By 24 h, the level of synthesis of β-spectrin appeared to have increased as compared with that at 12 h in both the control and EGTA-treated cells. At 36 h, when the EGTA block was removed, α-, γ-, and β-spectrin were synthesized in the control and EGTA-treated cells. Although it appears from the fluorograms that the amount of [^{35}S]methionine incorporation into the spectrin isoforms is greater in the control cells than in those treated with EGTA at 36 h, note that the total number of cells is less in the EGTA-treated culture than in the control due to rounding up and removal during changes of media. This is reflected in the difference in DNA (Fig. 5*A*). Indeed, quantitation of the amount of radioactivity incorporated into these three spectrin subunits normalized to DNA (Fig. 5*C*) showed no difference in normal and EGTA-treated cells (data not shown).

Accumulation of Ankyrin and α- and β-Spectrin Is Inhibited in Fusion-blocked Cells

The results on the incorporation of [^{35}S]methionine into spectrin isoforms described above demonstrate that blocking cell fusion with EGTA had little or no effect on the time-dependent induction of β-spectrin synthesis or, qualitatively, on the levels of synthesis of different spectrin isoforms compared with control cells. By contrast, analysis by immunoblotting of the steady state levels of ankyrin (Figs. 6,*A* and *B* and 7*B*) and α-spectrin (Figs. 6,*C* and *D*, and 7*C*) revealed significant differences between the control and EGTA-treated cells. In control cells, the apparent rate of accumulation of ankyrin (Fig. 7*B*) and α-spectrin (Fig. 7*C*), respectively, was similar during early myogenesis. Cells treated with EGTA exhibited a rate of accumulation of these proteins which was similar to that in control cells for the first 19 h. However,

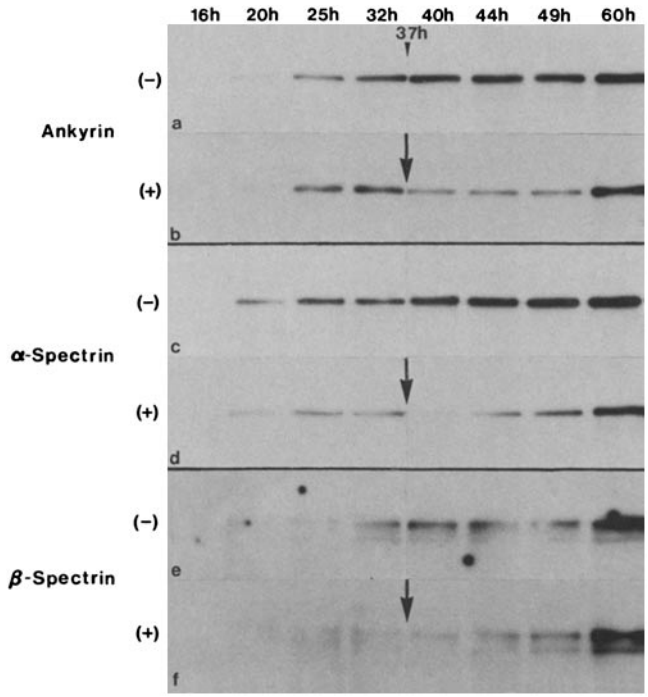

FIGURE 6 Analysis of protein levels of ankyrin and α- and β-spectrin in control and EGTA-treated myogenic cells. Chick myogenic cells were prepared as before and cultured in the absence (−) and presence (+) of 2 mM EGTA as described in Materials and Methods. At given times after initiation of the culture (16, 20, 25, 32, 40, 44, 49, and 60 h), cells from both cultures were processed for SDS PAGE and then immunoblotted with antibodies specific for ankyrin (*a* and *b*), α-spectrin (c and *d*), and β-spectrin (e and *f*). The resulting autoradiograms are presented; exposures were for 0.25–36 h. The arrow marks the time (37 h) of removal of EGTA from the culture medium.

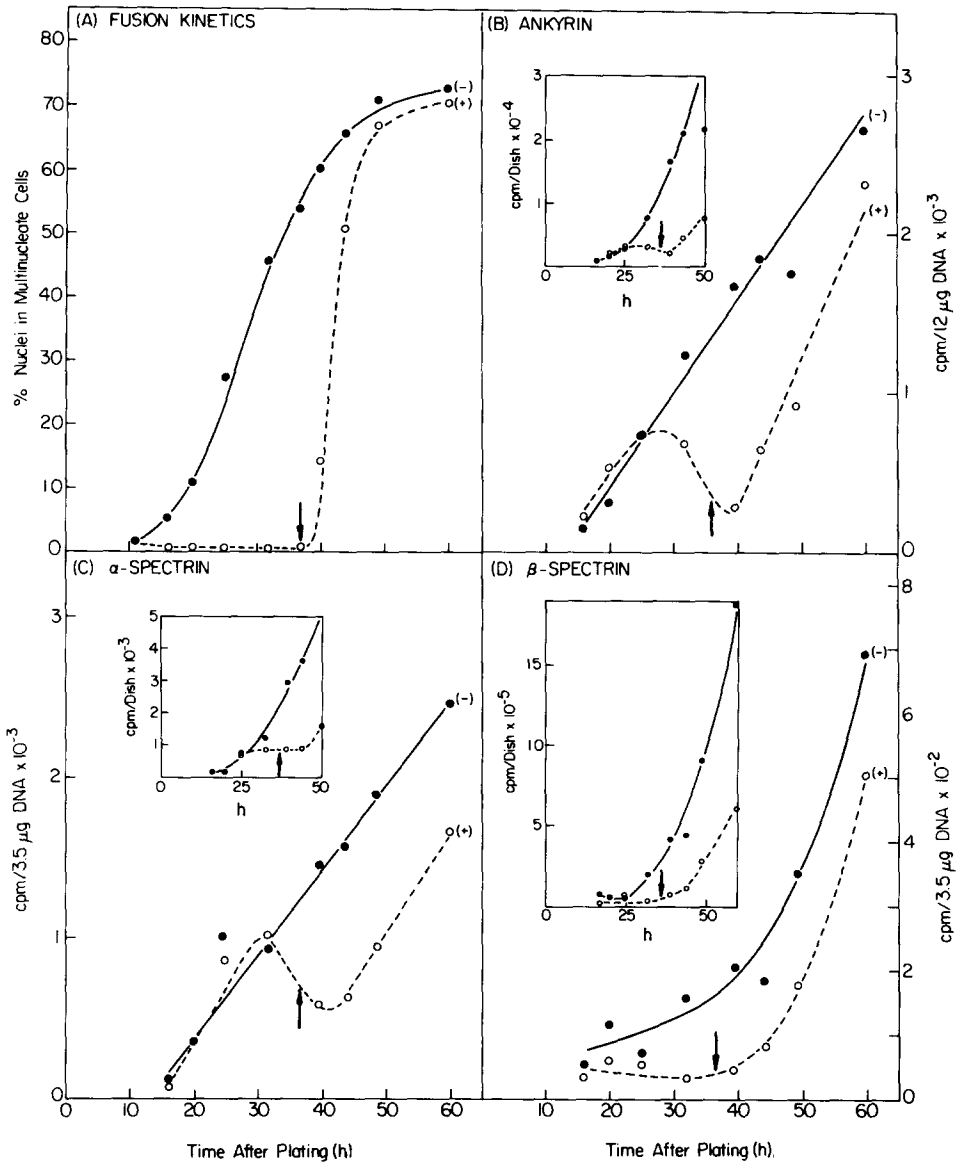

FIGURE 7 Graphic presentation of the protein levels of ankyrin and α- and β-spectrin in control and EGTA-treated cells. For quantitation, the areas of the nitrocellulose sheets that corresponded to the bands on the autoradiograms presented in Fig. 6 were carefully excised and counted in a gamma counter. The data are presented as counts per minute normalized to DNA (main panels), or per petri dish (insets); all lines were drawn by eye. The arrows mark the time of removal of EGTA from the culture medium. Fusion kinetics in control (−) and EGTA-treated cells (+) were calculated as described in Materials and Methods.

after this time, the kinetics of accumulation changed (Fig. 7, *B* and *C*). When the data were normalized to DNA, the relative amounts of ankyrin and α-spectrin appeared to decline. However, when the total relative amount of each protein per dish was calculated (see inserts Fig. 7, *B* and *C*), the amounts of ankyrin and α-spectrin were shown to remain constant between ~30 h and the time of release from the EGTA block; during this period, there was an approximately twofold increase in DNA (for example, see Fig. 5*A*).

Immediately after removal of EGTA from the culture medium (marked by an arrow in Fig. 7), the relative amounts of ankyrin and α-spectrin remained constant (Fig. 7, *B* and *C*, insets). Accumulation of these proteins was not detected until 3–5 h after release from the EGTA block (Fig. 7, *B* and *C*). This point of re-induction corresponded to a time when ~50% of the cells have already fused. Interestingly, the subsequent rate of accumulation of ankyrin and α-spectrin in the EGTA-treated cells was similar to that in the control cells (Fig. 7, *B* and *C*).

The accumulation of β-spectrin in early myogenesis exhibited kinetics different from those of ankyrin and α-spectrin (Fig. 7*D*; see also Fig. 1). In control cells, the apparent rate of accumulation of β-spectrin was relatively slow until ~25–30 h after initiation of the culture. Then the relative amount of β-spectrin increased significantly (Fig. 7*D*). Cells treated with EGTA accumulated only small amounts of β-spectrin (see Fig. 6*F*). After removal of EGTA from the culture medium, the amount of β-spectrin remained low for ~5 h, whereupon there was a significant induction in the accumulation of this protein (see Fig. 6*F*). This apparent rate of accumulation was similar to that observed in control cells after 30 h in culture (Fig. 7*D*).

Accumulation of Spectrin Subunits (α- and β-) in Fusion-blocked Cells: Analysis by Indirect Immunofluorescence

We analyzed cultures of control and EGTA-treated cells by immunofluorescence to determine whether the accumulation of spectrin isoforms in the absence of cell fusion was characteristic of all cells, or only a subpopulation. Immunofluorescence with α-spectrin antibodies demonstrated that all cells were positive in both 37-h control cultures (Fig. 8, *A* and *C*) and in cultures treated for 26 h with EGTA (Fig. 8, *B* and *D*);

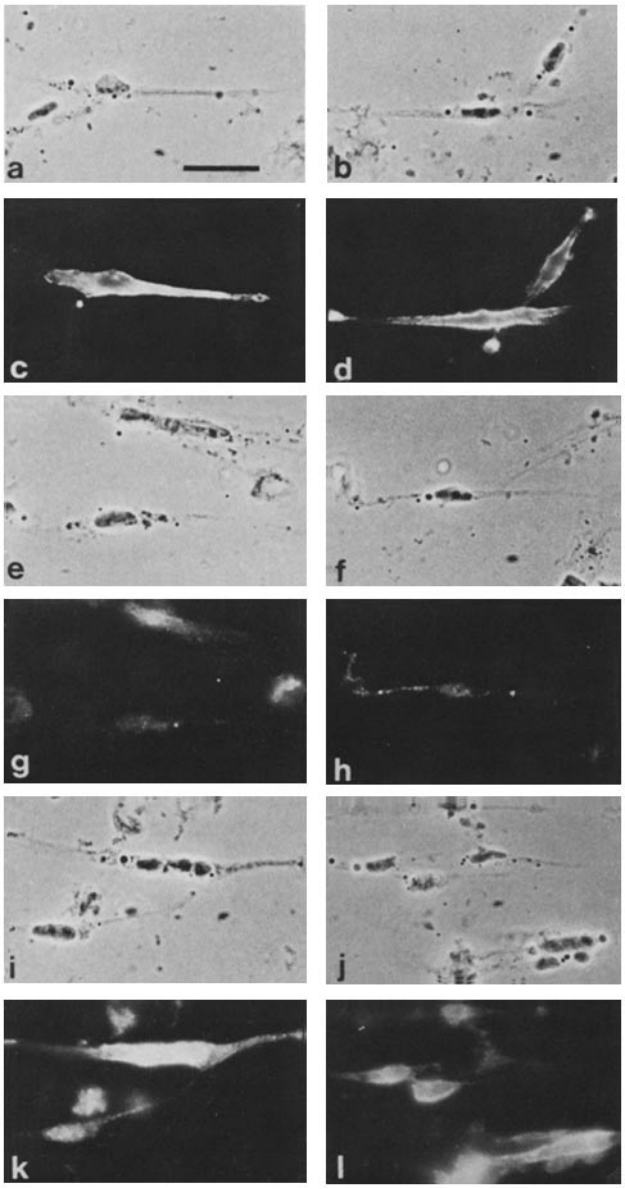

FIGURE 8 Immunofluorescence of control and EGTA-treated cells with α- and β-spectrin-specific antibodies. Cultures of chick myogenic cells were grown on collagen-coated coverslips in the absence (control; *a*, *c*, *e*, *g*, *i*, and *k*) and presence (*b*, *d*, *f*, *h*, *j*, and *l*) of EGTA as described in Materials and Methods. Micrographs are of corresponding phase-contrast and fluorescent images of 37-h culture of cells (26 h in EGTA) stained with α-spectrin antibodies (control, *a* and *c*; EGTA, *b* and *d*); of a 21 h culture of cells (10 h in EGTA) stained with β-spectrin antibodies (control, *e* and *g*; EGTA, *f* and *h*); and of a 37 h culture of cells (26 h in EGTA) stained also with β-spectrin antibodies (control, *i* and *k*; EGTA, *j* and *l*). Bar, 20 μm. × 500.

note that in both cases the α-spectrin fluorescence was localized to the perimeter of the cell, presumably in association with the plasma membrane. Indirect immunofluorescence with β-spectrin antibodies on cells from 21-h control cultures (Fig. 8, *E* and *G*) and cultures treated for 10 h with EGTA (Fig. 8, *F* and *H*) showed that most cells exhibited weak staining which, however, was above background staining with the preimmune serum. However, cells from a 37-h control culture (Fig. 8, *I* and *K*) exhibited a more intense fluorescence than did those from 21-h cultures (Fig. 8, *E* and *G*); in particular, multinucleated cells exhibited strong fluorescence, whereas the intensity of fluorescence in single cells was markedly less (Fig. 8*K*). Similarly, cells from 37-h cultures that had been treated with EGTA for 26 h exhibited a degree of heterogeneity in staining with the β-spectrin antibodies (Fig. 8, *J* and *L*); some cells showed intense staining, similar to the multinucleate cells from the control culture (Fig. 8*K*), whereas other cells exhibited relatively weak fluorescence, similar to that of the cells from the 21-h control cultures (Fig. 8 *G*).

Rapid Accumulation of Desmin in Early Myogenesis Is Not Inhibited by Blocking of Cell Fusion

Analysis by immunoblotting of protein levels of desmin in 12-h myoblasts and 8-d myotubes (Fig. 1, *G* and *H*) demonstrated that there was also a dramatic accumulation of this muscle-specific intermediate filament protein together with ankyrin and α- and β-spectrin during myogenesis. Furthermore, the main burst of desmin accumulation occurred within the first 96 h in early myogenesis (Figs. 2 *D* and *3*). Recent results showed also a dramatic increase in desmin mRNA levels during this period (12). Since desmin, ankyrin, and α- and β-spectrin appeared to accumulate concurrently and with similar kinetics, we sought to determine whether or not the accumulation of desmin was also inhibited by the blocking of myogenic cell fusion. Aliquots of SDS-solubilized cell extracts, from the same samples that were used in the analysis of ankyrin and spectrin (see Figs. 6 and 7), were processed for immunoblotting with desmin-specific antibodies (Fig. 9), and quantitation was performed as described above (Fig. 10). The results demonstrate clearly that the accumulation of desmin is similar if not identical in control cells and cells treated with EGTA. These results contrast sharply with the observed inhibition of accumulation of ankyrin and α- and β-spectrin in EGTA-treated cells (compare Fig. 10 with Fig. 7).

DISCUSSION

Accumulation of Ankyrin and α- and β-Spectrin and the Decline of γ-Spectrin Are Early Events in Myogenesis

Analysis by immunoblotting of the amounts of ankyrin and α- and β-spectrin during the first 96 h of muscle differentiation in vitro revealed that the accumulation of these

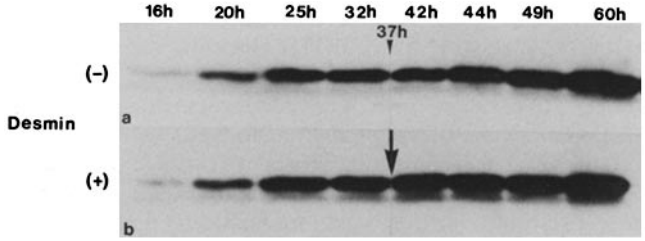

FIGURE 9 Analysis of protein levels of desmin in control and EGTA-treated cells. Myogenic cells were treated with (+) or without (−) EGTA and processed for immunoblotting with desmin antibodies in parallel with ankyrin and α- and β-spectrin antibodies as described in the legend to Fig. 6. The resulting autoradiograms are presented. Exposure times were for 30–60 min.

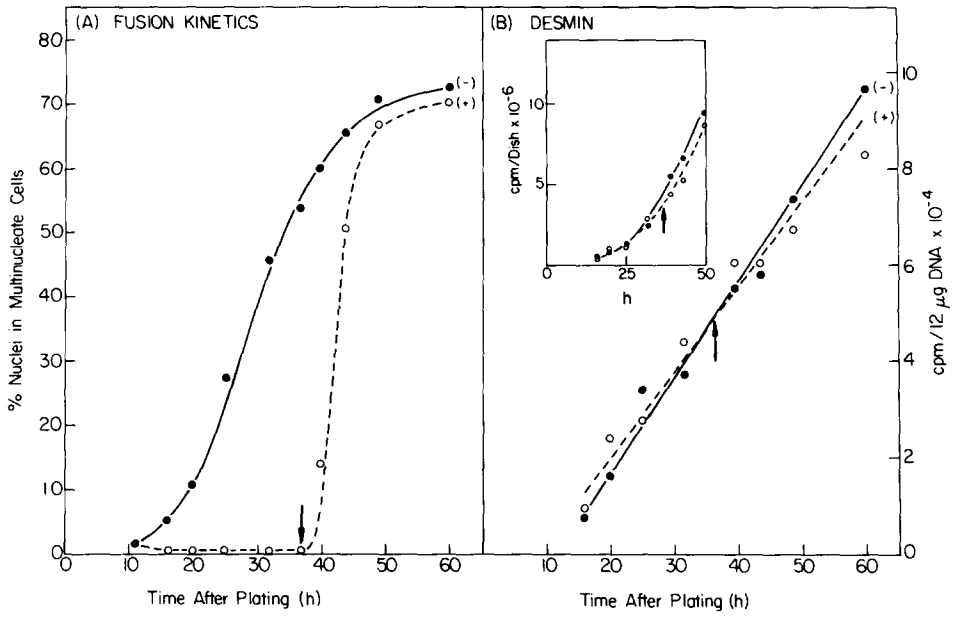

FIGURE 10 Graphic presentation of the protein levels of desmin in control and EGTA-treated cells. Relative amounts of desmin in cultures of control (−) and EGTA-treated cells (+) were quantitated from the nitrocellulose filters after the immunoblotting procedure as described in the legend to Fig. 6 and in Materials and Methods, and are presented (*B*) as counts per minute normalized to DNA (main panel) or per petri dish (inset). *A* shows the analysis of the fusion kinetics in the presence (+) and absence (−) of EGTA. The arrow marks the time of removal of EGTA from the culture medium. All lines were drawn by eye.

proteins occurs early in myogenesis. During this period, the presumptive myoblasts undergo a limited number of cell divisions and then fuse with one another to form postmitotic, multinucleated myotubes. With the high cell inoculum used in the present study, the main burst of myogenic cell fusion occurred between 20 and 47 h after initiation of the culture. Analysis of SDS-solubilized extracts of cells during this time period by immunoblotting demonstrated that the time of induction in the accumulation of ankyrin and α-spectrin was delayed 5–12 h after the main burst of cell fusion; the lag period for β-spectrin was ~24 h. A more exact determination of the temporal relationship between cell fusion and the onset of the accumulation of ankyrin and spectrin was obtained by an analysis of the re-induction of protein accumulation after release of cells that had been inhibited from fusing by EGTA. Previous studies have shown that EGTA treatment results in increased synchrony for cell fusion (14, 53, 54, 60–62), and the data presented here confirm this. The main burst of cell fusion was initiated within 3 h after release from the EGTA block, and was essentially complete 7 h later (as compared with a period of 27 h in the control culture). Analysis by immunoblotting indicated a lag period of 3–5 h in the onset of accumulation of ankyrin and α-spectrin after release of cells from the EGTA block, which suggests that the induction in the accumulation of these proteins may coincide closely with cell fusion and the onset of muscle terminal differentiation. Interestingly, the induction in the accumulation of β-spectrin also appeared to coincide closely with cell fusion after release of the cells from the EGTA block. This contrasts with the delay of ~12–24 h in the onset of accumulation of β-spectrin after the main burst of cell fusion in control cultures (see next section for further discussion).

The close temporal relationship between cell fusion and the induction in the accumulation of ankyrin and spectrin parallels that shown previously for other muscle-specific proteins, including myosin heavy chain (53, 63), α-actin (59, 63), acetylcholine receptor (31, 54), acetylcholinesterase (54), and creatine phosphokinase (14, 61). As in the case of these muscle-specific proteins, synthesis of α- and β-spectrin was detected before the main burst of cell fusion in control cells and EGTA-blocked single cells, which indicates that cell fusion per se is not an obligatory step in the initiation of spectrin synthesis and accumulation (see below).

The period of cell fusion and initiation of muscle terminal differentiation is marked also by the onset of the decline in the amount of γ-spectrin. This decline was first detected 36 h after initiation of the culture, which corresponded to a lag of ~12 h after the main burst of cell fusion. This time lag was similar to that observed for the induction of accumulation of α- and β-spectrin. This suggests that there may be a temporal relationship associated with the onset of terminal differentiation, between the accumulation of the muscle-specific spectrin subunits (α- and β-spectrin) and the decline in the spectrin subunit characteristic of presumptive myoblasts (γ-spectrin). Indeed, a similar relationship has been shown for the differential expression of muscle and nonmuscle isoforms of actin during myogenesis (20, 59, 63, 66).

A temporal relationship between the onset of accumulation of ankyrin and α- and β-spectrin, and terminal differentiation observed here in muscle cells, has also been found in neurons of the chick retina and cerebellum (48). In neurons, however, distinct complexes of $\alpha\gamma$- and $\alpha\beta$-spectrin are present in the mature cell, which are distributed in different parts of the cell; $\alpha\beta$-spectrin is present exclusively in the perikarya and dendrites and is actively segregated from $\alpha\gamma$-spectrin, which is a component of axonal transport (29). The asymmetric distribution of these two spectrin complexes in neurons is paralleled by the steady state disribution of two isoforms of ankyrin; α-ankyrin (M_r 260,000) is developmentally expressed and co-distributed with $\alpha\beta$-spectrin upon terminal differentiation, whereas β-ankyrin (M_r 237,000) is expressed in postmitotic cells and is co-distributed with $\alpha\gamma$-spectrin (48). Using the same antiserum that was used in the analysis of neuronal ankyrin (46), we detected a single reactive protein in muscle cells that has a similar apparent molecular weight in myoblasts, mature myotubes, and adult chicken muscle (not shown; see reference 46). At present, we do not know whether muscle ankyrin is structurally related to either of the neuronal isoforms of ankyrin, or whether it has a higher binding affinity for $\alpha\beta$-spectrin than for $\alpha\gamma$-spectrin, as has been shown recently for erythrocyte ankyrin (7, 11). However, our results do show clearly that the accumulation of this isoform of

ankyrin in early myogenesis coincides with the accumulation of $\alpha\beta$-spectrin and the decline in the amounts of γ-spectrin. Hence, by analogy to the different binding affinities of erythrocyte ankyrin for $\alpha\gamma$- and $\alpha\beta$-spectrin, muscle ankyrin may play an important role in the differential stabilization of newly synthesized $\alpha\beta$-spectrin on the membrane, and the subsequent accumulation of this spectrin isoform during early myogenesis.

Synthesis and Assembly of the Membrane-Skeleton (Ankyrin and Spectrin) in Early Myogenesis Are Not Coupled Events

The increases in the amounts of mRNA (39), rates of synthesis (reference 44 and this study), and accumulation at steady state (this study) of ankyrin and α- and β-spectrin during early myogenesis could be interpreted as evidence for a coupling among the three stages of protein expression, namely transcription, translation, and protein accumulation. Indeed, a coupling of this nature has been inferred from previous studies on the accumulation of other muscle-specific proteins in early myogenesis, including myosin heavy chain (13, 53, 63) and α-actin (59). However, by analyzing the effect of the inhibition of cell fusion on the accumulation of ankyrin and spectrin, we have demonstrated clearly that after ~19 h, accumulation of these proteins ceases abruptly, whereas there is no significant difference in the level of synthesis of α- and β-spectrin between the control and EGTA-treated cells.

That the accumulation of ankyrin and $\alpha\beta$-spectrin, but not their levels of synthesis, was affected by the inhibition of cell fusion strongly suggests that the synthesis of these proteins and their subsequent accumulation as a complex, although concurrent, are not coupled events, and that the accumulation of these newly synthesized proteins may be determined by control mechanisms operative at the posttranslational level. Presently, the exact nature of these control mechanisms is unknown. However, the fact that these proteins are synthesized but do not accumulate at steady state indicates that a critical step in the stable assembly of newly synthesized ankyrin and α- and β-spectrin onto the membrane is blocked. This might result in the rapid degradation of unassembled proteins in the cytosol, and hence the observed cessation of protein accumulation. An implicit assumption is that newly synthesized proteins that have assembled onto the plasma membrane resist intracellular catabolism. This is supported by the observation that the absolute amounts of ankyrin and α-spectrin that accumulate in cells within the first 19 h of EGTA treatment then remain constant in the absence of further accumulation. However, we cannot rule out the possibility that there is an exchange equilibrium between assembled and unassembled proteins, although previous studies on the assembly of the membrane-skeleton in erythrocytes indicate that it is unlikely (8, 37, 38). Furthermore, the fact that newly synthesized and assembled spectrin ($\alpha\gamma$) is relatively stable to intracellular catabolism has been demonstrated in myoblasts (44) and fibroblasts (W. J. Nelson, and E. Lazarides, unpublished results). In addition, recent studies on the assembly of the chicken erythrocyte membrane-skeleton have demonstrated that the fraction of ankyrin and spectrin subunits synthesized in excess of available binding sites on the plasma membrane are rapidly degraded in the cytosol, whereas those assembled are relatively stable (8, 37, 38). An important concept derived from these studies is that the stable assembly of the erythrocyte membrane-skeleton as a whole ultimately depends on the synthesis and accumulation on the membrane of a specific protein(s) that acts as a binding site for the ankyrin-spectrin complex (for a review, see reference 31); in the case of the erythrocyte, this protein is thought to be the anion transporter (band *3*), to which ankyrin has been shown to bind with high affinity (3, 6, 24). By analogy to the assembly of the membrane-skeleton in erythrocytes, the accumulation of ankyrin and α- and β-spectrin in muscle cells may also be determined by the availability of a specific membrane protein. However, in muscle cells the identity of this protein is still unknown. That the accumulation of muscle-specific proteins may be determined posttranslationally is suggested also by the results of Munson et al. (42) and Olson et al. (50–52) on the expression of the acetylcholine receptor and creatine phosphokinase during differentiation of the BC_3H1 nonfusing muscle-like cell line.

Although the results of this study demonstrate the presence of a control mechanism(s) at the posttranslational level that determines the accumulation of elements of the membrane skeleton, they also show that a common regulatory mechanism at this level does not determine the accumulation of all structural proteins during early myogenesis. This is illustrated by the fact that the accumulation of desmin intermediate filaments is not affected by the inhibition of cell fusion with EGTA. This dramatic difference between the effect of EGTA treatment on spectrin/ankyrin and desmin accumulation may be due to the fact that desmin assembles in vitro in the absence of cofactors or other proteins, and hence its rate of accumulation may be limited only by the amount of unassembled subunits. Indeed, in vivo studies on the assembly of vimentin intermediate filaments in chicken embryo erythroid cells have shown that although newly synthesized vimentin enters a soluble pool of unassembled protein, the assembly of these proteins from this pool onto preexisting intermediate filaments is an extremely rapid process ($t_{1/2} < 5$ min) (8), and that once assembled, intermediate-filament proteins appear to be resistant to intracellular catabolism (8, 34). Hence, in early myogenesis the induction in the expression of desmin at the mRNA level (12) and at the level of synthesis (19) may result in the rapid assembly and accumulation of the protein in the cell. Since inhibition of cell fusion does not block desmin synthesis (18), accumulation at the protein level is also not affected, which indicates that accumulation of this cytoskeletal subunit in early myogenesis is not limited at the posttranslational level.

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