Vimentin Filaments Are Assembled from a Soluble Precursor in Avian Erythroid Cells

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ABSTRACT The synthesis and assembly of vimentin was studied in erythroid cells from 10-d-old chicken embryos. After various periods of [^{35}S]methionine incorporation, cells were lysed in a Triton X-100-containing buffer and separated into a soluble and an insoluble (cytoskeletal) fraction. Analysis of these two fractions by two-dimensional gel electrophoresis shows that vimentin is almost exclusively present in the cytoskeletal fraction and that newly synthesized vimentin is rapidly incorporated into this fraction. However, after a short pulse-labeling period, a prominent labeled protein at the position of vimentin is present in the soluble fraction. By immunoautoradiography and immunoprecipitations with vimentin antibodies, this protein was identified as vimentin. The vimentin in the soluble fraction is not sedimented by high speed centrifugation, suggesting that it does not consist of short filaments. After different pulse-labeling periods, assembly of newly synthesized vimentin in the cytoskeletal fraction increases linearly, while the radioactivity in the soluble vimentin remains constant. During a 2-h pulse-chase period, the vimentin in the soluble fraction is chased into the cytoskeletal fraction, with a half-life of 7 min. These results suggest that in chicken embryo erythroid cells newly synthesized vimentin is rapidly assembled into filaments from a soluble precursor.

Higher eukaryotic cells contain a class of cytoplasmic filaments with a diameter of ~100 Å, known as intermediate filaments. By immunological and biochemical criteria, at least five different classes of intermediate filament proteins can be identified. These include keratin, desmin, vimentin, neuro-, and glial filaments (for a review, see reference 13). However, very little is known about the function of these filaments. The spontaneous assembly of intermediate filament proteins, purified under denaturing conditions, into filaments has been extensively studied in vitro (4, 19, 21, 25), but the regulation of the assembly of intermediate filaments in vivo has received little attention. To investigate this question we have chosen chicken embryo erythroid cells as a model system.

Chicken erythrocytes are nucleated but contain very few organelles. Like mammalian erythrocytes, they contain a submembranous spectrin-actin network (3, 8, 20) but also a marginal band of microtubules (1). Recently, it has been shown that avian erythrocytes also contain intermediate filaments (8, 9, 23, 24). They are composed of vimentin (8, 9) and synemin, an intermediate filament-associated protein originally described in muscle cells (7). By electron microscopy the vimentin filaments appear to have a very close association with the plasma membrane as well as the nucleus, suggesting that these filaments form a three-dimensional network interlinking the nucleus and the plasma membrane, thus functioning in positioning the nucleus within the cell (8, 9, 10, 23, 24). Vimentin-containing filaments are also associated with purified membranes from these cells, further suggesting that vimentin is associated with some component of the plasma membrane (8, 9). Treatment of these cells with a nonionic detergent leaves an insoluble cytoskeleton composed of the nucleus, the spectrin-actin network, and intermediate filaments (9).

In this study we have investigated the synthesis and assembly of vimentin in chicken embryo erythroid cells. Cells were lysed in a Triton X-100-containing buffer to separate the intermediate filaments in the cytoskeletal fraction from soluble proteins (soluble fraction). Electrophoretic analysis shows that almost all of the vimentin is present in the cytoskeletal fraction. However, after a short pulse-labeling period of cells with [^{35}S]methionine, a large fraction of the newly synthesized vimentin appears in the soluble fraction. Furthermore, the amount of this soluble form of vimentin stays constant during a labeling period of 2 h and can be chased into the cytoskeletal fraction. These results show that vimentin assembles into intermediate filaments from a soluble precursor pool of newly synthesized vimentin.

MATERIALS AND METHODS

Preparation of Cells: Erythroid cells were isolated from 10-d-old chicken embryos. The embryos were bled in Earle's balanced salt solution, the resulting cell suspension was filtered through four layers of cheesecloth, and the erythroid cells were collected by centrifugation in a clinical centrifuge. The cells

were resuspended in 155 mM choline chloride, 5 mM HEPES, pH 7.1, and centrifuged again. This was repeated four times with removal of any buffy coat each time.

Labeling of Cells: The cells were washed twice in methionine-free minimal essential medium (MEM) and resuspended at a 10% (vol/vol) concentration in methionine-free MEM containing 10% dialyzed calf-serum prewarmed to 37°C. The cell suspension was incubated at 37°C for 15 min before the addition of [^{35}S]methionine (200–400 μCi/ml; 1,000–1,200 Ci/mmol, New England Nuclear, Boston, MA). The cells were then labeled for different periods of time (3–120 min). For each time point, an equal number of cells was used, usually 1.2–2.5 × 10^8 cells. For the pulse-chase experiment, further incorporation of [^{35}S]methionine was inhibited by the addition of cold methionine (0.4 mM) after a 3-min labeling period; the incubation was then continued for different time periods (5–120 min). At the end of the labeling period, 10 vol of 155 mM choline-chloride, 5 mM HEPES, pH 7.1, were added at 0°C, and the cells were harvested by centrifugation. The cells were lysed in 4 vol of lysis buffer containing 150 mM NaCl, 10 mM Tris-HCl, pH 7.2, 5 mM $MgCl_2$, 2 mM EGTA, 0.25 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoride (PMSF), and 1% Triton X-100. The lysates were then centrifuged at 10,000 *g* for 5 min. The supernatants were removed (soluble fraction) and the pellets resuspended with lysis buffer to the original volume (cytoskeletal fraction). Solid urea and β-mercaptoethanol were added to the soluble and cytoskeletal fractions to give a final concentration of 9.5 M and 2.5%, respectively. The samples were subsequently analyzed by two-dimensional gel electrophoresis (see below).

Quantitation of [^{35}S]Methionine-labeled Protein: The vimentin spots from dried two-dimensional gels were cut out and transferred to scintillation vials. The gel pieces were rehydrated in 1 ml of 100 mM NH_4CO_3, 0.1% SDS, and the proteins were eluted by incubating the vials at room temperature for 12–20 h with constant agitation. Thereafter, 10 ml of Aquasol-2 was added and the radioactivity determined by scintillation counting.

Total radioactivity incorporated into proteins was determined by spotting 10 μl of each sample on a filter paper (Whatman 3MM). The filter papers were immersed for 30 min in 10% trichloroacetic acid, for 30 min in 5% trichloroacetic acid, and twice, for 30 min each, in ethanol at 0°C. The filter papers were dried and counted, after addition of Aquasol-2.

PAGE: Proteins were separated by one-dimensional SDS PAGE based on the system of Laemmli (12) as modified and described previously (11). Two-dimensional isoelectrofocusing was performed according to the method of O'Farrel (18) with the modifications described previously (11), except that the gels from the first dimension were loaded directly onto the second dimension without equilibration in sample buffer. 12.5% polyacrylamide gels were used for separation in the second dimension as described above. The gels were processed for fluorography with En^{3}Hance (New England Nuclear), dried, and exposed to Kodak XAR-5 x-ray film. For the immunoprecipitation gel (Fig. 6), an intensifying screen was used.

Immunoautoradiography: Erythroid cells from 10-d-old chicken embryos were lysed in lysis buffer and separated into a soluble and a cytoskeletal fraction as described above. The two fractions were analyzed by two-dimensional gel electrophoresis and subjected to immunoautoradiography (2) as described previously (7). Gels were incubated with vimentin antisera (see below) diluted 1,000-fold, followed by ^{125}I-labeled protein A. The gels were dried and exposed to Kodak XAR-5 x-ray film with an intensifying screen for 24 h.

High Speed Centrifugation: Erythroid cells from 10-d-old chicken embryos (100 μl of packed cells) were labeled for 10 min at 37°C with [^{35}S]-methionine. The cells were lysed in lysis buffer (900 μl) and separated into a soluble and a cytoskeletal fraction as described above. The cytoskeletal fraction was resuspended in lysis buffer to the original volume (1,000 μl). Aliquots of the soluble fraction (180 μl) were subjected to high speed centrifugation in an airfuge (Beckman Instruments, Inc., Fullerton, CA) at 150,000 *g* for 5, 15, and 30 min, respectively. The supernatants were removed and the pellets resuspended in 180 μl of lysis buffer. All samples were brought to 9.5 M urea by adding solid urea and to 2.5% by adding β-mercaptoethanol. An equal volume (50 μl) of each sample was then subjected to immunoprecipitation with vimentin antisera (see below).

Immunoprecipitation: Antibodies raised against electrophoretically purified chicken skeletal muscle vimentin have been previously characterized (6). Samples (50 μl) were diluted 10 times with immunoprecipitation buffer containing 20 mM triethanolamine (pH 7.4), 1% NaCl, 5 mM EGTA, 1 mM EDTA, 1% deoxycholate, 1% Triton X-100, and 0.1% SDS, before the addition of antibodies; antibodies (2 μl) were then added and the samples incubated for 2 h (4°C). 50 μl of a 10% (vol/vol) suspension of fixed *Staphylococcus aureus* bacteria (Pansorbin, Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA) were then added and the samples incubated with constant rotation for 2 h at 4°C. The bacteria were pelleted by centrifugation in an Eppendorf microcentrifuge through 120 μl of 1 M sucrose, containing 20 mM triethanolamine (pH 7.4), 10 mM methionine, 20 mM EDTA, 1 mM EGTA, 1% deoxycholate, and 1% Triton X-100. The pellets were resuspended by vortexing in 500 μl of immunoprecipitation buffer and again centrifuged through a sucrose cushion. This was repeated twice. The bacteria were finally suspended in 70 μl of SDS sample buffer, heated for 5 min at 95°C, and pelleted in an Eppendorf microcentrifuge. The supernatants were directly applied onto a 12.5% polyacrylamide gel.

RESULTS

Identification of a Triton X-100 Soluble Form of Vimentin

Erythroid cells from 10-d-old chicken embryos were prepared as described in Materials and Methods. After a 60-min [^{35}S]methionine-labeling period, cells were lysed in a Triton X-100 containing buffer, and the lysate was immediately centrifuged. This resulted in a soluble fraction and an insoluble cytoskeletal fraction. Fig. 1 *A*, *D* show the protein pattern of these two fractions after analysis by two-dimensional gel electrophoresis and staining with Coomassie Brilliant Blue. Within the isoelectric point range examined here (pH 4.0–6.5), the cytoskeletal fraction has a relatively simple protein composition consisting of actin, vimentin, α-spectrin, and nuclear lamins (Fig. 1 *A*, see also reference 9). As can be seen, vimentin is one of the main proteins in the cytoskeletal fraction (Fig. 1 *A*). The soluble fraction on the other hand exhibits a more complex protein pattern (Fig. 1 *D*). A faint Coomassie Brilliant Blue spot is also present in the soluble fraction that migrates in the same position as vimentin in the cytoskeletal fraction (Fig. 1 *D*). The corresponding fluorograms show that vimentin is one of the main [^{35}S]methionine-labeled proteins in the cytoskeletal fraction. However, in the soluble fraction a prominently labeled protein also appears in the same position as vimentin.

To examine whether the protein in the soluble fraction at the position of vimentin was indeed vimentin or some other polypeptide that migrated at the same position, we analyzed two-dimensional gels identical to those described above by immunoautoradiography using vimentin antibodies. Indeed, the vimentin antiserum recognized a polypeptide with identical molecular weight and isoelectric point in both the soluble and the cytoskeletal fraction (Fig. 1 *C*, *F*). The series of polypeptides shown on the autoradiograms as having a slightly lower molecular weight and being more acidic than vimentin represent breakdown products of vimentin (5, 14). The nature of the two spots recognized by the vimentin antibodies having a slightly lower molecular weight and a more basic isoelectric point than vimentin is not known. However, similar polypeptides have been described from other cell types to react with these vimentin antibodies (6). The newly synthesized vimentin in the soluble fraction was also identified as vimentin by immunoprecipitation (see below). To examine whether this soluble form of vimentin was produced as a consequence of the lysis conditions in the presence of Triton X-100, we repeated the experiments shown in Fig. 1 after separation of the cells into a membrane-cytoskeletal and a soluble fraction by hypotonic lysis (9). The results obtained were similar to those described in Fig. 1 (data not shown). Taken together, the results suggest that a subfraction of the vimentin is soluble under the lysis conditions used here.

Soluble Form of Vimentin Serves as a Precursor to Vimentin Filaments

To follow the synthesis and the incorporation of vimentin into the soluble and cytoskeletal fraction, we pulse-labeled

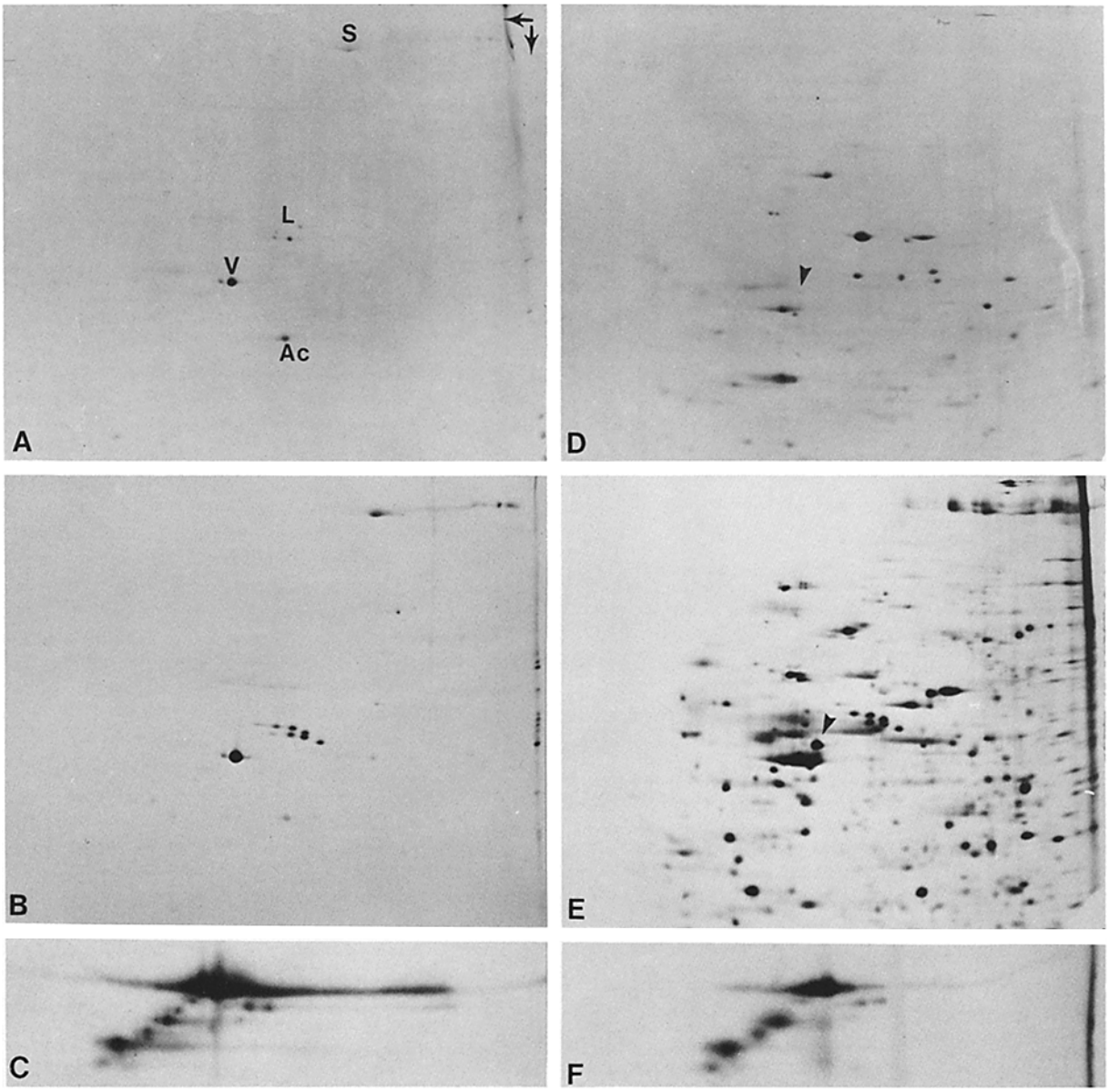

FIGURE 1 Distribution of vimentin between the Triton X-100 soluble and cytoskeletal fractions from chicken erythroid cells. Erythroid cells (2.5×10^8 cells) from 10-d-old chicken embryos were labeled with [^{35}S]methionine (200 μCi/ml) for 60 min. They were then lysed in 200 μl of lysis buffer and separated into a cytoskeletal and a soluble fraction. Urea and β-mercaptoethanol were added and 150 μl of each sample were each analyzed by two-dimensional gel electrophoresis. The dried [^{35}S]methionine-labeled gels were exposed for 4 d. Identical sets of cytoskeletal and soluble fractions were prepared from unlabeled cells, and these samples were analyzed by two-dimensional gel electrophoresis followed by immunoautoradiography with vimentin antibodies. The two-dimensional gels are: cytoskeletal fraction stained with Coomassie Brilliant Blue (*A*); corresponding fluorogram (*B*) and immunoautoradiography with vimentin antibodies (*C*); soluble fraction stained with Coomassie Brilliant Blue (*D*); corresponding fluorogram (*E*) and immunoautoradiography with vimentin antibodies (*F*). In *A* the arrows in the upper right-hand corner mark the directions of electrophoresis. Isoelectrofocusing was from right (basic) to left (acidic), and gel electrophoresis from top to bottom. *Ac*, actin, *V*, vimentin, *L*, lamins, *S*, α-spectrin. The arrowheads in *D* and *E* mark the position of vimentin.

(10–120 min) cells with [^{35}S]methionine. Subsequently, the cells were lysed in a Triton X-100-containing buffer and separated into a soluble and a cytoskeletal fraction and analyzed by two-dimensional gel electrophoresis. Fig. 2 shows the vimentin region of the resulting fluorograms, while Fig. 3 shows the quantitation of the radioactivity in the vimentin spots for the different time periods. Newly synthesized vimentin is rapidly incorporated into the cytoskeletal fraction, and the radioactivity in vimentin increases linearly for at least 2 h. The vimentin in the soluble fraction becomes also rapidly labeled with [^{35}S]-methionine, but after ~20 min of incubation the amount of radioactivity in this protein remains constant. This result shows that the concentration of the soluble form of vimentin is maintained at a constant level, and it suggests that this form serves as a precursor to vimentin filaments.

To further analyze whether the soluble form of vimentin serves as a precursor to vimentin filaments in the cytoskeletal fraction, we performed a pulse-chase experiment. Cells were labeled with [^{35}S]methionine for 3 min and then cold methionine was added to stop any further incorporation of [^{35}S]-

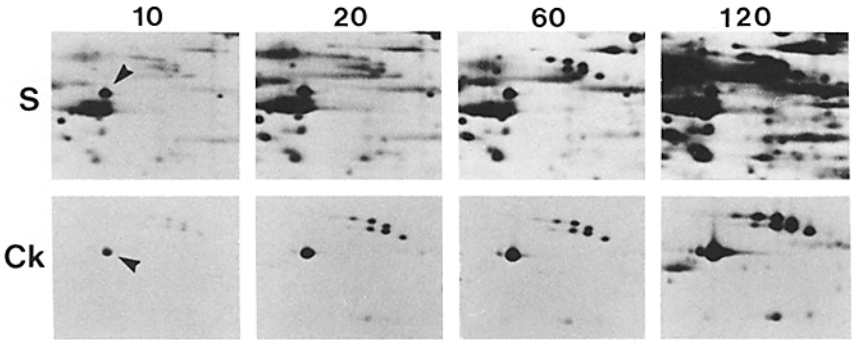

FIGURE 2 Two-dimensional gel electrophoresis of soluble and cytoskeletal fractions from erythroid cells labeled with [^{35}S]methionine for different time periods. An equal number of cells was labeled with [^{35}S]methionine for different time periods as indicated (10–120 min). Identical volumes of soluble (*S*) and cytoskeletal (*Ck*) fractions were analyzed by two-dimensional gel electrophoresis. The fluorograms were exposed for 4 d. The figure shows only the vimentin region of the fluorogram. The arrowheads mark the position of vimentin.

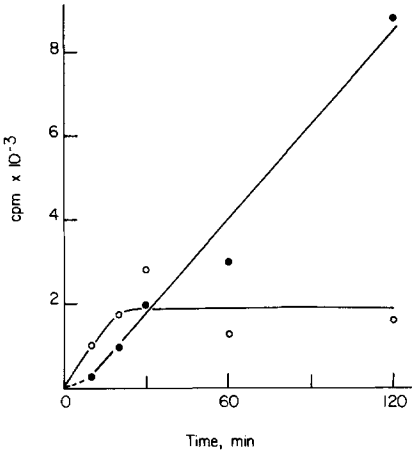

FIGURE 3 Synthesis and assembly of vimentin in erythroid cells. Cells were labeled with [^{35}S]methionine for different time periods (10–120 min) and soluble and cytoskeletal fractions were analyzed by two-dimensional gel electrophoresis as described in Fig. 1. The radioactivity incorporated into vimentin was quantitated as described in Materials and Methods. ●, amount of radioactivity (cpm) present in vimentin from the cytoskeletal fraction; ○, vimentin from the soluble fraction.

methionine. After different times of incubation, the cells were lysed in a Triton X-100-containing buffer and separated into a soluble and a cytoskeletal fraction. Fig. 4 shows the vimentin region of fluorograms of two-dimensional gels from cytoskeletal and soluble fractions from different time points of chase. As can be seen, the radioactivity in the soluble form of vimentin decreased during the chase period with a concomitant increase of radioactivity in the vimentin in the cytoskeletal fraction (Figs. 4 and 5). At 0 min after the addition of cold methionine, 60% of the radioactivity in vimentin appeared in the soluble fraction and this amount decreased exponentially during the chase period. After 2 h, only 10% of the radioactivity originally present in the soluble fraction could be recovered in this fraction. The increase of radioactivity in vimentin in the cytoskeletal fraction was complementary to its decrease in the soluble fraction (Fig. 5). The half-life of the vimentin in the soluble fraction, calculated from the time it takes to decrease 50% of its value from 0 min of chase, was ~20 min (see also Discussion). During the chase period, the total amount of radioactivity incorporated into total (i.e., cytoskeletal plus soluble) vimentin remained constant, suggesting that vimentin is not turned over during an incubation period of 2 h. Furthermore, the total amount of radioactivity incorporated into protein was constant during the chase period (data not shown). These results show that newly synthesized vimentin appears first in a soluble pool and that this pool serves as a precursor to the vimentin filaments. Similar results were obtained if the chase with cold methionine was performed in the presence of cycloheximide (3.6×10^{-4} M) to inhibit protein synthesis (data not shown).

High Speed Centrifugation of the Soluble Form of Vimentin

To analyze the sedimentation properties of the soluble vimentin, we performed a centrifugation experiment. Erythroid cells were labeled for 10 min with [^{35}S]methionine and separated into a soluble and a cytoskeletal fraction as described above. The soluble fraction was centrifuged at high speed (150,000 *g*) in an airfuge for different time periods (5, 15, and 30 min). The resulting pellets were resuspended to the original volume and all samples were subjected to immunoprecipitation with vimentin antisera. The preimmune serum did not precipitate any vimentin (Fig. 6, lane 1). As in the experiments described in Figs. 2 and 3, a large fraction of the newly synthesized vimentin was present in the soluble fraction (Fig. 6, lanes 2 and 3). All of the newly synthesized vimentin in the soluble fraction remained in the supernatant even after a centrifugation time of 30 min (lanes 4, 5, and 6), while negligible amounts of vimentin were sedimented (lanes 7, 8, and 9). We calculate that under the conditions of centrifugation used here complexes with an *S* value >20S (upper limit) should have been sedimented. This suggests that the newly synthesized vimentin in the soluble pool does not consist of filaments that could have derived from breakage of the cells and handling of the extract.

DISCUSSION

We have shown here that newly synthesized vimentin rapidly assembles to vimentin filaments from a soluble precursor pool in erythroid cells from 10-d-old chicken embryos. Newly synthesized vimentin appears first in a Triton X-100 soluble form and can be subsequently chased into the cytoskeletal fraction. The identity of this soluble form of vimentin, as vimentin, stems from the observations that vimentin antibodies react with it by immunoautoradiography and by immunoprecipitations. The newly synthesized vimentin in the soluble fraction also shares the property of vimentin to bind to single-stranded DNA (reference 15, unpublished observations). However, the soluble vimentin is not sedimented by centrifugation at high speed, suggesting that it is not composed of short filaments. At present we do not know, however, whether it is an oligomer, a monomer, or whether vimentin is in a complex with other polypeptides that retains it in a soluble state.

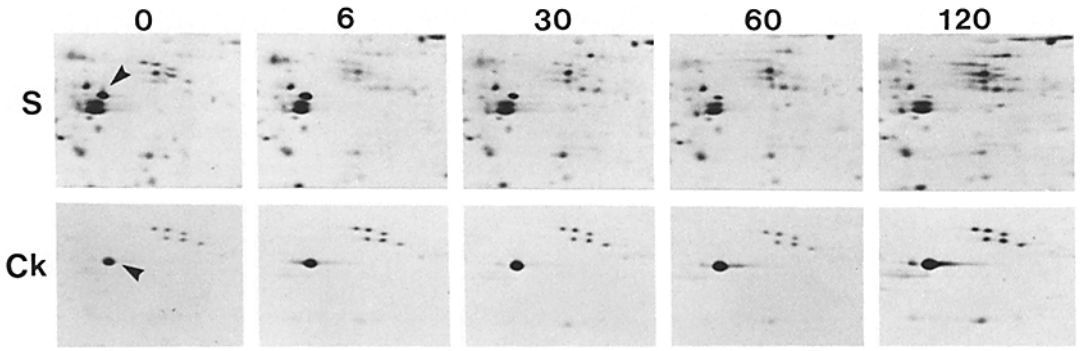

FIGURE 4 Two-dimensional gel electrophoresis of soluble and cytoskeletal fractions from erythroid cells labeled with [^{35}S]-methionine and chased with cold methionine. Erythroid cells were labeled with [^{35}S]methionine for 3 min before the addition of cold methionine. After different time periods (0–120 min), equal numbers of cells were harvested and lysed in a Triton X-100-containing lysis buffer. The lysate was separated into a soluble (*S*) and a cytoskeletal fraction (*Ck*), and an equal volume of each sample analyzed by two-dimensional gel electrophoresis. The fluorograms were exposed for 6 d. The arrowheads mark the position of vimentin. Vimentin decreases in the soluble fraction and increases in the cytoskeletal fraction during the chase period. Only the vimentin region of the fluorogram is shown.

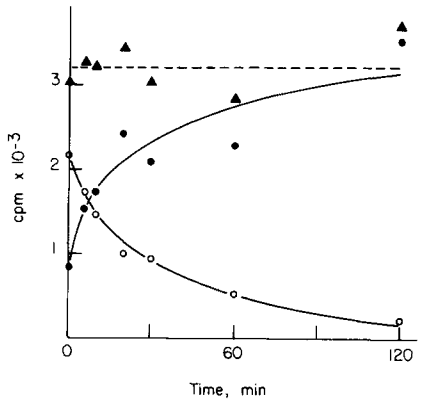

FIGURE 5 Quantitation of vimentin in soluble and cytoskeletal fractions after a pulse-chase with methionine. The amount of radioactivity (cpm) incorporated into vimentin was measured as described in Materials and Methods, from the experiment in Fig. 4. ○, vimentin in the soluble fraction; ●, vimentin from the cytoskeletal fraction. The total amount of radioactivity incorporated into vimentin was calculated from the sum of the vimentin in the soluble and cytoskeletal fraction (▲). The 0-min time point represents the time when cold methionine was added.

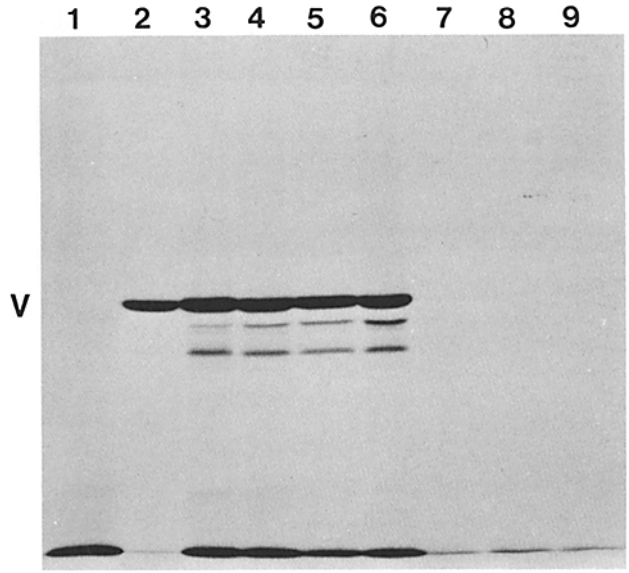

FIGURE 6 Immunoprecipitations of newly synthesized vimentin (*V*) from samples centrifuged at high speed. The samples were prepared as described in Materials and Methods and subjected to immunoprecipitations with preimmune sera (lane 1) and vimentin antisera (lanes 2–9). The different lanes on the fluorogram are: cytoskeletal fraction (2); soluble fraction (3); supernatants from the soluble fraction centrifuged at 150,000 g for 5 min (4); 15 min (5); 30 min (6), respectively, and the corresponding pellets from centrifugation at 5 min (7); 15 min (8); and 30 min (9), respectively.

The newly synthesized vimentin in the soluble fraction could be chased into the cytoskeletal fraction within 2 h, with a half-life of 20 min. This half-life was determined by calculating the time it takes for the radioactivity in the soluble vimentin to decrease to 50% of its value at time 0 min of chase. However, assuming that all of the newly synthesized vimentin in the cytoskeletal fraction at this time point originates from the soluble fraction, the cross-point between the curves from the soluble and the filamentous vimentin should represent the actual half-life (see Fig. 5). This gives a value of 7 min for the half-life of the soluble vimentin.

During a chase period of 2 h, the vimentin did not exhibit any turnover. However, during a longer chase period, the amount of radioactivity in vimentin started to decrease in the cytoskeletal fraction after approximately a 10-h period of chase. This was not followed by a concomitant increase of radioactivity in the vimentin from the soluble fraction, suggesting that vimentin was being degraded at this time (unpublished observations). The rapid assembly of vimentin filaments and the slow turnover of vimentin suggest that the filaments are growing steadily, with very little turnover. This is consistent with what has been observed in these cells by electron microscopy (8).

The more acidic variants of vimentin visible on Coomassie Brilliant Blue-stained two-dimensional gels most likely represent phosphorylated variants of vimentin (16, 17, 22). Adult avian erythrocytes and erythroid cells from embryos contain a large fraction of vimentin in these variants, which can be shown to incorporate $^{32}PO_4$ (8, unpublished observations). However, newly synthesized vimentin in the soluble fraction is composed only of the most basic unphosphorylated variant (see Figs. 2 and 4). Furthermore, newly assembled vimentin in the cytoskeletal fraction contains very little of the phosphorylated variants. However, after prolonged times of incubation,

vimentin in the cytoskeletal fraction contains progressively more of the phosphorylated variants (see Figs. 2 and 4). This result suggests that phosphorylation of vimentin does not play any role in the conversion of the soluble form of vimentin to the cytoskeletal fraction but that vimentin is phosphorylated after it is incorporated into the filaments.

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