The Association of Prosomes With Some of the Intermediate Filament Networks of the Animal Cell

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Abstract. The small RNP complexes of defined morphology and biochemical composition termed prosomes, first isolated from the cytoplasm associated with repressed mRNA (Martins de Sa, C., M.-E Grossi de Sa, O. Akhayat, F. Broders, and K. Scherrer. J. Mol. Biol. 1986. 187:47-493), were found also in the nucleus (Grossi de Sa, M.-E, C. Martins de Sa, E Harper, O. Coux, O. Akhayat, P. Gounon, J. K. Pal, Y. Florentin, and K. Scherrer. 1988. J. Cell Sci. 89:151-165). Immunofluorescence, immunoelectron microscopy, and immunochemical studies using mAbs directed against some of the prosomal proteins of duck erythroblasts indicate that in the cytoplasm of HeLa and PtK cells, prosome antigens are associated with the intermediate filament network of the cytokeratin type.

The prosomes, a class of small cytoplasmic RNP complexes first observed in HeLa cells (Spohr et al., 1970), were characterized in duck and mouse erythroblasts as a subcomplex of repressed mRNP (Schmid et al., 1984). Since then, prosomes were isolated from several other eukaryotic cell systems, such as Drosophila cells (Arrigo et al., 1985; Schuld and Kloetzel, 1985), HeLa cells (Martins de Sa et al., 1986), sea urchin eggs and embryos (Akhayat et al., 1987a), and tobacco plants (Kremp et al., 1986), indicating that they are highly conserved ubiquitous RNP particles.

The prosome is a highly stable RNP complex (85% protein, 15% RNA) sedimenting at ~19 S and showing a distinct raspberry-shaped morphology on EM pictures. Biochemically, it contains a group of ~25 proteins with molecular weights of 21,000-35,000 (with an additional 56,000-mol wt component characteristic of avian species) and some low molecular weight RNAs of ~80-150 nucleotides in length (Schmid et al., 1984; Arrigo et al., 1985; Martins de Sa et al., 1986).

Comparative biochemical studies have shown that 16 of the ~25 prosomal polypeptides have identical pIs and molecular weights in globin mRNPs of duck and mouse cells, and also in the heterogeneous 10-40-S mRNP of HeLa cells containing a very heterogeneous population of repressed mRNA (Martins de Sa et al., 1986). Immunological comparison of the prosomes in several species indicated a strong conservation in evolution of some antigenic epitopes of prosomal proteins. The 27,000-mol wt polypeptide in particular seems to be present in all eukaryotic cells tested, from human to plants; others were found to be more species specific (Grossi de Sa et al., 1988; and unpublished observations). The small RNA constituents (pRNA) differ with respect to both the cell system and the types and complexity of the mRNA in the mRNP fraction from which they were isolated (Martins de Sa et al., 1986). A particular “21K prosome” was found to be built of multiple copies of a single protein with a molecular weight of 21,000 (Akhayat et al., 1987b); preliminary experiments indicate that others contain larger sets of different proteins. Taken together, all the data available suggest that prosomes are a population of RNP whose individual particles are made up of variable sets of proteins and pRNA corresponding to the cell type and mRNA population with which they are associated. Some of its constituents seem to be highly conserved and others diverge in function of the species and the type of differentiation of a given cell type (Pal et al., 1988).

To investigate the cellular distribution of this novel ubiquitous RNP complex, we produced a series of mAbs directed against proteins of prosomes isolated from repressed duck globin mRNP (Grossi de Sa et al., 1988). A first investigation by immunofluorescence and immunoelectron microscopy of the intracellular distribution of the prosomal proteins was carried out on avian erythroblasts in differentiation. It showed that all tested prosomal antigens are localized not only on cytoplasmic structures, but also to various extents in the nucleus, with quantitative variation in their distribution depending on the stage of erythroblast differentiation (Grossi de Sa et al., 1988). The majority of the reacting nuclear and cytoplasmic polypeptides were found to have the same molecular weight and to be present exclusively in mRNP complexes of 10-40 S (Martins de Sa et al., 1986). No prosomal antigen was found in the soluble protein fraction.

In this paper we report that, in the cytoplasm of HeLa and
Preparations and Immunological Analysis of the Prosome and Postmitochondrial Supernatant (PMS)

PMJs and prosomes were prepared from different species as described by Martins de Sa et al. (1986). Proteins were analyzed by 13% SDS-PAGE (Laemmli et al., 1970), transferred onto nitrocellulose paper (Towbin et al., 1979), and immunoreacted with the anti-p27K and anti-p31K mAbs. After washing in PBS (7 mM Na2HPO4, 1.5 mM KH2PO4 [pH 7.4], 137 mM NaCl, 2.7 mM KCl), the protein blots were further reacted with rabbit anti-mouse IgG coupled to peroxidase (Miles Laboratories, Ltd., Slough, England). After washing in PBS, the immunoreaction was detected by incubation in the presence of 4-chloro-l-naphthol and hydrogen peroxide.

Indirect Immunofluorescence Microscopy

Cells grown on coverslips were fixed and processed for indirect immunofluorescence microscopy by two different procedures.

**Method/Acetone Fixation.** Cells were rinsed twice with PBS, and treated for 5 min with methanol/acetone (3:7 vol/vol) at room temperature. The coverslips were air dried for 15 min and rinsed in PBS; cells were preincubated for 5 min with methanol/acetone (3:7 vol/vol) at room temperature and treated for 5 min with 1% normal goat serum and 1% BSA in PBS (to reduce background staining) and then incubated with the prosomal mAbs (anti-p27K and anti-p31K as ascitic fluids diluted 1:50) in PBS/0.1% BSA (incubation buffer) for 2-3 h at room temperature in a humid chamber. The cells were then washed three times for 30 min and the fixed antibody was revealed by FITC-conjugated goat anti-mouse IgG (Cappel Laboratories, Cochraneville, PA; 1:50 in PBS). The cell pellet was washed twice with PBS, and mounted in 50% glycerol in PBS (pH 8.5). Observations were made by a fluorescence photomicroscope (Zeiss, Oberkochen, FRG), and photography was done on HPS film (400 ASA; Ilford Ltd., Basildon, Essex, England). Controls were carried out by the identical procedure, except that the antibody was replaced by mouse immunoneutral serum (diluted 1:50).

**Triton Extraction Followed by Paraformaldehyde Fixation.** Cells attached to slides were briefly rinsed with cytoskeleton (CSK) buffer (100 mM NaCl, 300 mM sucrose, 10 mM Pipes [pH 6.9], 3 mM MgCl2, and 1.2 mM phenylmethylsulfonyl fluoride) at room temperature and treated for 5 min with CSK buffer containing 0.5% Triton X-100. Cells were carefully washed, fixed for 20-30 min in 2% paraformaldehyde in CSK buffer, washed three times for 30 min in PBS, and then processed for immunofluorescence as described above.

In the case of double-label indirect immunofluorescence, the prosomal mAbs were used in combination with rabbit antivimentin and anticytokeratin antibodies (Biozol, Lyon, France), and with rhodamine-conjugated phalloidin (Molecular Probes, Inc., Junction City, OR; 0.1 μM in PBS). In the case of staining by a secondary antibodies, they were further incubated with rhodamine-labeled goat anti-mouse IgG (for prosomes) and FITC-labeled goat anti-rabbit IgG (for vimentin and cytokeratin) antibodies. When double labeling was done with rhodamine-conjugated phalloidin, FITC goat anti-mouse IgG antibody was used to reveal the prosomal antigens.

**Materials and Methods**

**Cell Culture**

Monolayer cultures of HeLa cells and of mouse 3T3 fibroblasts were grown in DME (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS (Boehringer Mannheim GmbH, Mannheim, FBC) and antibiotics (100 IU/ml penicillin, 50 μg/ml streptomycin). HeLa cells in suspension were pretreated for 5 min with 2% glutaraldehyde for 15 min, washed in PBS, fixed for 30 min with 2% osmium tetroxide, dehydrated, and embedded in Epon/Araldite, while still attached to the plastic culture dish. Ultrathin sections were cut and examined after staining with uranyl acetate.

**Direct Single Immunogold Labeling**

For direct immunogold staining, PtK1 cells were processed as described above. The coupling of the gold particles (20-nm) to the anti-p27K mAb was done as described by De Mey (1983). The anti-p27K prosome mAb coupled to 20-nm colloidal gold was used at 20 μg/ml in presence of 1% BSA.

**Cell Fractionation**

The cell pellet was washed twice with isotonic wash buffer (10 mM Hepes [pH 7.0], 140 mM NaCl, 5 mM KCl, 1.5 mM MgCl2) and packed again by low-speed (500 rpm) centrifugation for 10 min. After washing of the cells at room temperature, all further operations were carried out in the cold (0-4°C). 4 vol of hypotonic buffer (10 mM Tris(hydroxymethyl) amino methane, 1 mM MgCl2, 5 mM 2-mercaptoethanol) was added. Isotonicity to the cells was restored 3 min later by addition of sucrose (0.25 M final) and the lysis was performed as described by Martins de Sa et al. (1986). Nuclei and mitochondria were removed by centrifugation (2,500 rpm/10 min and 10,000 rpm/20 min, respectively). The PMS was removed carefully so as not to stir up lysosomes from the pellet. Differential ultracentrifugation yielded preparations of polyribosomes and postribosomal particles as described in Vincent et al. (1980). The nuclear fraction was purified according to Reynaud et al. (1980), taking all precautions to prevent cytoplasmic contamination. The mitochondrial fraction was washed twice with isotonic buffer (hypotonic buffer in 0.25 M sucrose) and centrifuged for 20 min at 10,000 rpm. All final pellets (nuclei, mitochondria, polyribosomes, and postpolyribosomal particles) were resuspended in equal volumes of TES buffer (10 mM Tris(hydroxymethyl) amino methane, 50 mM KCl, 5 mM 2-mercaptoethanol) and aliquots were analyzed by SDS-PAGE (Laemmli, 1970), after protein quantification (Bradford, 1976).

**Triton-soluble and Cytoskeletal Fractions**

HeLa cells were washed twice in CSK buffer. After centrifugation, pellets were resuspended in CSK buffer containing 1 mM phenylmethylsulfonyl fluoride and 5 μM RNase to inhibit proteases and RNases, respectively, and Triton X-100 was added to 0.5%. Cells were stirred gently for 5 min at 4°C and centrifuged at 1,000 rpm for 5 min. The resulting soluble fraction was removed. After fractionation, 30 μg of each fraction was immediately applied to a 15-33% sucrose gradient in low salt buffer (10 mM Tris-HCl [pH 7.4], 50 mM NaCl, 5 mM MgCl2) and layered over a 1-mL cushion of 2 M sucrose at 4°C. The absorbance profile was directly recorded at 254 nm in a flow spectrophotometer, 50 μl from each of two subsequent fractions (Fig. 3) or equivalent amounts of proteins (Fig. 5) were precipitated with 10% (final) TCA for 30 min on ice, washed with acetone, dried, and analyzed by SDS gel electrophoresis.

**Immunoelectrophoresis (Western Blotting)**

After SDS-PAGE was carried out according to Laemmli (1970), proteins were electrophoretically transferred onto nitrocellulose paper (0.45 μm) according to Towbin et al. (1979), in the presence of 0.1% SDS in the transfer buffer. The protein blot was saturated overnight at 4°C with 3% BSA in PBS, in order to block nonspecific adsorption sites. The papers were then...
Prosomes Are Ubiquitous in Higher Eukaryotes

Results

Prosomes Are Ubiquitous in Higher Eukaryotes

Fig. 1 shows that the prosome particles (Fig. 1 A) are truly ubiquitous. The mAbs directed against the duck prosomal proteins p27K and p31K cross-react with proteins of purified prosomes isolated from duck, mouse, human, and Drosophila cells. The same epitope is present in proteins of the PMS of amphibian (Pleurodeles walti) oocytes, and in plant cells (Datura). All peptides reacting are in the 27,000-30,000-mol wt range except in Datura where an ~75,000-mol wt polypeptide reacts. On the basis of this experiment, and the fact that we never found a higher eukaryotic cell devoid of the typical particles or antigens, we conclude that prosomes are truly ubiquitous.

Differential Distribution of the Prosomal Antigen p27K in Different Subcellular Fractions

In this investigation we were interested in exploring in more
Figure 2. Localization of the p27K prosome antigen in different cell fractions. HeLa cells were fractionated as described in Materials and Methods. (A) 30 μg each of the protein fractions was separated by electrophoresis on a 13% polyacrylamide gel and stained by Coomassie Blue. (B) Western blot analysis of the proteins separated as shown in A, electrophoretically transferred to nitrocellulose paper, and immunoreacted with the mAb anti-p27K. Molecular weight markers indicated in the left margin were phosphorylase b (92,000), BSA (66,000), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,000), and lactalbumin (14,000). The lanes show (1) total lysate; (2) PMS; (3) nuclear fraction; (4) mitochondrial fraction; (5) polyribosomal fraction; (6) postribosomal particle (free mRNP) fraction; (7) postribosomal particle supernatant (cytosol). The small arrow points to a faint 38,000-mol wt band in the nuclear and polyribosomal fraction.

detail the cytolocalization of prosomal antigens in various cells and in particular that of the most ubiquitous p27K protein, chosen as a representative of the prosomal proteins. We first used the prosomal anti-p27K mAb to study by immuno-electrophoresis the subcellular distribution of a prosomal protein in HeLa cells. The results shown in Fig. 2 indicate that this antigen is also present in the nucleus (Fig. 2 B, lane 3), confirming earlier data (Grossi de Sa et al., 1988). In the cytoplasm it is most abundant in the postpolyribosomal particle fraction including the "free" mRNP (Fig. 2 B, lane 6). No reaction was found in the mitochondrial fraction and the cytosol (Fig. 2 B, lanes 4 and 7). A very faint reaction was observed in the polyribosomal fraction (Fig. 2 B, lane 5). The level of antigen p27K in the nucleus is less than in the cytoplasm, as seen in Fig. 2 B (lane 3). In both compartments, most of the antigen has the same molecular weight, but an additional polypeptide of ~38,000 mol wt was consistently detected in the nucleus and the polyribosomal fraction (small arrow, Fig. 2 B); the latter observation was independently made by Kreutzer, C., and H.-P. Schmid (unpublished observations) who found, furthermore, that the 38,000-mol wt component includes the oligopeptide pattern of the p27K protein (cf., Discussion below). The main point to be retained is that these results confirmed our previously reported observations indicating that the prosomes are predominantly associated with the cytoplasmic free mRNPs (Schmid et al., 1984; Martins de Sa et al., 1986).

Figure 3. Localization of the p27K prosomal antigen in the Triton-soluble and cytoskeletal fractions. HeLa cells were fractionated as described in Materials and Methods. (A) 30 A260 units of the cytoskeletal fraction (CSK) were loaded on a 15-33% sucrose gradient in low salt buffer, layered onto a 1-ml 2-M sucrose cushion, and centrifuged (40,000 rpm for 2 h at 4°C; model SW41; Beckman Instruments, Inc.). (B and C) Proteins from each of two subsequent pooled fractions from the cytoskeletal and soluble fractions, respectively, were separated by electrophoresis, blotted onto nitrocellulose paper, and immunoreacted with the anti-p27K mAb.

To investigate the possibility that prosomes might be associated with Triton-insoluble structures, lysates of Triton-treated HeLa cells, prepared as described in Materials and Methods, were sedimented through a sucrose gradient (Fig. 3). Every two pooled fractions were subjected to electrophoresis, blotted onto nitrocellulose paper, and reacted with the anti-p27K mAb. In both fractions, Triton-soluble and -insoluble, the bulk of the prosomes sediment in a zone in 10–40 S (Fig. 3, A and B; P). Interestingly, the 38,000-mol wt polypeptide is present only in the cytoskeletal fraction; it sediments in both, the polyribosome and prosome regions,
while the p27K antigen is restricted to the latter (Fig. 3, CSK).

Localization of the Prosomal p27K Antigen by Single Label Immunofluorescence

Some of the mAbs raised against duck prosomal proteins cross-react with specific polypeptides found in cells from a vast variety of eukaryotic species (Fig. 1; and Grossi de Sa et al., 1988). Analysis by indirect immunofluorescence of avian erythroblasts and Avian Erythro Glastosis Virus-transformed erythroleukemic cells using antiprosomal mAbs indicated that these antigens are differentially distributed in both the cytoplasm and the nucleus (loc cit). Since prosomes are mRNA-associated structures, and as it was known for a long time that cytoplasmic mRNA remains structurally bound after Triton X-100 extraction (Cervera et al., 1981), we were

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Figure 4. Immunofluorescence pattern of the p27K prosomal antigen, in HeLa (A, C, and E) and PtK-1 (B, D, and F) cells. Cells were exposed to the anti-p27K mAb (C–F) and nonimmune serum (A and B) both in 1:50 dilutions and then stained by FITC-conjugated goat anti-mouse IgG. C and D show reaction with the anti-p27K mAb after methanol/acetone fixation, and E and F after treatment by Triton X-100 before paraformaldehyde fixation. The cells shown in A and B were reacted with nonimmune serum after methanol/acetone fixation. Observation was by a Zeiss photomicroscope. Bars, 20 μm.
particularly interested in identifying the insoluble structures involved in this association.

The cytolocalization of prosome antigens was thus analyzed in detail and particularly with respect to the CSK using immunofluorescence techniques. Two different methods of fixation were explored: methanol/acetone, which was used to show the physiological status quo of prosomal antigen distribution; while treatment by Triton X-100 followed by parafomaldehyde fixation (see Materials and Methods) was done to show the insoluble cytoskeletal structures. Fig. 4 shows the immunofluorescence patterns obtained using the anti-p27K mAb on two types of epithelial cells, human HeLa and rat Kangaroo PtK 1 cells. Similar results were obtained using the mAbs specific for the p31K, p29K, and p28K antigens (see below and data not shown). When the cells were simultaneously fixed and permeabilized by a methanol/acetone mixture leaving in place soluble proteins, a relatively diffuse stain forming strings of punctuated dots was observed in the cytoplasm against a background of unresolved structures. Interestingly, the stain was often more intense at the perinuclear membrane (Fig. 4 C); patterns of strings can be observed that seem to extend from these to the protruding edges of plasma membrane (Fig. 4). Staining was often asymmetrical, leaving large areas of the cytoplasm relatively unstained (upper left corner of 4 D).

A very different pattern of staining was observed when the cells were processed to show the insoluble CSK after Triton X-100 extraction. In this case, the pattern showed characteristic filaments extending from the nuclear periphery toward the cells' plasma membrane (Fig. 4, E and F). This suggests the presence of the prosomal antigen possibly associated with structural components of the cytoskeletal networks of the cytoplasm. Here also the distribution of the antigen is not uniform but more intense in certain sectors of the cell (Fig. 4 F), and in particular at the nuclear periphery in HeLa cells (Fig. 4 E).

To understand the variable pattern observed in function of the procedure applied, it has to be recalled that in the living cell IF are bundled together with microfilaments and microtubules into cytoplasmic channels which include the mitochondria and where the latter move (De Brabander, 1982). It seems possible that such strings containing the filaments and the prosomal antigen (cf. Fig. 4, C and D) might be relaxed when the hydrostatic pressure changes and lipids and soluble components are released from the cytoplasm. Another possibility was that prosomal antigens, released upon dissolution of the lipid layers, might randomly associate to the cytoskeletal structures. We therefore investigated the location of the prosomal antigen in relation to the microfilament containing actin and the IFs including, in these cells, the cytokeratin and vimentin networks.

The data shown in Fig. 5 indicate that a substantial fraction of the p27K antigen is released from the cell when the lipids and cytosol fractions are extracted by Triton X-100. The molecular weight of the protein reacting with the p27K mAb in the soluble and cytoskeletal fraction is identical (Fig. 5 B); it is quite different from that of the proteins identified as cytokeratins by a (polyclonal) antibody (Fig. 5 C), excluding the possibility that the prosomal antibody might cross-react with cytokeratins.

Identification of the Networks Containing the Prosomal Antigen by Double-Label Immunofluorescence and Immunogold Labeling

To check the possibly random association of the prosomal antigens with the cytoskeletal structures and to compare the networks stained by the anti-p27K and anti-p31K mAbs with some of the known cytoplasmic structures, double-labeling by antiactin antibodies specific to the microfilamental network, and by antibodies specific to the IF proteins cytokeratin and vimentin, was carried out on HeLa and PtK 1 cells. Figs. 6 and 7 (HeLa cells and PtK cells; respectively) show that the p27K antigen is extensively located on networks that are superimposable onto those of the cytokeratin-type IFs (Figs. 6, A and A'; 7, A, A', B, and B'). Little if any coincidence of the prosomal networks to those of vimentin and F-actin can be detected (Figs. 6, B, B', C, and C'; 7, C and C'). Similar results were obtained when other mAbs specific to the prosomal proteins p29K and p31K were used (Fig. 8).

The specificity to the cytokeratin-type IF and the absence of the prosomal antigen on the vimentin (Figs. 6 B and 7 C) and actin (Fig. 6 C) networks make unlikely a random association of free mRNP (containing prosomes) to the CSK upon Triton extraction of the lipids and cytosol. Furthermore, the patterns observed cannot be attributed to cross-reaction with the cytokeratin-type IF proteins since these have molecular weights quite different from those of the prosomal proteins (Fig. 5 C). As can be noticed in all the micrographs comparing the prosomal to the IF networks, although
Figure 6. Cyto-localization in HeLa cells of the p27K antigens by double-label immunofluorescence staining. HeLa cells were double labeled with the following combinations of primary antibodies: anticytokeratin (A) alone or with prosomal anti-p27K (A'); antivimentin (B) alone or with anti-p27K (B'); rhodamine-conjugated (actin-specific) phalloidin (C) alone or with anti-p27K (C'). The fixed prosome antibody anti-p27K was secondarily detected by rhodamine-labeled goat anti-mouse IgG (A' and B') and by FITC-labeled goat anti-mouse IgG (C'). The IF antibodies were detected by FITC-labeled goat anti-rabbit IgG. Bars, 20 μm.

The fibers can be superimposed extensively, the pictures are not identical. Indeed, if the anticytokeratin antibodies sharply label the fibers, the prosomal mAbs label in addition to the fibers some particulate material which seems attached to them. This is particularly obvious at high magnification (Fig. 7, B and B'). It is hence evident that the target antigen cannot be the same in the two immunoreactions, using the anticytokeratin and the antiprosome antibodies. This observation, on top of the controls shown in Fig. 5, seems to exclude definitely the possibility of a mere cross-reaction of the prosomal antibodies with both the antigens.

To confirm the results obtained by double-label immunofluorescence, cells were doubly labeled with colloidal gold particles of different sizes, specific for prosomal antibodies on one hand, and on the other for the IF antibodies cytokeratin and vimentin. The anti-p27K mAb labeled directly by 20-nm gold particles had shown the presence of the p27K antigen on filamentous structures of the cytoplasm.
Figure 7. Cytolocalization in PtK1 cells of the p27K antigens by double-label immunofluorescence staining and direct immunogold labeling. PtK1 cells were double labeled with the following combinations of primary antibodies: anticytokeratin (A and B) alone or with prosomal anti-p27K (A' and B'), and antivimentin (C) alone or with anti-p27K (C'). B and B' are high magnifications to emphasize the granular structures attached to the fibers labeled by the prosomal mAb, and undetected by the cytokeratin-specific antibody. The prosomal anti-p27K and IFs antibodies were detected as described in Fig. 4. (D) For direct immunogold labeling, cells were incubated with anti-p27K coupled to 20-nm colloidal gold particles. Bars: (A-C and A'-C') 20 μm; (D) 0.2 μm.
Figure 8. Cytolocalization in HeLa and PtK1 cells of the p31K and p29K antigens by double-label immunofluorescence staining. HeLa (A, A', B, and B') and PtK1 (C, C', D, and D') cells were double labeled with the following combinations of primary antibodies: anticytokeratin (A and B) alone or with prosomal anti-p31K (A' and B'); anticytokeratin (C and D) alone or with prosomal anti-p29K (C' and D'). The fixed prosome-specific primary antibodies were secondarily detected by rhodamine-labeled goat anti-mouse IgG. The IF antibodies were detected by rhodamine-labeled goat anti-rabbit IgG. Bars, 20 nm.

(Fig. 7 D). Examination by EM of double-labeled preparations (Fig. 9, A–D) shows that the prosomal antigens p27K and p31K (arrowheads) are found on filaments labeled also by the cytokeratin-specific antibodies (arrows). The antivimentin antibodies revealed a quite different pattern: this network seems not to coincide with those of the prosome antigens, which most often occupy different areas in the micrographs (Fig. 9 E). It seems likely that, after Triton X-100 extraction, the vast majority of the prosomal antigens tested are on the cytokeratin and not on the vimentin fibers.

Prosomal Antigen Distribution in 3T3 Cells
In the two types of epithelial cells investigated which contain two types of IFs, it was found that the antiprosome mAbs reacted with a fibrous network that seems to coincide, after Triton X-100 extraction, with that of the cytokeratins. It was
Figure 9. Double-label immunogold labeling of HeLa cells. Cells were reacted with anti-p27K or anti-p31K (mouse mAbs) and anticytokeratin and vimentin (rabbit polyclonal antiserum) antibodies. The primary antibodies reacted were detected by 5-nm gold particles (GAM-G5)
of interest, therefore, to explore the prosome location in relation to the CSK of a cell of nonepithelial origin devoid of the cytokeratin-type network. To answer this question, we used the mouse fibroblast 3T3 cells which contain only vimentin fibers in their IFs. The results (Fig. 10 A) indicated that prosome antigens are present also in these cells. After methanol/acetone fixation, the pattern observed is similar to that seen in the epithelial cells. When the cells were treated with Triton X-100, the typical cytokeratin fibers seen in HeLa cells are not present, but there is a faint label in the cytoplasm and on nuclear patches (Figure 10, B and C'); sometimes the antigen seems to form faint strings (Fig. 10, C' and D'). Double-label immunofluorescence with antibodies against anticytokeratin and vimentin show that in these cells, the pattern ob-

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**Figure 10.** Immunofluorescence staining of 3T3 cells. (A) Cells stained with anti-p27K (after methanol/acetone fixation). (B) Cell stained with anti-p27K after treatment by Triton X-100 followed by paraformaldehyde fixation. (C and C') Cells double labeled by antivimentin/anti-p27K. (D and D') Cells double labeled by anticytokeratin/anti-p27K. The prosome antibody anti-p27K was detected by rhodamine-labeled goat anti-mouse IgG (C' and D'), and the cytokeratin antibody by FITC-labeled goat anti-mouse IgG (C and D). Bars, 20 μm.

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tained (Fig. 10, C, C', D, and D') is quite different from that observed in epithelial cells; there is relatively little coincidence of the prosomal antigen pattern to that of vimentin (Fig. 10, C and C'), and the cytokeratin networks are clearly absent (Fig. 10 D).

Discussion

Prosomes are a novel class of small RNP particles found associated with repressed free mRNP in the cytoplasm of avian and mammalian cells (Schmid et al., 1984; Martins de Sa et al., 1986). They contain characteristic sets of proteins partially conserved among several species, and small RNA (pRNA) constituents which seem to differ with respect to the cell system and the type of repressed mRNA population with which they associate. Recent studies have shown that prosomes are also present in the cell nucleus; their cytodelocalization varies in keeping with their function of cell differentiation (Pal et al., 1988; Gauthier et al., 1988; Grossi de Sa et al., 1988).

The biochemical data on HeLa cells reported here show that the prosomes in the cytoplasm are particularly abundant in the repressed free mRNP relative to other cellular fractions; prosomes are almost completely absent in the other cytoplasmic fractions and in particular from polyribosomes (cf. Figs. 2 B and 3). In duck erythroblasts (Grossi de Sa et al., 1988), prosomal antigens were observed exclusively in the 19-S prosome peak, after dissociation of the 20-S-free globin mRNP in 0.5 M KCl, whereas no prosomal antigens were observed in the fraction of soluble proteins. All these data confirm the previous results (Martins de Sa et al., 1986) which showed that prosomes are subcomplexes of repressed free mRNPs from which they can be dissociated in conditions identical to those that split ribosomes (0.5 M KCl or EDTA).

However, a substantial fraction of the p27K antigen can be extracted from the cell by Triton X-100, which leaves behind the largest fraction of mRNA attached to insoluble structures (Cervera et al., 1981). Since in the total lysate all prosomal antigen is in the particulate form of prosomes, this result indicates that some of them are either kept in place by lipids at the plasma membrane or the reticulum, or that free (i.e., unattached) forms do exist. Whether all the other prosomal antigens behave the same as antigen p27K is not yet known. Moreover, to sectors of a given cytoplasm in embryonic non-transformed cells, genuine prosomes also exist as particles in the nucleus. Although Kleinschmidt et al. (1983) and Castano et al. (1986) give no evidence for the presence of RNA in their particles, the possibility exists that they observed "preprosomes". Indeed, the association of proteins with the prosomal RNAs might allow the particles to exit from the nucleus.

The cytoskeletal framework in the cytoplasm of animal cells contains a complex array of proteinous filaments, known as microtubules, microfilaments, and IFs (Goldman et al., 1975; Osborn and Weber, 1977); all these structures are thought to be involved in cell shape and mobility (Brinkley, 1981). Traub (1985) speculated, however, that the IFs might serve some function other than architecture and might be involved in gene control. He demonstrated that vimentin in particular has RNA binding properties (loc cit). The results of our investigation raise the question of why the p27K and p31K prosomal antigens, which might illustrate Traub's suggestion, are found on the cytoskeletal network only and not on all types of IF. We show elsewhere (Pal et al., 1988) that a given prosomal antigen, part of a multicomponent system, is restricted to given types of cells and, moreover, to sectors of a given cytoplasm in embryonic non-transformed cells. This holds true even for the p27K epitope, in spite of its evolutionary conservation. Since all types of cells seem to contain prosomes, it follows that a given prosomal antigen is specific to a given status of differentiation and to subnetworks of the IF, possibly occupied by given types of mRNA.

This latter possibility is particularly intriguing, in view of the fact that given types of mRNA were found to occupy topologically defined sectors of the cytoplasm (Colman et al., 1982; Lawrence and Singer, 1986). Possibly other yet undetected prosomal antigens might occupy other types of IF in cells different from those that were used to produce our prosomal mAbs (chicken erythroblasts and globin mRNP-specific prosomes). Work is in progress to create mAbs against other types of prosomes, in particular from muscle and nerve cells, to explore this possibility.

The immunofluorescence and immunogold experiments...
documented here demonstrate the association of prosomes with one of the three major cytoskeletal networks, the IF. After double-labeling individual cells, we observed that the anti-p29K and the anti-p31K prosomal antibodies and antibodies specific for cytokeratin stained the same filaments in the cytoskeletal system of human HeLa and rat kangaroo PK1 cells (cf., Figs. 6, A and A'; 7, A, A', B, and B'; and 8, A-D and A'-D'). Little if any superposition of the patterns obtained with the anti-prosomal mAbs was detected in immunofluorescence and gold labeling when antivimentin and antiaxin (phalloidin labeled by rhodamine) were used as a counterstain (Figs. 6, B, B', C, and C'; 7, C and C'; and 9 E). The latter observation makes it likely that the association of prosomes to the cytoskeleton network is specific and not merely due to the random sticking of previously free mRNP and prosomes to the cytoskeleton.

The results obtained by immunofluorescence were confirmed by immunoelectrophoresis of the proteins in the Triton-soluble and cytoskeletal fractions. Although prosomes are quite abundant in the cytoskeleton, prosomes ot the cytoskeleton. Furthermore, Zumbe et al. (1982) have shown that the 50,000-mol wt cap-binding protein is associated with the IFs. On the basis of these and our data, one might consider that mRNP, translated or not, is bound to the cytoskeleton, and that prosomes represent one of the structural elements in the cytoplasm specific to the repressed mRNP, which is operational in posttranscriptional or posttranslational control mechanisms operating on the cytoskeletal networks.

Two populations of repressed free mRNP can be defined, as described by Vincent et al. (1981); one containing mRNA species having a translated polyribosomal counterpart (possibly in equilibrium with each other), and another one containing the fully repressed mRNA. This conclusion is based on equilibrium labeling and pulse-chase experiments carried out on HeLa cells, which did show that according to chosen conditions, from 40 to 60% of labeled mRNA is in free mRNP particles (Spohr and Scherrer, 1972). The bulk of repressed mRNA in free mRNP decays, like polyribosomal mRNA, with its own intrinsic half life (Spohr and Scherrer, 1972; Mauron and Spohr, 1978; McMullen et al., 1979). The factors, which must therefore define qualitatively and quantitatively the mRNA fraction in either the active polyribozomal or the repressed free mRNP form, are unknown at present. It is possible that the prosomes, found as cofactors of free mRNP and capable of inhibiting translation in vitro (Akhayat et al., 1987), might represent one of the mechanisms by which specific mRNA types are identified and withdrawn from the translation machinery. Further work will be necessary to fully characterize the relation of prosomes, mRNP, and the IFs of the cytoskeleton which, as suggested by Traub (1985) and shown here, are most likely to be somehow involved in posttranscriptional mechanism of gene expression.

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