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The prosomal protein of 27 kDa and a nuclear 38 kDa protein are immunologically related

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A monoclonal antibody, recognizing the prosomal 27 kDa protein, was used to detect prosomal proteins in nuclear fractions. Protein gel electrophoresis and immunoblotting revealed large amounts of a 38 kDa protein and in very low amount a 27 kDa protein reacting with the monoclonal antibody. Immunoblot analysis of V8 proteolytic fragments demonstrated that both proteins are intimately related and suggest that the nuclear 38 kDa protein could be a precursor of the 27 kDa protein (pros P 27) which is a structural component of cytoplasmic prosomes.

Prosome, Pros P 27, Nuclear 38 kDa protein, Peptide mapping

1. INTRODUCTION

Prosomes were first identified as cytoplasmic ribonucleoprotein complexes containing small RNA in mouse and duck erythroblasts [1]. In the meantime they have been isolated from the cytoplasm of various vertebrate and invertebrate cells [2–5] from tissues of higher plants and archaebacteria [6–9]. In all cases they consist of a specific set of proteins with molecular masses ranging from 19 to 35 kDa. Some of them, e.g. the 27 kDa protein, were highly conserved during evolution, while others vary from species to species. Similarly, the small RNAs of prosomes are related but not identical. However, RNA is an intrinsic part of prosomes. We have recently shown that the prosomal proteins protect RNA fragments with size of approximately 76–80 nucleotides against nuclease digestion [10]. Furthermore we like to remember that they resist the strong detergent lauroylsarcosyl-Na [1]. The cellular function of prosomes is still an open question. We demonstrated recently that prosomes are involved in the repression of viral mRNA [11]. Others showed that they have specific proteinase and peptidase activity [12,13]. Anyway, their existence in all eucaryotic organisms investigated so far, suggests an important physiological function for the cell. Immunocytological studies of amphibian oocytes showed that high amounts of prosomal antigens are localized in the nucleus [14]. After fertilization during cell cleavage, they gradually occur in the cytoplasm and later in the blastula; prosomal antigens accumulate again in large amounts in the nucleus. In *Xenopus* oocytes particles similar to prosomes were isolated and characterized from the nucleus [15]. However, in HeLa cells or other differentiated cells, only small amounts of prosomal antigens were detected in the nucleus as investigated by immunofluorescence microscopy; more than 90% are in the cytoplasm [16,17]. Thus we were interested to investigate nuclear subfractions of HeLa cells more closely for the distribution of prosomal antigens. For this approach we used a monoclonal antibody against the highly conserved prosomal protein 27 kDa (pros P 27).

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2. MATERIALS AND METHODS

2.1 *HeLa cell culture and labeling techniques*

HeLa cells were grown in suspension at 4×10^5 cells·ml with a generation time of about 24 h in Eagle's Minimum Essential Medium supplemented with 6% (v/v) newborn calf serum.

RNA labeling. Identification of poly(A) sequences in mRNA was by hybridization with [^{3}H]poly(U) as described in [18].

Protein labeling. HeLa cells were resuspended at 4×10^6 cells per ml in methionine-free medium and incubated for 3 h at 37°C with [^{35}S]methionine (10 μCi ml; 1 Ci = 37 GBq).

2.2. *HeLa cell fractionation and isolation of prosomes*

HeLa cells were resuspended in hypotonic buffer TBK 10 (TBK-X buffers = 20 mM Tris-HCl, pH 7.4; 3 mM $MgCl_2$, 7 mM 2-mercaptoethanol, X mM KCl where X = 10, 100 mM) and lysed by 10 strokes in a Dounce homogenizer. Nuclei were centrifuged out at low speed (7 min at $700 \times g$), they were resuspended in TBK 100 and Triton X-100 was added to a final concentration of 0.5%. After centrifugation through a layer of 2 ml 10% sucrose in TBK 100 (Sorvall rotor HB 4, 15 min, 500 rpm, 4°C) the nuclei were washed twice in TBK 100 and sedimented again through 10% sucrose layers to remove the detergent and low molecular weight components. Purified nuclei were resuspended in 2 vols of TBK 100 and sonicated (Branson B-12 sonifier, 5×15 s at 50 W). Thereafter chromatin and nucleoli were removed by centrifugation through 30 ml 30% (w/w) sucrose in TBK 100 (rotor SW 28, 5000 rpm, 15 min, 4°C). The opalescent supernatant (nucleoplasm) was analyzed by centrifugation in

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15–45% (w/w) sucrose gradients in TBK 100. The gradients were centrifuged at 27000 rpm in a SW 27 rotor at 4°C for 3.5 h.

After centrifugation, gradients were collected through a flow-cell with continuous measurement of the absorbance at 254 nm. Fractions were pooled as indicated in Fig. 1 and analyzed by protein gel electrophoresis and immunoblotting.

2.3. *Protein gel electrophoresis*

One-dimensional electrophoresis of proteins was performed according to [19] and in two-dimensional gels (isotachophoresis-SDS-PAGE) as described by [20]. Molecular mass markers were phosphorylase *b* (94 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20 kDa) and lactalbumin (14 kDa).

2.4. *Electrophoretic blotting onto nitrocellulose and immunological detection of transferred proteins*

After gel electrophoresis, proteins were transferred from the gels onto nitrocellulose membrane (0.45 μm) according to [21]. Thereafter the membrane was soaked for 1 h at 37°C in PBS containing 0.5% Tween 20 (w/v) to eliminate nonspecific reactions, then in-

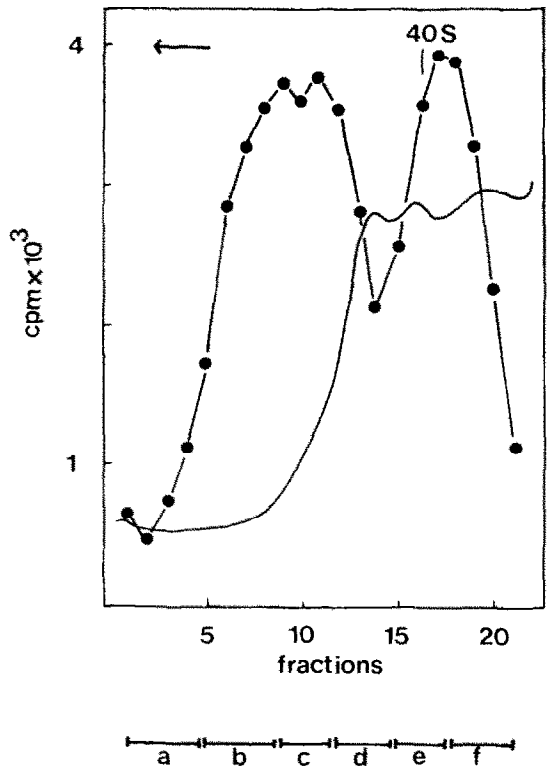

Fig. 1. Localization of pros P 27 in nuclear fractions (330 S–10 S) Purified HeLa cell nuclei were sonicated, chromatin and nucleoli were removed from the nucleoplasm by centrifugation through sucrose cushions. Then 50–60 A_{254} units of nucleoplasm were sedimented through 15–45% (w/w) sucrose gradients in TBK 100 (SW 27, 16500 rpm, 15 h, 4°C). Gradients were fractionated and 100 μl of each fraction was hybridized with [^{3}H]poly(U) to detect poly(A)$^+$ mRNA. Fractions a–f were precipitated with ethanol and analyzed by Laemmli protein gel electrophoresis. Proteins were transferred to nitrocellulose membranes and prosomal antigens were probed by a monoclonal anti pros P 27 antibody (IB 5) [16]. (P) Markers were FPLC-purified HeLa prosomes analyzed by gel electrophoresis and immunoblotting as described above. (——) Absorbance at 254 nm; (●) poly(A)$^+$ mRNPs.

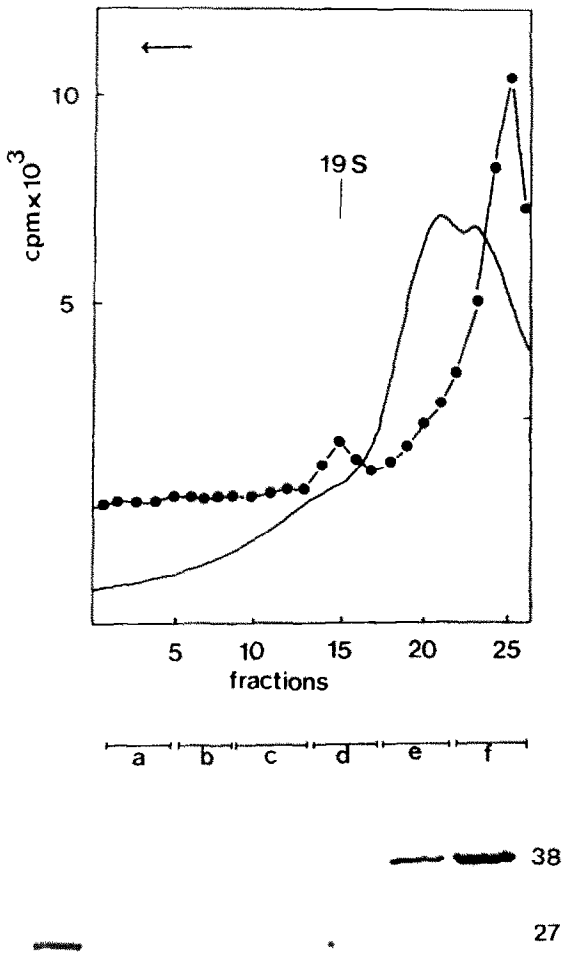

Fig. 2. Localization of pros P 27 in nuclear fractions treated with lauroylsarcosyl-Na. HeLa cells were labeled with [^{35}S]methionine, cell nuclei were purified, sonicated and the nucleoplasm was sedimented through 15–45% sucrose gradients as described in the legend to Fig. 1. Fractions e–f were pooled from 12 gradients and concentrated by high-speed centrifugation (Beckmann rotor Ti 60, 18 h, 48000 rpm, 4°C). Pellets were resuspended exposed to 1% lauroylsarcosyl-Na (final concentration) and approximately 5 A_{254} units of this suspension were loaded on 10–50% sucrose gradients in detergent buffer as described in [1]. After centrifugation (Beckman rotor SW 40, 18 h, 36000 rpm, 4°C) the gradients were fractionated, with continuous measurement of the absorbance at 254 nm. All fractions were precipitated with 10% TCA (final concentration) to measure the radioactivity in a liquid scintillation counter. (– – –) Absorbance at 254 nm; (●) particles and proteins labeled with [^{35}S]methionine; 19 S sedimentation rate of cytoplasmic prosomes.

cubated for 2 h (37°C) with monoclonal antibodies (IB 5) [16], diluted in PBS (1:500) containing 10% fetal calf serum After these procedures the membrane was washed 3 times with PBS, followed by incubation for 3 h at room temperature with a peroxidase-labeled antibody (goat anti-mouse IgG) diluted in PBS (1:1000) with 10% fetal calf serum. Finally the membrane was washed again several times with PBS and thereafter developed with a mixture of H_2O_2/4-chloro-1-naphthol [2].

2.5. *Peptide mapping*

Protein spots were cut out from two-dimensional protein gels, layered into the slots of one-dimensional SDS-Laemmli gels and digested with *Staphylococcus aureus* V8 protease for 30 min at room temperature according to [22]. Peptides were detected by immunoblotting as described above.

3. RESULTS

3.1. *Localization of pros P 27 in nuclear mRNP fractions*

Purified HeLa cell nuclei were broken by sonication, chromatin and unbroken nuclei were separated by low-speed centrifugation and the nucleoplasm was analyzed by sucrose gradient centrifugation (Fig. 1). [^{3}H]Poly(U) hybridization revealed two distinct mRNP populations, one with a sedimentation rate between 80 S and 300 S and another population between 10 S and 60 S. The messenger population with the higher sedimentation rates consists of unprocessed hn RNPs while the mRNP population with the smaller sedimentation rates migrated in the same region as cytoplasmic mRNPs dissociated from polyribosomes engaged in translation (paper in preparation). Surprisingly when a 38 kDa protein was probed with the antibody on top of the gradients, none of the nuclear fractions revealed a 27 kDa protein. According to these results there exist two possibilities: genuine prosomes containing 27 kDa proteins are not nuclear constituents in HeLa cells or they exist in the nucleus in a very low concentration which is not detectable at this stage of purification. To solve this problem nucleoplasmic fractions in the range of 10–40 S were pooled from sucrose gradients (Fig. 1), concentrated by sedimentation and analyzed again by high-speed centrifugation in 10–50% (w/w) sucrose gradients containing 1% lauroylsarcosinate-Na. Under these conditions we obtained a very small amount of a distinct sarcosyl-resistant particle fraction which sedimented in the range of 19 S and the antibody stained a 27 kDa protein (Fig. 2). Investigation of this fraction by electron microscopy revealed a spherical morphology similar to cytoplasmic prosomes (data not shown). From these experiments we concluded that prosome-like particles do exist in HeLa cell nuclei.

3.2. *Comparison of pros P 27 and the nuclear 38 kDa protein by peptide mapping*

The existence of a 38 kDa protein reacting with anti-pros P 27 antibodies raised the possibility that the nuclear 38 kDa protein and the 27 kDa protein of prosomes isolated from the cytoplasm of HeLa cells are closely related and of common source or the 27 kDa antibody recognized a totally different protein with a common epitope. Therefore we analyzed the nuclear 38 kDa protein and the cytoplasmic pros P 27 by peptide mapping. For this purpose HeLa nucleoplasm was separated by centrifugation in sucrose gradients and fractions reacting with anti-pros P 27 were analyzed by two-dimensional gel electrophoresis (Fig. 3, left). In parallel, prosomal proteins of HeLa cells were subjected to the same procedure (Fig. 3, right). Spots of nuclear 38 kDa protein and spots of pros P 27 were detected by immunoblotting with monoclonal antibody anti-27 kDa, excised from the gels and analyzed by gel electrophoresis in SDS-Laemmli gels after digestion with *S. aureus* V8 protease. Peptides were detected

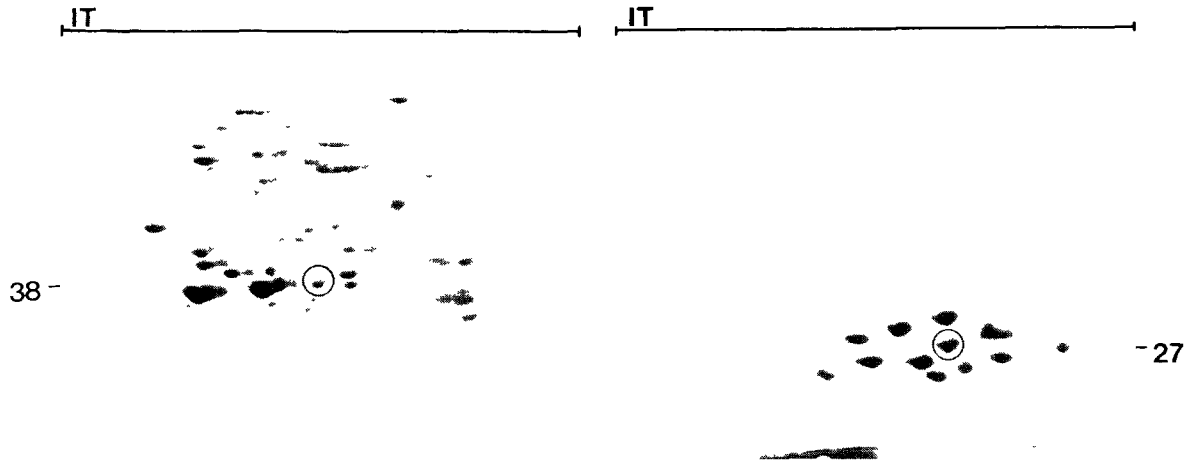

Fig. 3 Detection of nuclear and prosomal proteins reacting with monoclonal anti-pros P 27. Nucleoplasm (left) and FPLC-purified cytoplasmic prosomes of HeLa cells (right) were analyzed by two-dimensional gel electrophoresis, transferred to nitrocellulose filters and probed with the monoclonal anti-pros P 27 antibody (IB 5) [16]. Proteins were visualized by Coomassie blue stain. ( ) Protein spots which reacted exclusively with the antibody.

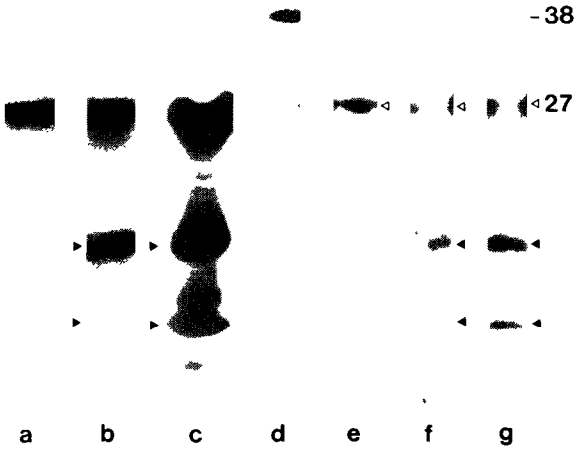

Fig. 4. Peptide digestion pattern of pros P 27 and the nuclear 38 kDa protein. Spots of nuclear 38 kDa protein and of pros P 27 were cut out from two-dimensional gels (see legend to Fig. 3), layered into the slots of one-dimensional SDS Laemmli gels (15% polyacrylamide) and digested with different quantities of *S. aureus* V8 protease. After electrophoresis peptides were transferred to nitrocellulose filters and probed with anti-pros P 27 antibodies. Filters were developed with a mixture of H_2O_2/4-chloro-1-naphthol. (a–c) Pros P 27; (a) without protease; (b) with 1 μg protease; (c) with 10 μg protease; (d–g) nuclear 38 kDa protein; (d) without protease; (e) with 1 μg protease; (f–g) with 10 μg protease.

again by immunoblotting with monoclonal antibody anti-27 kDa. We obtained in all cases the same peptide pattern (Fig. 4a–g) which demonstrates clearly that both proteins are intimately related. Finally it is interesting to note that the lowest concentrations of protease cleaved from the nuclear 38 kDa protein a peptide with exactly the same molecular mass as the cytoplasmic pros P 27 (Fig.4e). In addition, we observed that the nuclear 38 kDa protein degraded into a 27 kDa protein upon longer storage and repeated freezing and thawing.

4. DISCUSSION

We showed that the monoclonal antibody anti-27 kDa recognized two proteins with different molecular masses in the nuclear fractions of HeLa cells. The 27 kDa protein sedimented with a distinct sarcosyl-resistant particle fraction at 19 S with the similar immunological, morphological and biophysical properties as cytoplasmic prosomes [1]. However, we do not know if prosomes and nuclear prosome-like particles are identical, because we have no indications about their total protein and RNA composition. The 38 kDa protein sedimented on top of the gradient. If this protein is associated with other proteins or RNA-forming larger particles, or if its presence in the particle fractions with sedimentation rates of >5 S (Fig. 1 and Fig. 4) is simply an overloading effect has to be investigated.

The existence of two different proteins recognized by a highly specific antibody demonstrates clearly that detection of prosomal antigens by immunofluorescence and immunoelectron microscopy do not necessarily reflect the real distribution of prosomes or related particles in cellular compartments.

Nevertheless, peptide analysis and the fact that nuclear 38 kDa protein degrades easily into pros P 27 gave strong evidence that pros P 27 derived from the nuclear 38 kDa protein. Thus we would like to propose that processed pros P 27 could associate with other prosomal proteins and/or prosomal RNA in the nucleus. This means that partial or even total assembly of prosomes of HeLa cells takes place in the nucleus.

Our hypothesis is reinforced by the following facts.

(i) Prosomal antigens and prosome-like particles do exist in the nucleus of oocytes and undifferentiated cells ([14–17] and our results).

(ii) Prosomal proteins were never detected free as individual proteins in the cytosol [2,16].

(iii) Prosomal RNA is transcribed in the nucleus. In analogy to other RNPs, as for example ribosomes or messenger ribonucleoproteins [23], it seems unlikely that this RNA migrates free of proteins from the nucleus into the cytoplasm.

Unfortunately the concentration of prosomal antigens is too low in HeLa cell nuclei to characterize the protein and RNA composition of nuclear prosome-like structures or preprosomes of this cell system. We are continuing our experiments with calf liver and have isolated recently large amounts of prosomes from the cytoplasm [3] to solve these problems.

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