The Triton X-100 and High Salt Resistant Residue of *Saccharomyces cerevisiae* Nuclear Membranes, II

Isolation of the Nuclear Membrane Insoluble Residue from G1 Arrested Cells and Immunological Comparison with the Corresponding Vertebrate Nuclear Fraction

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The Triton X-100 and High salt resistant residue of the nuclear envelope of *Saccharomyces cerevisiae* cells arrested in the G1 phase of cell cycle with *a* mating pheromone was isolated. It showed the same electrophoretic protein pattern as the insoluble residue isolated from log-phase cells. Examination of nitrocellulose blots of nuclear protein fractions with antiserum raised against a vertebrate lamin failed to show any immunological cross-reaction.

1. Introduction

When nuclear envelopes of vertebrate cells were treated with Triton X-100 and high salt concentrations, there remained an insoluble residue (pore complex-lamina) which mainly consisted of a set of proteins with $M_r = 65000-75000$ [1]. These lamins were shown to be located in close contact to the inner nuclear membrane, where they form a fibrillar network or a dense lamina, depending on the organism used [2]. During the open mitosis of vertebrate cells the lamin network is depolymerized before or during nuclear envelope breakdown [3]. When we recently isolated the insoluble residue of rapidly dividing (log-phase) *Saccharomyces cerevisiae* cells (an organism with closed mitosis), no proteins with the characteristic M_r of vertebrate lamins were found to be enriched [4]. In this report we describe the extension of our studies to resting yeast cells, and the search for yeast nuclear proteins cross-reacting with antibodies raised against a vertebrate lamin.

2. Experimental

2.1. G1 arrest of yeast cells

The *Saccharomyces cerevisiae* strain *a* cat 1.S3–14A [5] was used to obtain crude *a* mating pheromone. The cells were grown at 30 °C in a medium containing 4% glucose, 2% casein peptone, and 1% yeast extract. After 40–45 h of growth, with continuous shaking, the cells were centrifuged at 6000 g for 10 min. The supernatant was concentrated 20 × by rotatory vaporation at 35 °C. The α cells to be arrested (strain SMC-19A [5]) were grown as above, harvested in the log-phase of growth, and suspended in an 1:1 mixture of fresh growth medium and *a* culture broth concentrate. The concentrate of 2 l was enough to arrest about 1 g of pelleted α cells. The α cells were incubated in the arresting medium at 30 °C with continuous shaking for 3 h. More than 95% of the cells were unbudded (arrested in the G1 phase of cell cycle) after this procedure. Unbudded cells were counted in a Thoma counting chamber under phase contrast. The cells were fixed in 10% formaldehyde and sonicated with a Branson B-12 sonifier at setting 2.5 for 15 s before counting. For some experiments *a* mating pheromone was partially purified according to [6].

2.2. Isolation of nuclear fractions

The arrested cells were converted to protoplasts with Zymolyase as described [7]. In some experiments partially purified *a* mating pheromone was added to the protoplasts medium. Nuclei were isolated from protoplasts as in [7] with two modifications: the protoplast homogenate was centrifuged for 20 min, and the final purification step was centrifugation through a discontinuous Ficoll gradient with zones of equal volume containing

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35%, 43%, and 50% Ficoll. Centrifugation was at 120000 g_{av} for 2 h. The nuclear membranes and the Triton X-100 and high salt resistant residue were isolated as in [4].

2.3. Analytical procedures

Polyacrylamide gel electrophoresis was done as in [8]. Protein was estimated as in [9] after precipitation as in [10]. RNA and DNA were measured as in [11] and [12]. Preparation of samples for electron microscopy was done as in [7].

2.4. Immunochemical procedures

The proteins of the nuclear fractions were transferred to nitrocellulose sheets (Millipore HAWP) from 10% polyacrylamide gels according to [13] as modified in [14]. Control blots were stained with amidoblack (0.1% in 45% methanol-10% acetic acid; destained in 90% methanol-2% acetic acid). The nitrocellulose sheets to be tested with antisera were washed in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) and 0.05% Triton X-100 for 15 min. Residual protein binding capacity of the nitrocellulose sheets was then blocked by bathing them in PBS containing 1% BSA for 8 h. The sheets were then incubated over night with anti-lamin serum raised in rabbits against the pore complex-lamina fraction of chicken erythrocytes. All antisera were a generous gift of R. Stick from the Max-Planck-Institut für Virologie, Tübingen. For further characterization of the rabbit anti-(chicken lamin) serum see references [14] and [15]. The rabbit serum was diluted 1:500 with PBS containing 0.1% BSA and 0.1% Triton X-100 before use. The nitrocellulose sheets (10 × 5 cm) treated with anti-(lamin) serum were then washed 4× in 50 ml of PBS containing 0.1% Triton X-100 for 3 h altogether. The washed sheets were treated with goat anti-(rabbit immunoglobulin) serum diluted 1:100 in PBS containing 0.1% BSA and 0.1% Triton X-100 for 2 h. After washing as above, the nitrocellulose sheets were finally incubated with peroxidase-antiperoxidase (Miles GmbH) diluted 1:100 in the usual PBS medium. After 2 h of incubation the sheets were washed as above and treated with a solution made up of 1 part of 3 mg 4-chloro-1-naphthol/ml ethanol and 5 parts of 0.1 M Tris buffer, pH 7.6. To start the peroxidase reaction, 5 µl of 30% H_2O_2 were added per ml of the above mixture. After 5–15 min the reaction was stopped by washing the nitrocellulose sheets in water. All steps were done at room temperature. Treatment of nitrocellulose sheets with antisera was done in sealed plastic bags to reduce the amount of antiserum needed. *Xenopus laevis* pore complex-lamina proteins provided by R. Stick were used as control [14].

3. Results and Discussion

3.1. G1 arrest of yeast cells

The reason for our experiments with resting *Saccharomyces cerevisiae* cells was the hope to identify some difference in the protein composition of the insoluble nuclear membrane residue between resting and dividing cells. This hope arose because we knew that the insoluble nuclear membrane residue (the pore complex-lamina) of vertebrate cells is depolymerized when the nuclear envelope breaks down during mitosis [3]. It is true that the nuclear envelope is not fragmented during the closed mitosis of ascomycetous yeasts, but during mitosis the nuclear envelope must rapidly extend. It seemed possible that this event would be accompanied by changes in the protein composition of a hypothetical lamina.

Our first problem was to find a method to arrest *Saccharomyces cerevisiae* cells in a way to prevent the disadvantageous changes in cell wall composition observed after cell cycle arrest in G1 phase by nitrogen starvation or treatment with cell cycle inhibitors like *o*-phenanthroline or 8-hydroxyquinoline. Cells arrested by any of these methods were too resistant against the lytic enzyme mixture used for protoplasting (Zymolyase), even after treatment with EDTA and mercaptoethanol, which usually make the cell wall less resistant. Initial experiments with such cells gave very poor recoveries (2–6% DNA recovery in the "nuclear" fraction). These troublesome changes do however not occur when yeast cells are arrested in G1 phase of cell cycle with the mating-type specific inhibitors of cell cycle excreted into the growth medium. Indeed this would be incompatible with the function of these mating pheromones [16]. With the crude *a* mating pheromone preparation, described in section 2, it was possible to arrest more than 95% of an α-cell population which showed about 25% unbudded cells in the log-phase of growth.

Because the protoplasting procedure was rather lengthy, we added partially purified *a* mating pheromone to the protoplasting medium in some experiments. Alternatively we used β-2-D,L-thienylalanine (500 µg/ml), which is known to keep mating pheromone arrested cells in G1 phase after removal of the pheromone [17]. These precautions were however unnecessary. They did not change the results obtained with pure protoplasting medium.

3.2. Isolation of nuclear fractions

Initially we tried to isolate nuclei from arrested cells with exactly the same procedure used for log-phase nuclei [7]. However, electron microscopic inspection of the isolated nuclei showed the presence of large smooth vesicles containing masses of ribosomes and endoplasmatic reticulum elements. These multi-organelle vesicles probably arose during the homogenization of protoplasts by fragmentation and resealing of the plasma membrane, which seemed to be more delicate than the plasma membrane of cells not treated with mating pheromone. The relative number of this vesicles could be reduced by reducing homogenization intensity, and by the change in the purification procedure described in section 2. However, electron microscopy as well as analytical data showed that the G1 nuclear fraction was much more contaminated than log-phase nuclear preparations. DNA recovery was 25–30% in both cases. Log-phase nuclei showed a DNA/protein ratio of 0.044 and a RNA/protein ratio of 0.22. The corresponding ratios for G1 phase nuclei were 0.028 and 0.27. DNA enrichment from homogenate to final nuclear pellet, measured in µg DNA/mg protein, was 10–11× for log-phase nuclei and 7–8× for G1 phase nuclei. The G1 phase nuclei did not contain long microtubules or spindles, as they were often seen in log-phase nuclei [7]. Because the contaminations of the G1 phase nuclear fraction were apparently removed during the isolation of the Triton X-100 and high salt resistant residue of the nuclear membranes, we did not try to purify the nuclei further.

Nuclear membranes and the insoluble residue could be isolated from the G1 phase nuclear fraction exactly as described for these fractions from log-phase nuclei [4]. When the polyacrylamide electrophoretic protein pattern of the insoluble membrane residues from both types of nuclei was compared, no qualitative or quantitative difference was visible. We concluded, that, if the yeast nuclear envelope Triton X-100 and high salt resistant residue would be the equivalent of the vertebrate nuclear lamina, it would have to grow continually when the nucleus becomes stretched and finally constricts and divides into two.

3.3. Immunochemical experiments

Because there were some minor (*i.e.* not enriched) proteins of $M_r = 50000-85000$ in the electrophoretic protein pattern of the yeast nuclear envelope insoluble residue [7] we decided to look for proteins showing immunological cross-reaction with vertebrate lamins. The antiserum we used had been prepared in rabbits against chicken lamins and had shown a good interspecies cross-reaction with rat, mouse, and *Xenopus laevis* tissue lamins [15]. We were however not able to detect any specific cross-reaction of this antiserum with electrophoretically separated proteins from *Saccharomyces cerevisiae* nuclei, nuclear membranes, or the Triton X-100 and high salt resistant residue of the nuclear membrans. This result, together with the results on the nuclear membrane residue protein pattern, indicate that this latter fraction is something different from the vertebrate pore complex-lamina. Therefore it is still an open question whether yeasts have any functional equivalent to the vertebrate lamina at all. Furthermore, we suppose that the lamins were an acquisition made at the earliest together with open mitosis on the path of evolution.

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