Biochimica et Biophysica Acta, 687 (1982) 57–62
Elsevier Biomedical Press

BBA 71092

THE ISOLATION OF *SACCHAROMYCES CEREVISIAE* NUCLEAR MEMBRANES WITH NUCLEASE AND HIGH-SALT TREATMENT

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(Received October 13th, 1981)

Key words *Nuclear membrane isolation, Nuclease digestion, High-salt treatment, Ficoll gradient centrifugation, Electron microscopy, (S. cerevisiae)*

Saccharomyces cerevisiae **nuclear membranes were prepared from isolated nuclei by digesting chromatin with deoxyribonuclease and ribonuclease, washing of residual nuclei with 0.5 M $MgCl_2$, and discontinuous gradient centrifugation in buffered Ficoll solutions. Electron microscopic examination of the preparations showed single membrane and double membrane vesicles and membrane sheets. Pores or residual pores were often visible. In double membrane profiles the two unit membranes were often separated by the remains of the perinuclear cistern. The nuclear membrane fragments contained 58% protein, 23.8% phospholipid, 6% sterols, 7.1% neutral acylglycerols, 4.8% RNA, and 0.3% DNA. The phospholipid content of the membrane preparations was influenced by a phospholipase activity with acidic pH optimum.**

Introduction

Recently we reported the isolation of *Saccharomyces cerevisiae* nuclear membrane fragments with sonication of nuclei and washing of the resulting membranes with buffered 10% potassium citrate solutions [1]. We obtained nuclear membrane vesicles of different sizes, some showing the double bilayer profile of nuclear envelopes. The two membranes were however always very close together and nuclear pores were not visible. Compared to most nuclear membrane preparations, which were mainly obtained from higher eukaryotes, the yeast nuclear membranes contained less protein and more lipid. It is however well known that morphology, composition, and also the enzyme activities of nuclear membrane preparations can vary considerably according to the method used to isolate them [2,3]. We therefore found it advisible to try an alternative method for comparison of results.

Materials and Methods

Culture conditions and protoplast preparation. The haploid *Saccharomyces cerevisiae* strain SMC-19A [4] was grown in a medium containing 1% yeast extract, 2% casein pepton, and 4% glucose at 30°C with continuous shaking. The cells were harvested in the log-phase of growth and protoplasted as described earlier [1]. The zymolyase (Miles GmbH, F.R.G.) was used two times. The supernatant of the first protoplast washing was centrifuged at $150000 \times g_{av}$ for 1 h and stored usually 1 week at −18°C for the next protoplast preparation.

Preparation of nuclei and nuclear membrane. Nuclei were prepared from protoplasts as described [1]. The nuclear pellet was taken up in 20 mM Tris buffer, pH 7.5, containing 0.2 mM $MgCl_2$, and suspended with a tightly fitting Dounce homogenizer to obtain a suspension with 2.5–5 mg protein/ml. This suspension was kept in ice, and after 10 min 5 mg of pancreatic

ribonuclease (RNAase) and 2–3 mg of pancreate deoxyribonuclease (DNAase) were added to 40–50 mg of nuclear protein (both enzymes from Boehringer mannheim GmbH, F.R.G.). After 30 min incubation at 18–20°C an equal volume of icecold 1 M $MgCl_2$ solution in the same buffer was added with gentle stirring. The mixture was kept in ice for 15 min before it was centrifuged at $40000 \times g_{av}$ for 30 min at 0°C. This whole procedure was repeated once again with 1 h incubation time with nucleases. The final pellet was suspended in 20 mM phosphate buffer, pH 7.5, containing 2 mM $MgCl_2$ and 20% Ficoll (w/v), and layered onto a discontinuous gradient of 35, 40, and 45% Ficoll in the same buffer. After centrifugation at $150000 \times g_{av}$ for 2 h at 0°C with a Beckman SW 65 Ti rotor, the nuclear membrane band was removed from the 35%/40% interface, diluted about five times with the same buffer without Ficoll, and centrifuged at $150000 \times g_{av}$ for 30 min at 0°C. 1 mM phenylmethylsulfonyl fluoride (PMSF) was added to all solutions from a freshly prepared stock solution of 80 mM in ethanol.

Analytical methods. Lipids, protein, and nucleic acids were extracted as described earlier [1]. Phospholipids were assayed as in Ref. 5. Sterols were measured according to Ref. 6. Neutral acylglycerols were estimated as in Ref. 7. RNA was measured as in Ref. 8 with yeast RNA as standard, and DNA was estimated by the absorbance of the hydrolysis products at 260 nm with calf thymus DNA as reference. Protein was determined before extraction of the other substances according to Ref. 9 after precipitation with the deoxycholate/trichloroacetic acid method of [10].

Polyacrylamide gel electrophoresis was done as in Ref. 11 with 4% stacking gel and a linear polyacrylamide gradient from 8 to 15% in the separating gel. The gels were about 10 cm long and 1.5 mm thick.

For electron microscopic examination the membranes were taken from Ficoll gradient or the last membrane pellet, and fixed in an equal volume of 5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.5, containing 5 mM $MgCl_2$, at 4–6°C over night. The sample was then postfixed with 1% OsO_4, contrasted with 1% uranyl acetate, and embedded according to Ref. 12. The thin sections were contrasted with lead citrate according to Ref. 13.

Results and Discussion

The isolation procedure

High concentrations of monovalent or divalent ions were often used to isolate nuclear membranes either alone [14], in combination with DNAase digestion of chromatin [15–18], with DNAase and RNAase treatment [19], or with sonication of nuclei [20,21]. Other groups preferred to avoid high salt concentrations because they were found to damage the pore complexes and to lead to extensive breakdown of the nuclear envelope [22–26]. Although these low-salt methods seemed indeed to preserve the nuclear envelope better than the high-salt methods, at least in some cases, there were nevertheless electron microscopic pictures showing high-salt treated nuclear membrane sheets with numerous well preserved pores [15,16] or even nuclear ghosts [19].

We began our search for an alternative method with the low-salt method of Ref. 24. This produced residual nuclei which easily penetrated 50% Ficoll solutions at centrifugation at $150000 \times g_{av}$ for 2 h. They contained about 30% RNA (weight% of the sum of compounds tested). We therefore turned to a high-salt method using 0.5 M $MgCl_2$ to wash the membranes after disruption of the nuclei by homogenization in a low salt buffer as described in materials and methods. $MgCl_2$ alone did however not suffice to remove as much of the chromatin as the citrate/sonication method in Ref. 1 did. To avoid sonication, we finally combined $MgCl_2$ washing with DNAase and RNAase treatment as described above. While DNAase was often used along with high-salt and low-salt methods, RNAase treatment could generally be avoided because the nuclei of higher eukaryotes used in most of these studies do not contain much RNA in relation to other compounds. The small yeast nuclei, however, isolated from rapidly dividing log-phase cells, contain a high proportion of RNA [27]. In one report the effect of RNAase on the nuclear membranes from pig liver was examined in detail [28]. RNAase was shown to remove some material of unclear significance from the interior of the pore complexes. The ultrastructural integrity of the membranes and the pore annuli were however unchanged. We therefore put up with this seemingly small deleterious effect to get pure membranes.

The nuclear membranes we obtain with the nuclease/$MgCl_2$ procedure usually formed a sharp band at the interface between the 35% and 40% Ficoll layers in the gradient centrifugation. Other bands, at the top of the gradient, and at the 20/35% interface, were too week to allow detailed analyses. They contained small single membrane vesicles. A pellet of nuclei with insufficiently degraded chromatin was always observed. Depending on the course of single preparations it contained from 20 to 40% of the material applied to the gradient. A single treatment with nucleases and $MgCl_2$ was not sufficient to obtain a reasonably strong membrane band in the Ficoll gradient. Up to 90% of the material was sedimented in this case. The pellet of residual nuclei contained 20–25% RNA and 1–1.5% DNA. After the second cycle of washing and nuclease treatment, the membrane band showed no remarkable chromatin in electron microscopic examination and the analytical values were satisfactory. A third washing cycle would perhaps have improved the membrane yield further. It was however not included in the purification procedure because, from the harvesting of cells to the final membrane pellet, it already takes about 14 h.

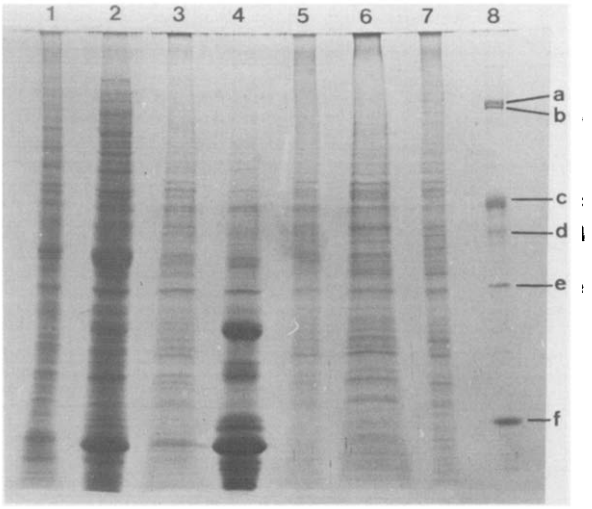

Fig 1 Electrophoretic analysis of nuclear protein distribution in different fractions of the isolation procedure and comparison of the protein pattern found with the different isolation method Lane 1, protein pattern of whole nuclei; lane 2, supernatant of the first nuclease/$MgCl_2$ treatment; lane 3, pellet of this same step, lane 4, supernatant of the second nuclease/$MgCl_2$ treatment, lane 5, pellet of the second nuclease/$MgCl_2$ step, lane 6, protein pattern of the final nuclease/$MgCl_2$-treated nuclear membrane, lane 7, protein pattern of sonicated and citrate washed membrane prepared according to Mann and Mecke (1980), lane 8, M_r standards: a, *E coli* RNA-polymerase, β'-subunit (165000), b, RNA-polymerase, β-subunit (155000), c, bovine serum albumin (68000), d, porcine brain tubulin (55000), e, RNA-polymerase, 2-subunit (39000), f, soybean trypsin inhibitor (21500)

The distribution of nuclear protein during the membrane preparation is shown electrophoretically in Fig. 1, lanes 1–6.

Electron microscopic examination

The nuclease/$MgCl_2$ method described here yielded a mixture of single membrane vesicles, double membrane vesicles, and membrane sheets (Fig. 2). In contrast to the sonicated and citrate-washed membranes described earlier [1], pores or residual pores, were frequently visible in thin sections. Some were empty, others showed variable amounts of electron scattering material within their lumina. When the double bilayer profile of the nuclear envelope was retained, the two membranes were often separated by a relatively wide space, seemingly representing the rest of the perinuclear cistern. Double membranes and pores are both markers for nuclear membranes. The low contamination of nuclei with other membranous organelles shown before [1], suggested that the single membrane vesicles arose by extensive breakdown of the nuclear envelope, which is almost always observable with comparable methods.

Composition of nuclear membranes

Table I shows, that the nuclease/$MgCl_2$-treated membranes contained more protein and neutral lipids, and less phospholipid and nucleic acids than the citrate membrane. The higher neutral lipid fraction of total measured lipid and the higher variability of phospholipid content of the nuclease/$MgCl_2$ membranes led us to suppose the presence of phospholipase activity. Addition of 0.4 mM nupercaine, a phospholipase A_2 inhibitor [29], did not change the membrane composition. Other inhibitors, NaF and EDTA, interfered with the nucleic acid digestion step. Added during the other steps of the isolation procedure, either alone or in combination with each other and nupercaine, they did not change the membrane composition. If the

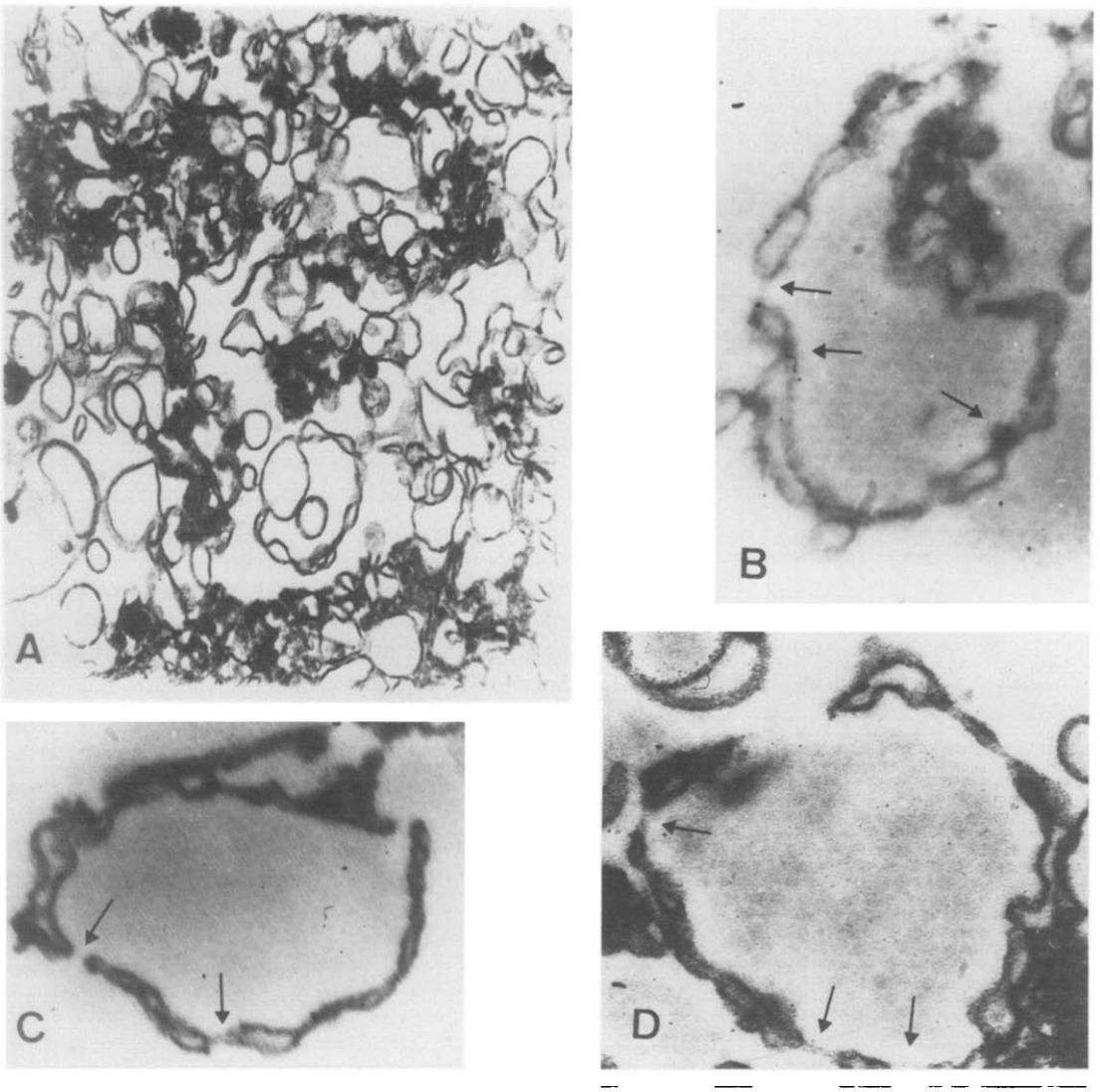

Fig. 2 Thin section of nuclear membrane preparations obtained with the nuclease/$MgCl_2$ procedure A, 30000×, B, 58000×, C, 55300×, D, 62000×; in B–D some pores or residual pores are marked with arrows They show varying amounts of electron scattering material extending between the membranes

nuclear membranes were prepared at pH 6.5 throughout, they contained 30–50% less phospholipid than those prepared at pH 7.5 A pH of 8.5 in all media did however not raise the phospholipid content of the membranes further. We conclude that there was phospholipase activity with acidic pH optimum acting in our preparation procedure. Phospholipase activity was also reported to occur during the preparation of nuclear membranes from onion tissues [21], but seems to be absent in preparations from animal tissues.

Table I also shows, that the nucleic acid content of the membranes was lower with the nuclease/$MgCl_2$ method. The very low DNA content of the yeast membrane as compared to most preparations obtained from higher eukaryotes, probably simply reflects the very much lower DNA content of yeast nuclei. In fact this very low value still represented a DNA recovery of about 3%.

The difference in protein content between the

TABLE I

COMPOSITION OF NUCLEI AND NUCLEAR MEMBRANE PREPARATIONS

The composition is given as the weight-percentage of the sum of compounds tested The mean of four preparations is shown with standard deviation A, nuclei; B, citrate-washed membranes; C, nuclease/$MgCl_2$-treated membranes

	Composition (weight-percentage)		
	A	B	C
Protein	73 5±0 7	50 5±2 6	58 0±3.5
Phospholipid	6 9±0 6	31 8±1 4	23 8±4 0
Sterols	1 8±0.2	5.0±0.7	6 0±0.7
Acylglycerols	1 8±0 3	4.3±0.4	7 1±0 5
RNA	14 6±1 2	7 9±0 6	4 8±0 5
DNA	1 7±0 2	0 5±0.15	0 3±0 1

two different membrane preparations coincided with a change from protein recovery from 5% with the citrate method to about 10% obtained in the most favourable cases with the new method.

The differences between the two membrane preparations were well within the range of differences found, when different groups prepared nuclear membranes with different methods from the same tissue. Despite this variation however, the yeast nuclear membranes were still richer in lipid than most nuclear membrane preparations from higher eukaryotes, although the difference to the average of these membranes has become smaller with the new method (see, for example Ref. 2 for compilation of data). But we do not know to which extent the phospholipid content of the nuclease/$Mgcl_2$ membranes was still lowered by the phospholipase activity described. the protein content of the yeast membrane remained correspondingly low.

The electrophoretic protein pattern

The electrophoretic protein pattern of nuclease/$MgCl_2$ membranes was very reproducible and was also exactly the same at all pH values we tried to improve the phospholipid content of the membranes. In contrast to the citrate method, omission of PMSF from the isolation buffers led to a diffuse protein pattern. We did not test in which part of the preparation this PMSF-sensitive protease activity acted. Fig. 1 shows that the protein pattern of the nuclear membranes prepared with the two different methods, citrate washing of sonicated nuclei or nuclease/$MgCl_2$ treatment, did not differ very strongly. The differences in the intensities of some bands could reflect the difference in pore frequency, an effect of the different treatments of the two membrane preparations, or a combination of both. In both preparations we found some material insoluble in the sample buffer, containing 5% sodium dodecyl sulfate and 4% mercaptoethanol, after boiling for 5 min. This material remained at the top of the gel (Fig. 1).

The enumeration of differences and similarities will be completed by a comparison of enzyme activities, which is under way in our laboratory. Preliminary results show considerable quantitative differences between the two types of membrane preparation procedure. Therefore both methods will probably be of use, depending on the aims.

Acknowledgements

We thank Professor Oberwinkler and Miss S. Dinkelmeyer for their kind help with the electron microscopic preparations, and Ciba Geigy GmbH for the gift of nupercaine.

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