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Isolation and Characterization of Nuclear Lamina From Ehrlich Ascites Tumor Cells

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We have developed a simple and rapid method for isolation of purified nuclear lamina from Ehrlich ascites tumor cells. The procedure employs chromatin structures prepared from whole cells at low ionic strength and is carried out under conditions that minimize the formation of artifactual protein-DNA complexes. When the isolation is performed in the presence of EDTA, nuclear lamina without distinct pore complexes is obtained. In the absence of EDTA, intact pore complexes and a large amount of vimentin 100 A filaments are seen associated with nuclear lamina. The main nuclear lamina proteins are characterized using gel electrophoresis, immunoblotting, and two-dimensional peptide mapping. An extensive structural homology is found between lamin A and lamin C, whose peptide maps differ by only one major spot, whereas lamin B has apparently unrelated pattern.

Key words: nuclear lamina, DNAse II digestion, lamins, vimentin, lamina-cytoskeleton association

The nuclear envelope consists of a double membrane and a proteinaceous layer apposed to its nuclear side known as the nuclear lamina (NL) [for review see refs. 1–4]. Nuclear lamina can be isolated from purified nuclei after removal of the membrane by detergents and extensive nucleolytic digestion of chromatin in the presence of Mg^{++} [5,6]. Under these conditions, the pore complexes remain associated with NL. In several types of cells, NL-pore complex has been found to consist of three main protein components (lamin A, B, and C) [5–7] and a number of unidentified polypeptides in smaller amounts. In rat liver, structural similarity has been found between lamins A and C; lamin B is apparently unrelated [8–10], although all three lamins have a common immunological determinant [11]. The morphology of nuclear pores has been extensively studied [1,4] but their protein composition remains uncertain [7,11,12].

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Functionally, NL-pore complex as part of the nuclear envelope is probably involved in the transport of molecules between nucleus and cytoplasm [1,4]. Nuclear lamina may also play a role in maintaining certain aspects of chromatin architecture [13,14]. A number of studies have suggested a close and, in some cases, a specific association between chromatin and nuclear envelope [15–18], but the molecular basis of this association is not yet known.

Another problem related to the nuclear envelope, which is just beginning to emerge, is that of association between nucleus and cytoskeleton. Electron microscopic observations have suggested a possible continuity between the cytoskeletal filaments and the intranuclear matrix [19].

Further study of NL and its possible association with chromatin or cytoskeleton requires an efficient yet mild method for isolation of this structure, free of possible protein-DNA aggregation artifacts [20]. We have recently shown that digestion of rat liver nuclei with DNAse II in the presence of EDTA yields empty shells devoid of intranuclear matrix and probably representing NL [21]. On the basis of these observations, we have now developed a simple and rapid method for isolation of purified NL from Ehrlich ascites tumor (EAT) cells and have characterized this structure in terms of morphology, protein composition, and possible association with cytoskeletal components. A preliminary account of the method has been published [22].

METHODS

Cell Labeling

Ehrlich ascites tumor cells were propagated in mice and used 6–8 days after transplantation. The whole ascites fluid was diluted in 10 volumes of serum-free medium 199 (Flow Laboratories) containing 50 mM HEPES·NaOH (pH 7.2), 0.5 units/ml heparin, 1–4 μCi/ml [^{3}H]thymidine or [^{3}H]uridine to label DNA or RNA and/or 0.1–0.5 μCi/ml [^{14}C] amino acid mixture to label the proteins. The samples were incubated for 2 hr at 36.5°C with gentle rocking.

Isolation of Chromatin Structures

Chromatin was isolated without prior isolation of nuclei as described earlier [23,24]. The cells were washed twice with 10 volumes of 0.25 M sucrose, 5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), pH adjusted to 8.0 with dilute NaOH before use. This and all subsequent operations were carried out at 0–4°C and the volumes refer to the original volume of ascites fluid. After centrifugation at 600g for 5 min, the cells were washed with 10 volumes of 0.25 M sucrose, 0.1 mM EDTA, 0.1 mM PMSF, pH 8.0, resuspended in 5 volumes of the same solution and mixed with 5 volumes of 0.5% Nonidet NP-40, 0.1 mM EDTA, 2 mM PMSF, pH 8.0. The sample was layered on a cushion of 10 volumes of 0.25 M sucrose, 0.1 mM EDTA, 1 mM PMSF, pH 7.0 and centrifuged for 15 min at 3,500g. The semitransparent pellet consists of discrete oval structures of decondensed chromatin, which retain the nuclear shape, whereas the nuclear membrane is removed by the detergent [23].

Electron Microscopy

Ultrathin sections were prepared as described previously [21]. For spreading, samples of the lamina preparations were layered on a cushion of 0.1 M sucrose

containing 2 M NaCl and 1% paraformaldehyde and centrifuged in a microcentrifugation chamber [25] for 5 min at 3,500g on freshly glow-discharged carbon-coated grids. The specimens were stained in 1% phosphotungstic acid in 70% ethanol and rinsed in absolute ethanol.

Polyacrylamide Gel Electrophoresis and Immunoblotting

For analysis of polypeptides in discontinuous 10% polyacrylamide SDS-containing gels [26], the material was dissolved in sample buffer and heated for 3 min in a boiling-water bath. Gels were stained with Coomassie blue. Samples for isoelectric focusing followed by SDS polyacrylamide gel electrophoresis in the second dimension were prepared and run as described by O'Farrell [27]. The pH gradient was measured either using marker proteins or with a surface pH electrode. A discontinuous 8% polyacrylamide/SDS gel was used in the second dimension.

The positions of lamin A, B, and C on two-dimensional gels were located by transferring the polypeptides from unstained gels onto nitrocellulose filter sheets followed by successive incubation of the filters with a 1/5,000 dilution of a monoclonal mouse antilamin IgM [11] and freshly prepared ^{125}I-labeled protein A [28], as described by Burnette [29].

Tryptic Peptide Mapping

The procedure described by Elder et al [30] was followed.

RESULTS AND DISCUSSION

Isolation of NL

The isolation of NL by existing methods [5,6,8] requires clean nuclei. However, at present there is no satisfactory method for isolation of purified nuclei from EAT cells. Disruption of the cells in solutions containing nonionic detergents and Mg^{++} results in so-called Triton X-100-resistant structures [31,32], in which nuclei are associated with large amounts of cytoskeletal filaments and boundary lamina. Our attempts to purify nuclei from these structures by treatment with EDTA, Tween-deoxycholate [33], and 2-mercaptoethanol in different combinations were unsuccessful. As an alternative, we used chromatin structures isolated by the method of Hancock [23] as modified for rat liver and Guerin ascites tumor cells [24]. Chromatin bodies isolated by this method lack nuclear membrane but retain a 250 $\pm$ 20-Å-thick NL layer that surrounds the extensively decondensed chromatin without morphologically distinct nucleoli and blocks of peripheral heterochromatin (Fig. 1). It should be noted that at this stage chromatin fibrils are seen apparently attached to NL in agreement with observations of other authors [15], but typical nuclear pores cannot be discerned.

We isolated NL from these structures in the following way. The material was suspended gently in 10% sucrose containing 2 mM Tris·Cl, 0.1 mM EDTA, pH 7.0 at a concentration of 1–2 A_{260}/ml (in some experiments up to 10 A_{260}/ml) and digested for 60 min at 25°C with 10–20 units DNAse II per A_{260}-unit plus 10 μg/ml pancreatic RNAse. The DNAse II reaction was terminated by addition of an equal volume of ice cold 4 M NaCl, 20 mM Tris·Cl, 2 mM EDTA, pH 8.0, and the sample was layered on a 10–20% sucrose gradient containing HSE (2 M NaCl, 10 mM Tris·Cl, 1 mM EDTA, pH 8.0) and centrifuged for 30 min at 3,500g. Because NL tended to stick

Fig. 1. Ultrathin section of a structured chromatin body from EAT cells (peripheral part), isolated in the presence of EDTA, showing the 250-Å lamina layer with chromatin fibers attached to it. The small interruptions in the lamina (arrows) probably mark the location of nuclear pores. Omission of EDTA from the isolation procedure does not change the ultrastructural appearance of the chromatin bodies (not shown). Bar denotes 0.25 μm.

severely to glass or plastic surfaces, bovine serum albumin (BSA) at 1 mg/ml was included in all sucrose gradients.

About 6% of the total nuclear protein (5.9 $\pm$ 1.1%, average from six experiments) as estimated from the radioactivity, sedimented to the bottom as a discrete fraction. The sediment consisted of oval fibrogranular structures appearing as empty shells, flattened on the grid (Fig. 2A), which resembled nuclear cages and related structures observed by others [16,34]. The shells consist mostly of a continuous layer 100–300 Å thick (Fig. 2B). No pore structures are seen free or associated with this layer. The NL structures are stable in HSE at 4°C at least for 3 weeks and are mechanically strong enough to be pipetted or vortexed. The material in the NL-fraction contained about 98% protein, 0.3% of the input DNA and 0.5% of the input RNA as determined from radioactivity data.

In the present experiments, as shown earlier for rat liver nuclei [21], we find no material corresponding to intranuclear matrix [35]. This could be due to several reasons: (a) We employ digestion with DNAse II, which does not require divalent cations. It is possible, therefore, to carry out the isolation under conditions of extensive chromatin decondensation (Fig. 1). This, together with the presence of sucrose during digestion [36], is likely to minimize the in vitro protein and protein-DNA aggregation [20]. (b) RNA is digested prior to dissociation of chromatin in 2 M NaCl, which has been shown to yield empty shells rather than conventional matrix structures [37]. It has been demonstrated [38] that adult chicken erythrocyte nuclei, which are inactive in RNA synthesis and contain no RNA, lack internal nuclear matrix, but the latter is acquired upon "reactivation" of RNA synthesis. Our present results are in agreement with the conclusions from earlier experiments [21] that a large part of irreversibly aggregated protein material, resistant to 2 M NaCl and EDTA and termed nuclear matrix [35], is an experimental artifact. However, this

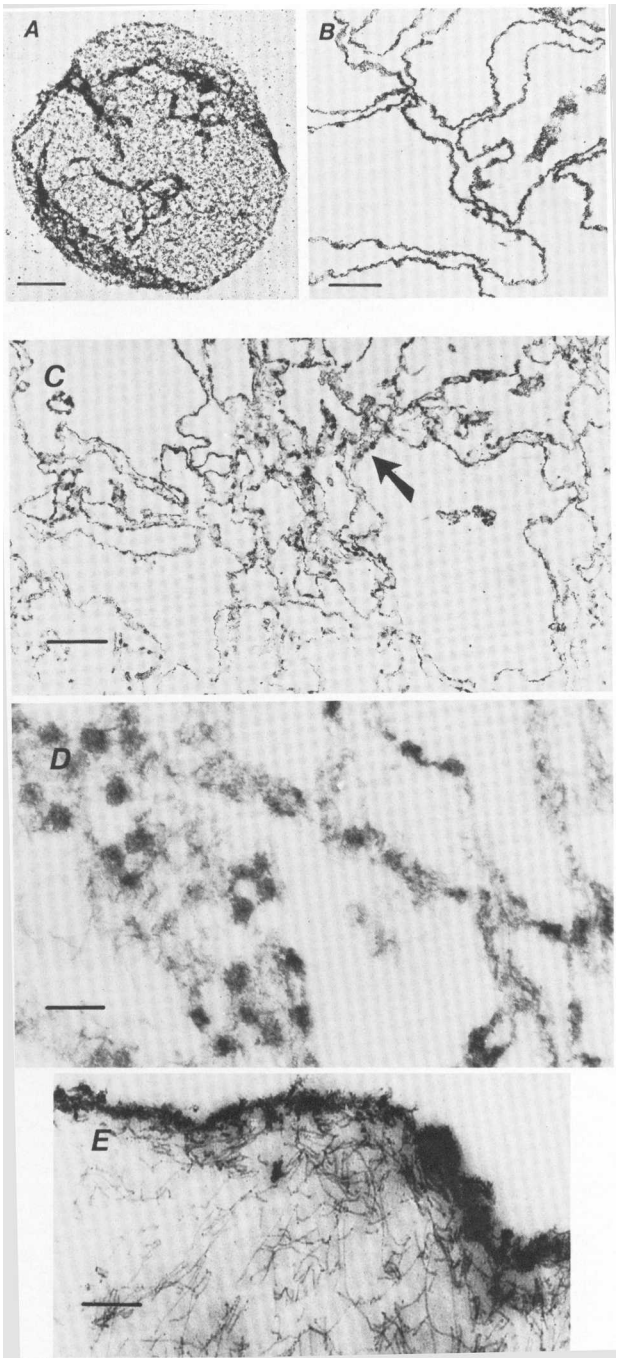

Fig. 2. A) Whole-mount preparation of nuclear lamina isolated in the presence of EDTA. Bar denotes 0.72 μm. B) Ultrathin section of NL-pellet isolated in the presence of EDTA. Bar denotes 0.35 μm. C) Ultrathin section of NL-pellet isolated in the absence of EDTA. Nuclear pores (arrow) are seen associated with the lamina layer. Bar denotes 0.1 μm. D) A higher magnification of the preparation in Figure 3C showing typical pore-complexes and vimentin filaments attached to NL. Bar denotes 0.2 μm. E) Ultrathin section of NL-pellet isolated without EDTA shows 80–100-Å vimentin filaments attached to one (presumably cytoplasmic) side of the lamina. Bar denotes 0.15 μm.

does not rule out the existence of a more delicate, as yet hypothetical skeletal network inside the nucleus, which is not apparent or is destroyed under the present conditions.

In SDS-polyacrylamide gels (Fig. 3), our NL preparations show three principle polypeptide components with mobilities corresponding to approximately 71, 68 and 64 kdaltons. These proteins, which amount to 73 ± 5% of the total Coomassie binding material, are present in proportion of 22, 28, and 50%, respectively, with the sum of the three taken as 100%. A number of other unidentified protein bands are present in smaller amounts.

The three major protein components of NL were identified as lamins A, B, and C by reacting blots of one-dimensional (Fig. 3) and two-dimensional (Fig. 4) protein gels with a monoclonal mouse antilamin IgM [11]. They were further characterized by their two-dimensional tryptic peptide maps, shown in Figure 5. The maps of lamin A and lamin C are nearly identical and differ in one major spot, present only in lamin A. Lamin B shows a different pattern. Structural homology between lamin A and lamin C has been reported earlier for other species [8–10]. It has been suggested that lamin C may be a product of proteolysis of lamin A [9,10]. Such a possibility seems compatible with our peptide mapping data and with the high content of lamin C. However, we have detected little in vitro proteolytic activity in our NL preparations: the SDS gel pattern and the ratio lamin A/lamin C do not change in the absence of

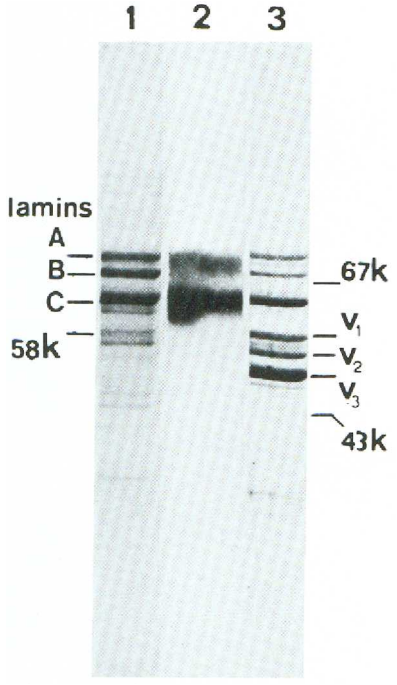

Fig. 3. Electrophoresis in 10% SDS-containing polyacrylamide gel of NL-fraction isolated in the presence (lane 1) and in the absence (lane 3) of EDTA; lane 2 shows a NL-preparation isolated in the absence of EDTA, electrophoretically transferred onto nitrocellulose paper and reacted with a monoclonal mouse antilamin IgM [11] as described in Methods. The positions of protein markers are indicated: BSA 67K, purified vimentin 58K, actin 43K.

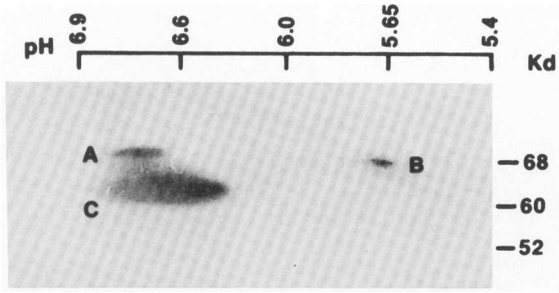

Fig. 4. Two-dimensional isoelectric focusing/SDS-polyacrylamide gel of the polypeptides from NL-fraction. Lamins are detected by reaction with a monoclonal mouse antilamin IgM [11] after transfer onto nitrocellulose paper.

PMSF or after prolonged incubation. Therefore, we consider it more likely that lamin A and lamin C are two different proteins, in agreement with the recent results of others [39].

NL Isolated in the Absence of EDTA

Omission of EDTA from the whole procedure of isolation of chromatin and NL results in significant changes in the final product. Under the same conditions of digestion, about twice as much (10–12L%) protein sediments in the pellet. Whole-mount preparations do not show much difference, but examination of ultrathin sections clearly demonstrates the presence of nuclear pore structures associated with NL (Fig. 2C, D). Another important difference is the presence of a large amount of 100-Å, presumably vimentin, filaments [31–33] associated with one side of NL, in the absence of free bundles of such filaments (Fig. 2D, E). The SDS gel pattern of NL isolated without EDTA (Fig. 3) shows, apart from the lamins, several strong protein bands with mobilities in the range 50–58 kdaltons. These polypeptides were identified as vimentin and its proteolytic cleavage products [40] by comparison of their tryptic peptide maps with that of purified vimentin isolated by the method of Traub [41] (Fig. 5).

Our results with NL isolated in the absence of EDTA (Fig. 3C–E) show that traces of divalent cations, perhaps tightly bound to proteins, are essential for the integrity of the pore annuli, in agreement with earlier observations [42]. However, at this stage it is not possible to decide whether the pores are disassembled and/or detached from NL, or simply distorted (Fig. 2B). These results also identify NL as the subnuclear structure that is directly associated with the cytoskeletal vimentin filaments in EAT cells. The observation may be relevant to the finding of cytoskeletal protein in preparations of nuclear matrices [10,43]. Preliminary results [Krachmarov et al, unpublished] have shown that a treatment of isolated NL-vimentin complexes with EDTA does not detach the vimentin filaments, suggesting that their absence from preparations isolated with EDTA is not due to disassembly [44] and also that NL-vimentin association undergoes stabilization during the 2 M NaCl step. A similar situation has been observed in the case of pore complexes.

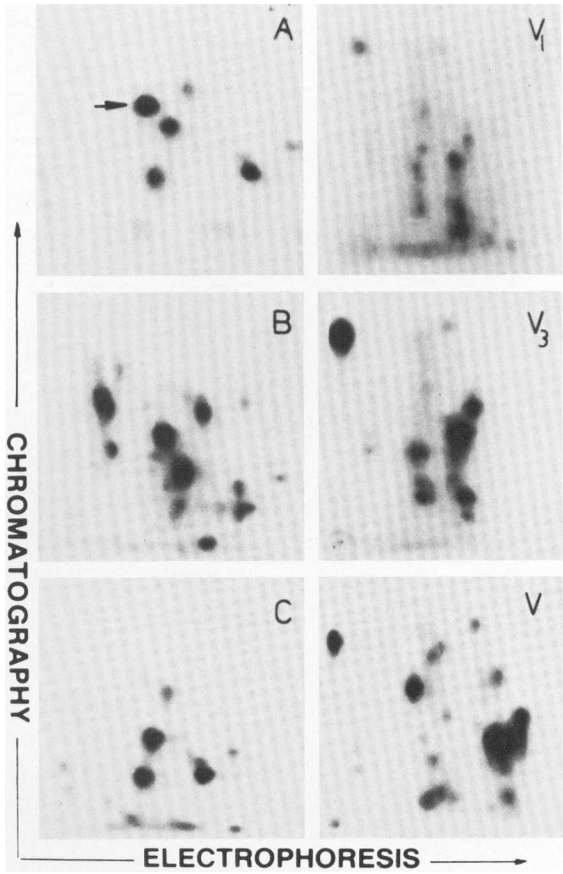

Fig. 5. Two-dimensional tryptic peptide maps of EAT polypeptides. The maps correspond to fractions shown in Figure 3. Identical results were obtained regardless of whether the polypeptide fractions were excised from one-dimensional or two-dimensional gels. Panels are labeled as follows: (A) lamin A, (B) lamin B, (C) lamin C, (V_1) vimentin fraction V_1, (V_3) vimentin fraction V_3, and (V) purified vimentin [36]. The arrow indicates the major spot representing the single difference between the maps of lamin A and lamin C. The map of vimentin fraction V_2 (Fig. 3) is almost identical to that of V_1 and V_3.

We consider the DNAse II method particularly suitable for studying the association between NL and DNA in vivo, since it is carried out under conditions that avoid certain artifacts related to protein-DNA aggregation and chromatin condensation [20].

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