Citric Acid Extracts a Specific Set of Proteins From Isolated Cell Nuclei

Uwe Rosenberger, Mehdi Shakibaei, Christoph Weise, Peter Franke, and Klaus Buchner

Arbeitsgruppe Neurochemie, Institut für Biochemie (U.R., C.W., P.F., K.B.) and Institut für Anatomie (M.S.), Freie Universität Berlin, 14195 Berlin, Germany

Abstract   The treatment of isolated cell nuclei with citric acid was described as a method for separating inner and outer nuclear membrane. Using cell nuclei from bovine cerebral cortex, we can show that citric acid does not cause a separation of the two nuclear membranes, but extracts a specific set of proteins from the nuclei. The extraction of proteins is not just an effect of damaging the nuclear membrane or destructing the cytoskeleton, but rather a specific effect of citric acid treatment. One of the extracted proteins, chosen as a marker for the putative outer nuclear membrane fraction, has an apparent molecular weight of 145 kDa and is located in the nucleoplasm as shown by immunofluorescence microscopy. By sequencing tryptic peptides it was identified as RNA helicase A, an abundant nuclear protein assumed to participate in the processing of mRNA.   © 1995 Wiley-Liss, Inc.

The nuclear envelope consists of two membranes which converge at the nuclear pore complexes, the nuclear pore complexes themselves and the lamina, a network of intermediate filament proteins lining the inner nuclear membrane [for review see Gerace and Burke, 1988; Wiese and Wilson, 1993]. Much interest has been focussed on the nuclear pore complex and considerable progress has been made in recent years in understanding this macromolecular structure [for review see Panté and Aebi, 1994]. However, the nuclear envelope is not only a carrier of the nuclear pore complexes, but is considered to be a dynamic structure, containing signal transduction components also [Dingwall and Laskey, 1992]. The outer nuclear membrane is continuous with the endoplasmic reticulum, and endoplasmic reticulum proteins (e.g., cytochrome P450) were shown to also be localized in the outer nuclear membrane [Matsuura et al., 1981]. For that reason, the outer nuclear membrane is regarded as a specialized region of the ER membrane. It is assumed that there are proteins specifically located at the outer nuclear membrane, but, up to the present, no such protein has been identified. The inner nuclear membrane contains proteins which bind to the lamina and to chromatin, such as the putative lamin B receptor [Worman et al., 1990], and a group of lamin attachment proteins (LAPs) [Foisner and Gerace, 1993]. A variety of methods for subfractionation of the nuclear envelope have been described, including the separation of the nuclear membranes from a pore complex-lamina fraction and the separation of the two nuclear membranes [for review see Agutter, 1991]. To separate inner and outer nuclear membranes, incubation with citric acid in a concentration range of 1–2.5% was used in several studies [e.g., Smith et al., 1969; Gilchrist and Pierce, 1993]. The initial aim of the present study was to separate inner and outer nuclear membranes in order to identify proteins specific for these membranes, and to localize protein kinase C isozymes present at the nuclear membranes [Buchner et al., 1992; Rosenberger et al., 1995] in more detail. It turned out that treatment

with citric acid is not suitable for separating the two nuclear membranes but, instead, leads to the selective extraction of a set of nucleoplasmic proteins, among them the RNA binding protein RNA helicase A.

MATERIALS AND METHODS
Antibodies

The monoclonal antibody against lamin B_2 was a gift from H. Henneckes, Institute Suisse de Recherches Experimentales sur le Cancer (ISREC), Epalinges, Switzerland. The antibodies against ribophorin I, p34 and α-SSR were a gift from D. Görlich, Max-Delbrück-Center for Molecular Medicine, Berlin, Germany. The peptides YTQVGPDHNR and EGETVEPYK of RNA helicase A were synthesized using a Neosystem NPS 4000 peptide synthesizer and coupled to keyhole limpet hemocyanin. Antibodies against these conjugates were raised in rabbits. All secondary antibodies were from Sigma Chemical Co. (St. Louis, MO). We used anti-mouse IgG coupled to FITC and anti-rabbit IgG coupled to TRITC for the immunofluorescence experiments, and both anti-mouse IgG and anti-rabbit IgG coupled to alkaline phosphatase for Western blotting.

Isolation of Cell Nuclei

The isolation of cell nuclei from bovine cerebral cortex was performed as described by Otto et al. [1992]. In brief, gray matter was homogenized using a glass-teflon-potter in NP 0.32 buffer, i.e., a buffer containing 0.32 M sucrose, 20 mM Hepes.Na, pH 7.4, 1 mM $MgCl_2$, 47 mM 2-mercaptoethanol, 10 mg/l leupeptin, 10 mg/l aprotinin, and 1 mM phenylmethylsulfonylfluoride. The homogenate was filtered through 125-mesh nylon gauze and centrifuged at $170g$ for 15 min. The pellet was resuspended in homogenization buffer and again centrifuged at $370g$ for 20 min. The resulting pellet was made up to 1.85 M sucrose and centrifuged at $70,000g$ for 60 min. The nuclear pellet was carefully resuspended and made up to 2.15 M sucrose. This solution was laid over a cushion containing 2.2 M sucrose and centrifuged at $70,000g$ for 60 min, yielding purified nuclei. The isolated nuclei were resuspended in NP 0.32 buffer and either frozen in liquid nitrogen or immediately used.

Incubation With Citric Acid

The suspension of nuclei (4 mg/ml protein) was added to 5 volumes of 1.5% citric acid. After incubation for 5 min, the suspension was homogenized by 5 slow strokes in a glass-glass Dounce-homogenizer (S-type). Then the suspension was centrifuged at $600g$ for 10 min, and the supernatant was centrifuged again for 10 min at $600g$ for a complete removal of nuclear debris. After the second centrifugation the supernatant was subjected to a $100,000g$ centrifugation for 75 min with the resulting pellet, taken up in NP 0.25 buffer, yielding the putative outer nuclear membrane fraction.

The pellet from the first centrifugation was washed by resuspending it in NP 0.25 buffer and subjected to another centrifugation for 10 min at $600g$. The resulting pellet (nuclei after citric acid extraction) was subjected to membrane isolation according to Thompson [1987], originally designed for preparing whole nuclear envelopes. In brief, the pellet was resuspended in NP 0.25 buffer to give the same volume as the original nuclear suspension before treatment with citric acid. DNAse I (Boehringer Mannheim, Mannheim, Germany; 0.2 mg/mg protein) was added and incubated for 8 min at 37°C. After cooling on ice the suspension was mixed with the 2.5-fold volume of NP 2.2, containing 0.5 M $MgCl_2$. This suspension was carefully overlaid with the same volume of NP 0.25 buffer and centrifuged for 30 min at $100,000g$. Material which accumulated at the interface between the two sucrose concentrations was removed with a pipette, diluted with the 10-fold amount of NP 0.25 buffer, and centrifuged at $10,000g$ for 20 min. The resulting pellet was taken up in a small volume NP 0.25 buffer yielding the putative inner nuclear membrane fraction.

Proteins which did not accumulate at the interface between the two sucrose concentrations were precipitated with 10% trichloroacetic acid and taken up in Laemmli sample buffer. This fraction was referred to as nucleoplasm. Unless noted otherwise, all steps were carried out at 4°C or on ice.

Affinity Purification of Polyclonal Antibodies

Five milligrams of the peptides, against which the antibodies were raised, were coupled to activated CH Sepharose (Pharmacia, Freiburg, Germany) according to the supplier's instructions. The sepharose was transferred into a column, loaded with 10 mg serum protein in PBS, and washed with 20 bed volumes of PBS. The specific antibody was eluted with 100 mM glycine, pH 2.0, containing 100 mM NaCl. The eluted

antibody was collected as fractions of 0.5 ml, and two fractions (F1 and F2) were used for immunofluorescence microscopy after being tested by Western blotting.

Western Blotting

Subcellular fractions were subjected to 10% (w/v) SDS-PAGE [Laemmli, 1970] and transferred to nitrocellulose membranes (Sartorius, Göttingen, Germany). Proteins were stained with Ponceau red to control the efficiency of blotting and equal loading of the gel. The membranes were then blocked with a solution of 5% (w/v) skimmed milk powder in TBS-T (overnight, 4°C). Incubation with primary antibody was performed for 2 h at room temperature or 4°C overnight, followed by 3 washes in TBS-T and incubation with secondary antibody coupled to alkaline phosphatase under the same conditions. All antibodies were diluted with TBS containing 5% (w/v) skimmed milk powder and 0.02% (w/v) NaN_3. After 3 washes in TBS-T and another wash in TBS, colour development was performed using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (p-toluidine salt).

Immunofluorescence Microscopy

Isolated nuclei were diluted in homogenization buffer to give a final protein concentration of about 0.2 mg/ml. Fifteen microliters of this suspension were loaded on single-wells of microscope slides (DUNN, 15 wells) and nuclei were allowed to sediment for 15 min. The buffer was carefully removed and for fixation a solution of 3.7% paraformaldehyde in PBS was added for 30 min at room temperature. Nuclei were permeabilized by incubating them with 80% methanol in PBS for 60 min at −20°C. Following three washes in PBS the incubation with primary antibody was done at room temperature for 1 h, followed by 3 washes in PBS and incubation with the secondary antibody under the same conditions. After another 3 washes with PBS, DNA was stained by adding 0.00004% DAPI for 5 min followed by a final wash with PBS. Cover slides were mounted with moviol (Polysciences, Warrington, PA) and allowed to dry overnight.

Protein Determination

Protein determination was done with the bicinchonic acid system (Pierce, Rockford, IL) using BSA as a standard.

Phospholipid Determination

Phospholipids were determined by a slight modification of the assay described by Simpson et al. [1987]. After extraction with chloroform/methanol (2:1 and 1:1) and evaporating the solvent, phospholipids were heated in a 1:1 mixture of 60% perchloric acid and concentrated sulfuric acid (200°C, 30 min; all mixtures v/v). The resulting inorganic phosphate was quantified by mixing 200 µl of the sample with 800 µl 1% ammonium molybdate and 60 µl of a solution of 15% sodium metabisulphite, 1% sodium sulphite, and 0.15% 1-amino-2-naphto-4-sulphonic acid (all w/v). The mixture was incubated 20 min at 90°C and the absorbance measured at 820 nm.

Identification of Proteins by Internal Sequencing

Protein bands were excised from Coomassie Blue stained gels. The proteins were digested in the gel matrix with trypsin and the resulting peptides eluted as described by Eckerskorn and Lottspeich [1989]. The eluted peptides were separated by reversed phase HPLC using a C-18 column (Vydac, Hesperia, CA). The solvent system used was 0.1% (v/v) TFA in water (solvent A) and 0.1% (v/v) TFA in acetonitril (solvent B). A gradient from 2 to 90% B was run in 77 min using a flow rate of 0.6 ml/min. Below 30% B the increase of B was 0.6%/min, afterwards 2%/min. Fractions were collected manually.

Mass spectrometry was performed with a Finnigan TSQ 700 electrospray mass spectrometer. Amino acid sequence analysis was performed using a 473 A gas phase sequencer (Applied Biosystems, Foster City, CA).

Electron Microscopy

Fractions were centrifuged, resuspended in PBS containing 2 mM $MgCl_2$, and centrifuged again. The resulting pellet was fixed in 1% glutaraldehyde and 1% tannic acid in 0.1 M phosphate buffer, pH 7.4. Subsequently, it was postfixed in a 2% OsO_4 solution. After rinsing and dehydration in the ascending alcohol series, the preparations were embedded in Epon and cut with an Ultracut E (Reichert, Vienna, Austria). The ultrathin sections were contrasted with 2% uranyl acetate and lead citrate and investigated with a Zeiss EM10 transmission electron microscope.

RESULTS

Cell nuclei were prepared from bovine cerebral cortex by a series of sucrose-density centrifu-

gations. Phase contrast microscopical observation showed that the nuclei were intact and not aggregated. Measuring the activity of marker enzymes as described earlier [Otto et al., 1992] revealed very small contaminations of nuclei with plasma membrane (below 3%) and cytosol (below 1%). The nuclei were used for a series of experiments aimed to separate inner and outer nuclear membrane. The starting point was a method described by Virtanen et al. [1977] who treated isolated nuclei from rat liver with 2.5% citric acid and collected the putative outer nuclear membrane by a 143,000g centrifugation step. We used this method in several variations, and also tried to isolate the inner nuclear membrane by applying a method for nuclear envelope preparation to the citric acid treated nuclei. While the nuclei show an intact double membrane structure before the treatment with citric acid (Fig. 1a), the nuclei widely lost at least one of the surrounding membranes after that treatment (Fig. 1b). Based on these electron microscopic pictures, which closely resembled pictures obtained by others [Virtanen et al., 1977; Gurr et al., 1963], it was assumed that indeed the outer nuclear membrane was removed from the nuclei.

Comparison of the protein patterns after SDS-PAGE showed that a specific set of proteins was highly enriched in the ONM fraction (Fig. 2). To further characterize the ONM (and INM) fractions, the distribution of marker proteins was analyzed by immunoblotting. Lamin B_2 was found predominantly in the INM and only in traces in the ONM fraction (Fig. 3). Surprisingly, p34, a membrane protein of the endoplasmic reticulum [Tazawa et al., 1991], was mainly found in the INM fraction and only to a very small extent in the ONM fraction (Fig. 3). This was not the result of destruction of most of the antigens by citric acid, as citric acid had no effect on the strength of the signals obtained by immunoblotting (data not shown). In contrast to this, we observed that treatment of isolated nuclei with citraconic anhydride, which was also reported to be useful for separating inner and outer nuclear membranes [Schindler et al., 1985], led to a complete loss of the antibody binding properties of both lamin B_2 and p34 (data not shown). Additionally, already low concentrations of citraconic anhydride caused an overall destruction of nuclei with a massive release of DNA (data not shown).

To confirm the distribution of the outer nuclear membrane proteins after citric acid treatment, two other endoplasmic reticulum proteins, ribophorin I [Kreibich et al., 1978] and α-SSR [Görlich et al., 1990], were investigated. Western blotting analysis revealed that the distribution of these proteins is identical with the
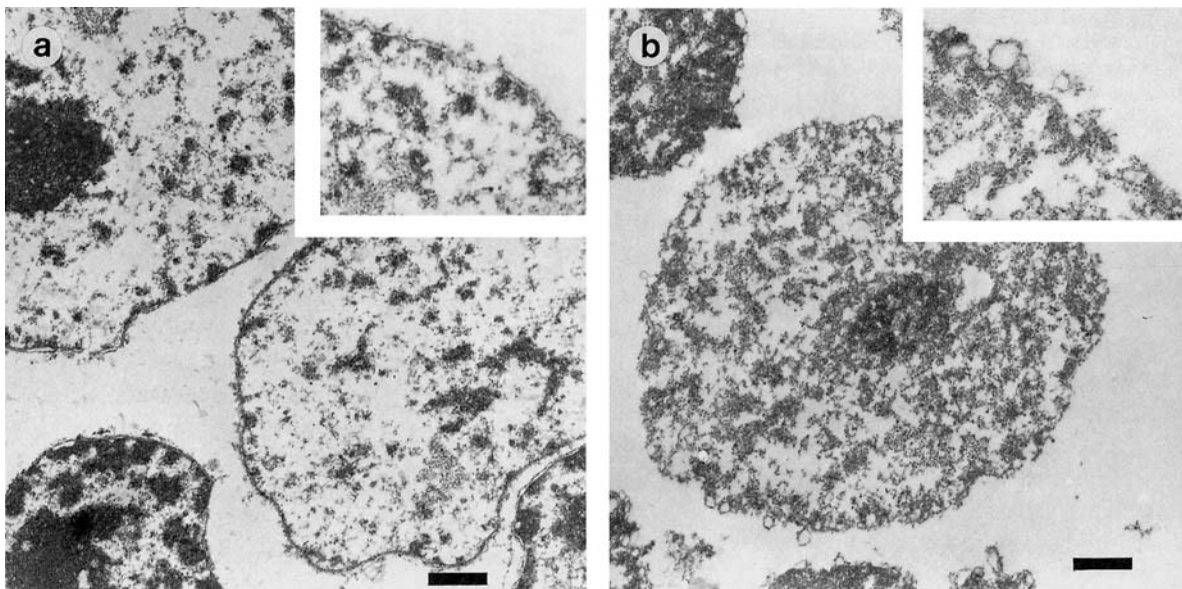
Fig. 1.   Electron microscopic picture of isolated cell nuclei before (a) and after (b) citric acid treatment. The insets show the nuclear envelopes in higher magnification. While the nuclei possess an intact double membrane structure before treatment with citric acid, at least one of the surrounding membranes seems to be lost after the treatment. Bars = 1 μm (2 μm within the insets).
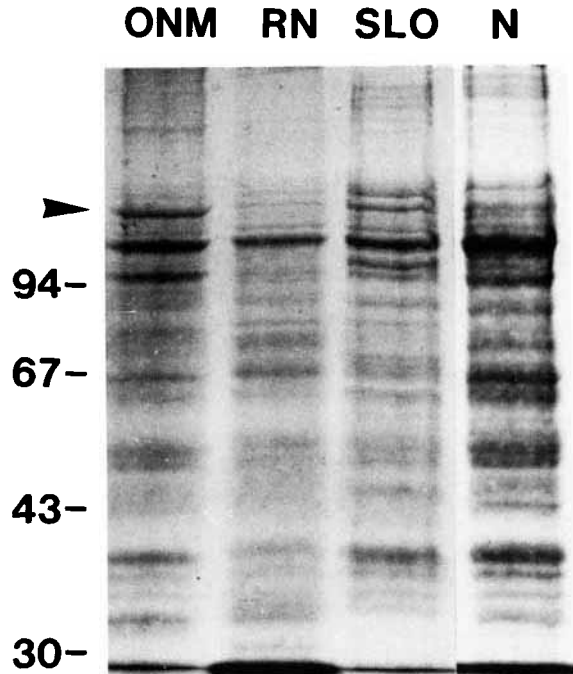
Fig. 2. Protein composition of nuclear fractions. ONM: putative outer nuclear membrane fraction; RN: nuclei after the extraction with citric acid; SLO: proteins which left the nuclei after the incubation with Streptolysin O; N: untreated cell nuclei. The arrowhead marks the 145 kDa protein chosen as a marker for the ONM fraction. Streptolysin O, which generates large pores in biological membranes, causes a different set of proteins to leave the nuclei than citric acid.
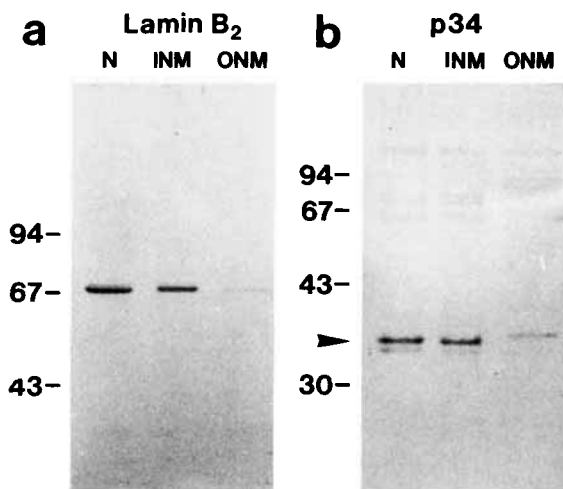
Fig. 3. Western blot of nuclear fractions with antibodies against lamin B₂ (a) and the ER-protein p34 (b). Both proteins are hardly extracted with citric acid.

distribution of p34, i.e., they could be detected mainly in the INM fraction (data not shown).

The protein patterns and the immunoblotting results suggested that the putative ONM fraction obtained after citric acid treatment con-

tained a specific set of nuclear proteins, but did not represent the outer nuclear membranes.

This was confirmed by electron microscopic analysis of ONM and INM fractions. Only a minor part of the ONM fraction shows membrane structures, while most of the material resembles chromatin or other structures of the nucleoplasm (Fig. 4a). On the contrary, the INM fraction contains mainly membranous material resembling pictures from preparations of the whole nuclear envelope (Fig. 4b).

To quantify the membrane content in the different fractions, we carried out a phospholipid determination. Compared to the phospholipid content of a nuclear envelope preparation from untreated nuclei, the putative INM fraction contains about 84%, the ONM fraction 15% of the phospholipids (means of two experiments).

To further characterize the "origin" of the nuclear proteins enriched in the putative ONM fraction, experiments were undertaken to identify and localize these proteins. First, it was investigated whether ONM proteins represent proteins, merely released nonspecifically from the interior by membrane and/or cytoskeleton damage. Apparently this is not the case, since analysis of the proteins, released after incubation of intact nuclei with either digitonin alone or digitonin and cytochalasin B, revealed a pattern different from that after citric acid (not shown). In both cases only a minor part of lamin B₂ and nearly no p34 left the nuclei. Finally, we tried an incubation of nuclei with Streptolysin O, a bacterial protein generating pores in biological membranes. This treatment lead to the loss of 30% of the nuclear proteins, but again the protein pattern was different from that after citric acid (Fig. 2). One of the prominent proteins of the putative ONM fraction with an apparent molecular mass of 145 kDa was chosen for further analysis. The relevant bands were cut out of several lanes and the protein was digested in the gel matrix. Thereafter, peptides were eluted and separated by reverse phase HPLC as described in Materials and Methods. Sequence and mass spectrometric data obtained for four of the peptides led to the identification of the 145 kDa protein as RNA helicase A (Table I). To confirm its subnuclear localization we used affinity-purified antibodies against one of the peptides.

From affinity-purification we did not obtain any antibody fractions which, on a Western blot,
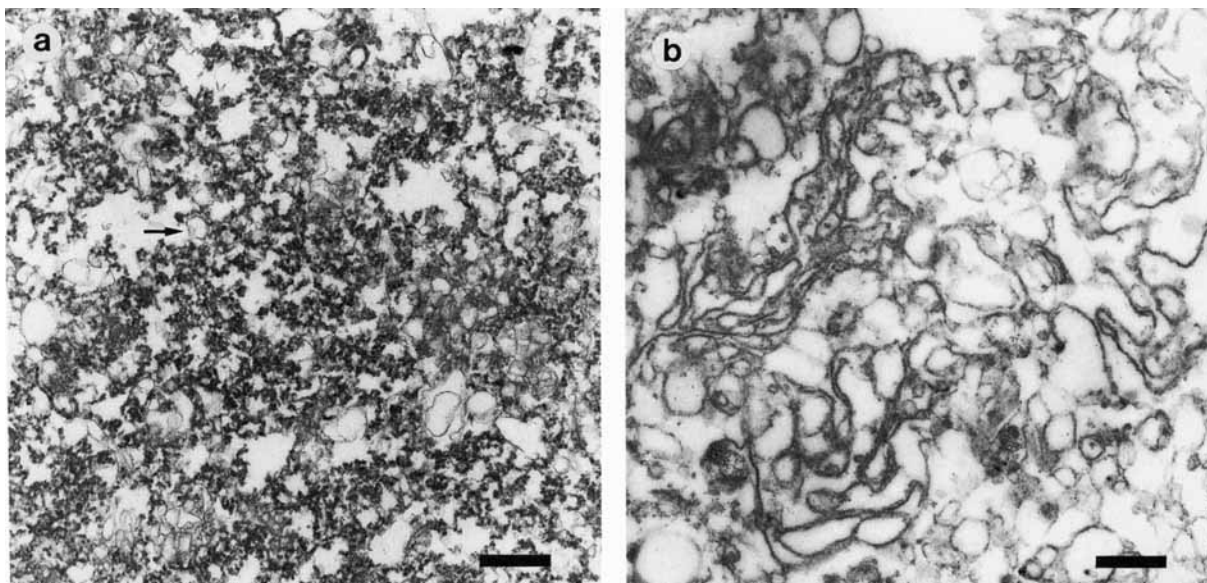
Fig. 4. Electron microscopic picture of the putative outer (a) and inner (b) nuclear membrane fractions. In the putative outer nuclear membrane fraction only a small amount of membranous material is visible (*arrow*); most of the fraction resembles internal nuclear structures (see Fig. 1). The putative inner nuclear membrane fraction consists mainly of membranous material, resembling pictures of complete nuclear membranes. Bars = 0.5 μm.

TABLE I. Internal Sequences From the Putative ONM Marker*

Peak no.	Mass determined by MS	Obtained sequence	Calculated mass	Sequence and residues of RNA helicase A
1	1050.8	EGETVEPYK	1051.1	EGETVEPYK (267–275)
2	1185.6	YTQVGPDHNR	1186.3	YTQVGPDHNR (200–209)
3	990.8	MGGEEAEIR	991.1	MGGEEAEIR (929–937)
4	n.d.	VFDPVPSGV	916.0	VFDPVPVGV (697–705)

*Peptide sequences obtained from the 145 kDa protein chosen as a marker for the ONM fraction and corresponding peptides from RNA helicase A. Only the peptide from peak 4 has a different amino acid in one position (S instead of V). This may be due to the different species examined (human/bovine). n.d. = not determined.

showed exclusive binding to the 145 kDa protein chosen as a marker for the ONM fraction (RNA helicase A). For immunolocalization of this protein we therefore used two antibody fractions: F1 bound to the 145 kDa protein and an unknown protein with an apparent molecular weight of 56 kDa (Fig. 5). F2 bound only to this 56 kDa protein (Fig. 5). We used both antibody fractions in immunocytochemical analysis and found that antibodies from F1 strongly stained the nucleoplasm with no clear intranuclear structures (Fig. 6a), whereas F2 gave a strong staining mainly for the nucleoli and, additionally, some form of punctate staining (Fig. 6b). These findings suggest that the 56 kDa protein is located predominantly in the nucleoli, whereas RNA helicase A is located in the nucleoplasm.

Its presence in the nucleoli, however, cannot be excluded. Staining could be blocked by the addition of the peptide of RNA helicase A in both Western blot and immunofluorescence microscopy.

To confirm that the extraction of RNA helicase A is not a specific effect for cell nuclei from the cerebral cortex, we also applied the citric acid treatment to cell nuclei isolated from bovine liver. Here the extraction with citric acid resulted in a similar enrichment of RNA helicase A in the putative outer nuclear membrane fraction as observed for brain nuclei (data not shown). In nuclei from mouse neuroblastoma Neuro 2a-cells we could not detect RNA helicase A with our antibody, but the distribution of lamin B_2 and ribophorin in subnuclear fractions
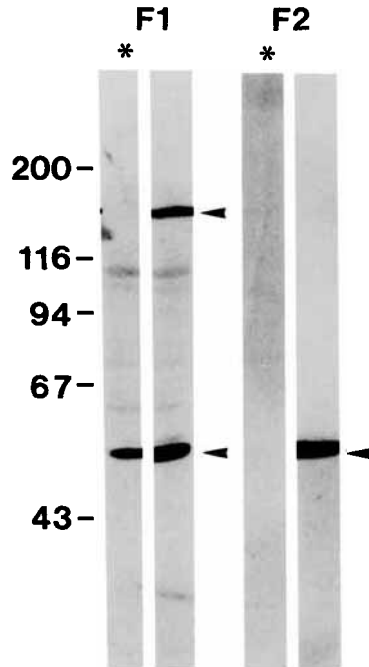
Fig. 5. Western blot of whole cell nuclei protein with antibodies raised against the peptide EGETVEPYK from RNA helicase A. Two antibody fractions obtained by affinity-purification were used: Antibodies of fraction 1 (**F1**) bind to two nuclear proteins with apparent molecular weights of 56 and 145 kDa (*arrowheads*). Antibodies of fraction 2 (**F2**) bind to the same 56 kDa protein (*arrowhead*), but not to the 145 kDa protein, which was chosen as a marker of the putative outer nuclear membrane fraction. In the lanes indicated with asterisks, specific binding was inhibited by the addition of peptide.

resembled the distribution in fractions obtained from bovine brain.

DISCUSSION

A reliable method for the separation of the inner and outer nuclear membranes would be of great importance, as it would allow localization and identification of proteins specific for these membranes. In addition to this, it would be possible to exactly determine the location of signal transduction components shown to be present at the nuclear envelope, e.g., inositoltrisphosphate-receptors [Matter et al., 1993] or protein kinase C [Buchner et al., 1992; Rosenberger et al., 1995].

One of the described methods to separate the two membranes is the treatment of isolated nuclei with citraconic anhydride [Schindler et al., 1985]. However, this method leads to a chemical modification of proteins [Gertler, 1971] and in our hands resulted in a complete loss of the ability of proteins to bind antibodies. Therefore it could not be controlled whether an effec-

tive separation of the two membranes had taken place, making this method not suitable, at least for our purpose.

Triton X-100 has widely been applied to remove both the outer and inner nuclear membrane from isolated nuclei or nuclear envelopes [Agutter, 1991]. Nevertheless, there are reports claiming the preferential removal of the outer nuclear membrane by low concentrations of Triton X-100 [e.g., Whittle et al., 1968]. However, Aaronson and Blobel [1974] showed, by testing a wide range of Triton X-100 concentrations, that there is no plateau in the Triton X-100 concentration at which approximately half of the lipid content is removed from nuclei. Therefore, the applicability of this method for separation of inner and outer nuclear membranes remains uncertain.

Of all reported methods the treatment with citric acid seemed the most reliable and has been applied repeatedly [e.g., Gilchrist and Pierce, 1993]. Electron microscopic comparison of nuclei before and after the treatment with citric acid alone suggests successful removal of the outer membrane, but the removed material is predominantly not membranous, as we showed using electron microscopic analysis. This observation was then confirmed by immunoblotting analysis of marker proteins, revealing that proteins of the outer nuclear membrane are hardly extracted. Since the phospholipid determination also shows that most of the nuclear membrane can be found in the putative INM fraction, it is somewhat astonishing that in the EM pictures citric acid treated nuclei appear to have lost at least one of the surrounding membranes. Perhaps parts of the nuclear membranes vesiculated but stayed attached to the nuclei in such a manner that they were lost during sample preparation for electron microscopy. This could also explain the appearance of most of the nuclear membranes in the putative INM fraction.

One protein of the putative ONM fraction was identified as RNA helicase A. RNA helicase A is homologous to the *Drosophila* MLE-protein involved in dosage compensation and was characterized and cloned only recently [Lee and Hurwitz, 1992, 1993]. Using anti-peptide antibodies we could confirm the nucleoplasmic location of this enzyme. Since this enzyme is strongly enriched in the putative ONM fraction, citric acid apparently does not merely release proteins from the nuclear interior nonspecifically by damaging
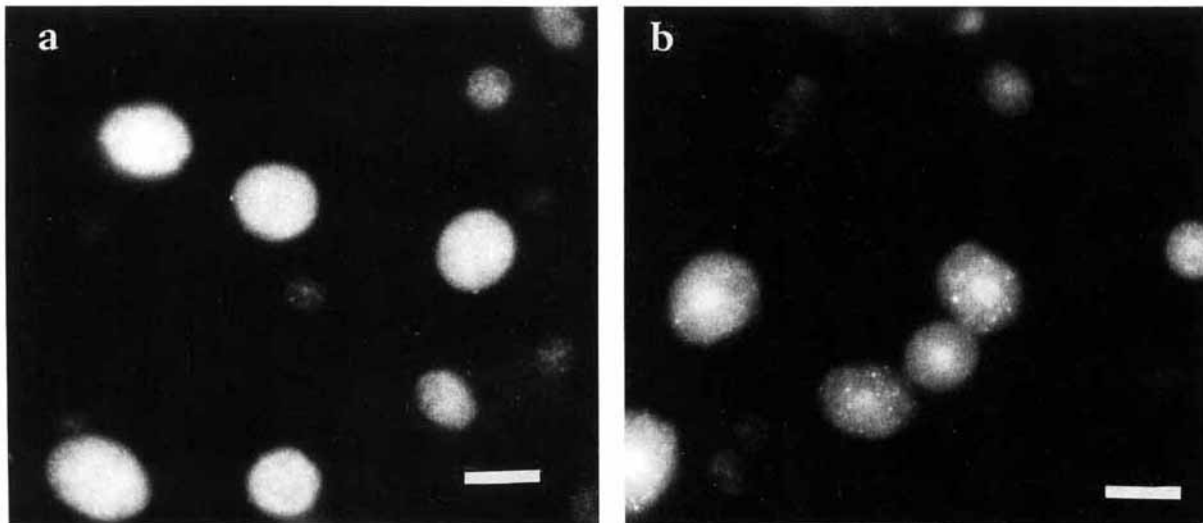
Fig. 6. Immunofluorescence microscopy of isolated nuclei with the same antibody fractions as in Fig. 5. Fractions 1 and 2 stain the cell nuclei with a markedly different pattern: Fraction 1 (**a**) evenly stains the whole nucleoplasm. In contrast to this fraction 2 (**b**) mainly stains the nucleoli, with the rest of the nuclei showing a very weak staining compared to fraction 1. Bars = 10 μm.

the membranes or the nucleoskeleton, but rather leads to the release of a specific set of proteins. The effect of citric acid seems to be mainly due to the acidic pH rather than to the chelating properties of the molecule (Rosenberger and Buchner, unpublished observations). A part of the released proteins could be pelleted at 100,000g. These proteins obviously are not tightly connected to the nucleoskeleton, which is not affected by citric acid, but must be part of other high molecular weight structures, presumably RNA-containing particles. On the other hand, a strong binding to DNA quite probably makes proteins resistant to the citric acid extraction. RNA helicase A as one of the proteins released by citric acid binds neither to DNA nor to the nuclear envelope, which may explain its extraction from the nucleus under relatively mild conditions. After leaving the nucleus, its binding to RNA may be responsible for its appearance in the pellet after the 100,000g centrifugation. Another protein, which can be extracted with citric acid, is the nuclear pore complex protein p62 first described by Davis and Blobel [1986] (not shown). Unlike RNA helicase A, however, this protein could not be found in the pellet after the 100,000g centrifugation. Supposedly, the nuclear pore complexes are partly destroyed by the citric acid incubation, but p62 leaves the nuclei separated from other proteins of the pore complex.

Taken together, our results strongly suggest that the incubation with citric acid is not a useful method for separating inner and outer nuclear membrane. Nevertheless, it may be a useful method for isolating nuclear RNA binding proteins.

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