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Immunofluorescence Microscopy and Biochemical Characterization of Two Nuclear Envelope Proteins of *Amoeba proteus* by Using a Monoclonal Antibody

Tae In Ahn, Si-Uk Yoo, and Yang R. Cho

Department of Biology Education, Seoul National University

Distribution of the antigens during the cell cycle of amoebae was followed by immunofluorescence microscopy using a monoclonal antibody against the nucleus as a probe. While the cells were in the interphase, the antigen was localized on the nucleus membrane. But it was dispersed all over the cytoplasm during mitosis and cytokinesis. The molecular weights of the immunoreacted antigens were 210 KD and 190 KD as determined by SDS PAGE and western blotting of the purified nuclei. The antigens were not soluble in non-ionic detergent, but were released from the nucleus by incubation with 0.05 M sodium carbonate, pH 10.6 or with 8 M urea at serial chemical extraction. Thus the antigens appeared to be peripheral proteins of the nucleus envelope. The isoelectric point of both antigens was 7.64 as determined by 2 D PAGE and transfer blotting. Considering the peripheral association with the nucleus membrane and the dispersed distribution during mitosis, the antigens could be lamin like proteins. However, it appears also possible that they are the component molecules of the unusually structured fibrous lamina of amoeba nucleus since they have the large molecular weight and the basic pI.

KEY WORDS : Nucleus envelope, Peripheral protein, *Amoeba*, Monoclonal antibody

All protozoan nuclei basically have the same structure of the cell nuclei of other eukaryotes (Raikov, 1982). Among Sarcodina, however, the nuclear envelope is of a typical structure only in forms with rather small nuclei. As a rule, the nuclear envelope of large sarcodinians with large nuclei (i.e. foraminiferans, radiolarians and large uninucleate amoebae) had various complications. *Amoeba proteus* has a large uninucleus in ovular shape. Within the nucleus it has many nucleoli dispersed in the periphery.

The nuclear envelope (NE) of *A. proteus* shows an extreme structural complexity which is no analogues among metazoans. The structure of the classic NE is usually preserved, but extra laminae is added to it from inside. The fibrous lamina on the inner side of the NE, which are usually thin and hardly noticeable, developed greatly and distinctly structuralized in this amoeba. The interwinding bundles of microfibrillar material form the walls of regular hexagonal alveoli that are up to 500 nm high, 150-180 nm wide and resemble a honeycomb; they are therefore called the 'honeycomb layer'. The alveoli are perpendicular to the nuclear envelope and each of them has a nuclear pore at the bottom (Leeson and Bhatnagar, 1975a; Gromov, 1985). The molecular nature of the lamina has not been studied yet. Thus the characterization of the distinctly fibrous and structured lamina of amoebae would be significant not only for comparison with those of other organisms but also for deducing the role of the nuclear lamina.

The enhancement of the mechanical strength of

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the nuclear envelope is usually regarded as a probable function of the honeycomb layer of the envelope, although it may also participate in transport of particles and macromolecules. The fibrils that form the alveolar walls were considered contractile (Leeson and Bhatnagar, 1975b).

Amoeba undergoes a semiopen mitosis with an intranuclear spindle rather than open mitosis (Gromov, 1985). In metaphase the NE loses its pore complexes and transforms into a system of rough endoplasmic reticulum cisternae (ERC). The nucleoli and the honeycomb layer disappear completely (Gromov, 1985). The NE becomes reconstructed from ERC in the late anaphase, but its formation being completed by the end of telophase only.

Biochemical composition and behavior of the protein components of the NE and nuclear skeleton have been well documented in vertebrates (Hubert and Bourgeois, 1986, Krohne and Benavente, 1986). But very little is known about the biochemical nature of NE in amoebae. Recently Kim *et al.* (1989) followed the behavior of spectrin-like 220 KD protein of NE in amoebae by using an mAb as a probe.

In this study by utilizing another mAb to the NE of *A. proteus*, the behavior and biochemical nature of the two NE proteins, 210 KD and 190 KD, were studied.

Materials and Methods

Cell Culture

TD strain of *Amoeba proteus* was cultured in a modified Chalkley's medium (Ahn, 1983) at 23°C. Amoebae were fed daily with cultured *Tetrahymena* as food organism (Goldstein and Ko, 1976).

Selection of Division Sphere

By following the criteria of Chalkley and Daniel (1933) and Dawson *et al.* (1937), the amoebae in mitosis (division sphere) were selected under the dissecting microscope, and transferred to the precooled Chalkley's in 24-well plate to stop further progress of mitosis. Then, the selected division spheres were studied under the phase contrast microscope to observe the progress of cell division, or processed for immunofluorescence microscopy.

Immunostaining

Amoebae were fixed for 30 min in 3% paraformaldehyde, pH 7.5, incubated for 1 min in 0.05% Triton X-100, and washed 3 times with phosphate buffered saline (PBS), pH 7.4 at 15 min intervals. Then the cells were incubated for 30 min in PBS containing mAb against amoeba nucleus (1/50 dilution of the ascitic fluid), washed again with PBS and incubated for 30 min in PBS containing the secondary antibody (Goat antibody to mouse IgG coupled with DTAF, Jackson Immunoresearch Lab. Inc.), washed 4 times in 20 min. Stained cells were mounted with a solution containing 10% p-phenylene-diamine and 90% glycerol, and observed under the Balplan epifluorescence microscope (Bausch and Lomb). Pictures were taken using Kodak Tri-X pan film (ASA 400). The clone for mAb against the nucleus of amoeba was obtained from Dr. Kwang W. Jeon (University of Tennessee at Knoxville, Tn) and ascitic fluid was obtained by peritoneal injection of the hybridoma cells.

DAPI Staining

As a tracer for the chromatin or the condensed chromosomes during the cell cycle, amoebae fixed in paraformaldehyde were stained with 0.1 μg/ml of 4′, 6-diamino-2-phenylindole (DAPI-)(Coleman *et al.*, 1981) for 15 min, washed in PBS for 30 min and examined under the fluorescence microscope using 300-400 nm exciting filter (Corning 5840).

Subcellular Fractionation

Subcellular fractionation was done at 4°C, otherwise noted. Amoebae starved for 3 days to eliminate any contaminating food organism in the phagosomes, were collected and washed by spinning for 30 seconds at 150 × g. Cells were homogenized in 20 mM Tris, 1.5 mM $MgCl_2$, 10% sucrose, 0.03% spermidine, pH 7.2 (TMSS-buffer). The homogenate was filtered through 45 nm-nylon screen. Plasmalemma was purified from the residue left on top of the screen by 2 cycles of differential centrifugation at 1000 × g for 2 min.

The nuclei were purified from the filtrate by Percoll step gradient (15, 25, 35, and 45% in TMSS-buffer) centrifugation for 7 min at 550 × g. Nuclei were banded at the boundary of 25% and 35%. The boundary fraction was collected and diluted in 10 volume of the TMSS-buffer. Then nuclei were pelleted by spinning for 2 min at 550 × g.

Fractionation of Nucleus Envelopes

Purified nuclei (50 μl of packed volume) were vortexed and mixed with equal volume of nucleases, 2.5 μg each of DNase and RNase. After incubation for 15 min at 24°C, the incubation was spun for 10 min at 12000 × g. Then the pellet was washed with 0.5 M NaCl in 20 mM Tris, 1.5 mM $MgCl_2$, 10% sucrose, pH 7.2 (TMS). To the pellet 500 μl of nucleases were added and incubated for 15 min at 24°C. After spinning, NE was obtained as pellet and processed for chemical fractionation.

Chemical Fractionation of Nucleus Envelopes

To obtain nuclear pore-lamina complex (Dwyer and Blobel, 1976), isolated NE pellet was resuspended either in 1% NP-40 or 2% Triton X-100 with either 0.02 M or 0.15 M KCl and incubated for 15 min in an icebox (Senior and Gerace, 1988). Then, they were spun and the pellet was washed with 0.5 M NaCl in TMS buffer. The nuclear pore lamina complex was obtained as pellet fraction.

For chemical fractionation of NE (Gerace *et al.*, 1982), the isolated NE pellet was vortexed and either (a) shortly resuspended in 0.1 M NaOH or (b) incubated in 6 M urea in 20 mM Tris, 1.5 mM $MgCl_2$ pH 7.2 for 30 min. They were then centrifuged at 12000 × g for 10 min to yield supernatants and pellets.

Extraction of nucleus envelopes with Triton and salt were carried out according to Senior and Gerace (1988) with slight modifications. NE were incubated with DNase I, washed in 0.5 M NaCl, incubated again with DNase I, then extracted with 2% Triton X-100 either in 0.02 M KCl or in 0.15 M KCl. After an incubation of 15 min each, samples were centrifuged for 15 min, and the proteins solubilized in supernatant or left in the pellet were analyzed by SDS PAGE and immunoblotting.

Gel Electrophoresis

Proteins of the amoebae homogenate or the purified subcellular fractions were analyzed by SDS-PAGE (Laemmli, 1970) or two dimension gel electrophoresis (O'Farrell, 1975). Separating gel in SDS PAGE was made 3-12% polyacrylamide gradient. The gel was stained with Brilliant Coomassie Blue (BCB) (Diezel *et al.*, 1972). for two dimensional separation 5-12% separating gel was used, and the gel was stained by silver (Merril *et al.*, 1981).

Immunoblotting

Separated proteins in acrylamide gel was electrophoretically transferred to nitrocellulose (NC) paper by following Towbin *et al.* (1979). On completion of transfer non-specific binding of the antibody on NC paper was blocked by incubating the paper for an hour in 3% (w/v) non-fat dry milk. And the paper was washed 3 times at 30 min intervals in 0.3% Tween 20-PBS (TPBS). Then the paper was immunostained by incubating for 1 hour with mAb (1/500 dilution of ascitic fluid in PBS), followed 3 washes with TPBS, and incubated with secondary goat antibody to mouse IgG coupled with horseradish peroxidase (Jackson Immunoresearch Lab. Inc.). Color reaction was carried out using 4-chloro-1-naphtol as the substrate (Fujita *et al.*, 1986).

Results

Behavior of the Nucleus Antigen During Mitosis

To localize the nucleus antigen, the selected division sphere or the amoebae in interphase were processed for immunostaining. During the interphase the nucleus antigen was concentrated on the NE (Fig. 1). But it was dispersed all over the cytoplasm during mitosis and cytokinesis (Figs. 1C, 2E and 2F). For comparison with the nucleus chromatin, amoebae were counter stained with DAPI. By DAPI staining it was possible to follow the behavior of the nucleus chromatin (Figs. 2G, 2H, and 2I). The DAPI fluorescence of amoebae nuclei started condensing at prophase, then the dense

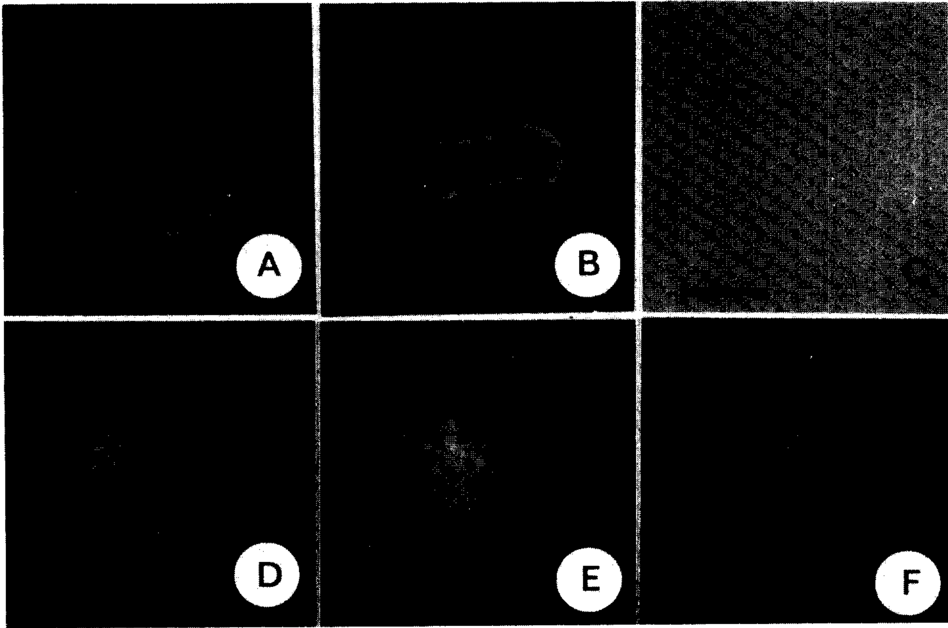


Fig. 1. Immuno- (A, B, and C) and DAPI-fluorescence (D, E, and F) micrographs of the amoebae nuclei at interphase (A, B, and D), prophase (C, E) and metaphase (F). The interphase nucleus was either circular (A) or disk (B) in morphology. Immunofluorescence was prominent at the periphery of the nucleus (A, B). The discrete immunofluorescence shown in interphase nucleus was dispersed all over the cytoplasm during mitosis (C) and cytokinesis. The pictures were taken and printed at the same exposure and magnification. The bar in C represents 25 μm for all prictures.

granular form was aligned at the mid plane of the dividing nucleus zone and migrated to the opposite pole as the cell cycle proceeded mitosis and cytokinesis.

On completion of cytokinesis the nucleus envelope regained the interphase immunofluorescence. Thus the behavior of the NE antigens was confirmed. They are localized on the nucleus envelope during the interphase and dissociate from the NE by the onsẽt of mitosis, disperse in the cytosol during mitosis and cytokinesis, and reassemble into NE at the end of cytokinesis.

Characteristics of the Antigen

By the procedure using Percoll gradient and subsequent NE fractionation, 1.5 mg of nucleus envelope protein was obtained from 20 ml of packed amoebae. In phase-contrast microscopy, the purified nucleus fraction did not contain any significant contaminant (Fig. 3). Whole homogenate and the subcellular fractions were run in parallel and processed for BCB or immuno-staining with the monoclonal antibody. The apparent molecular weight of the immunostained bands of the nucleus fraction were 210 KD and 190 KD (Fig. 4, Lane N).

For further characterization of the protein, purified nuclei were incubated for 30 min in 2% Triton X-100 at room temperature or in 0.05 M sodium carbonate, pH 10.6 at 4°C. Then each of the incubation mixture was spun for 1 hour at 12,000 $\times$ g. The supernatant and the pellet fractions were analyzed by SDS PAGE and transfer blotting (Fig. 5). Two percent of Triton X-100 did not dissolve or release the antigen from the nucleus. The antigen was left in the pellet fraction. But the antigen dissociated from the nucleus by treatment with 0.05 M sodium carbonate (Fig. 5B).

Solubility of the two proteins were further tested by dissolving the NE in 0.1 M NaOH or in 6 M urea (Fig. 5). The 190 KD antigen was mostly solubilized in 0.1 M NaOH, and completely solu-

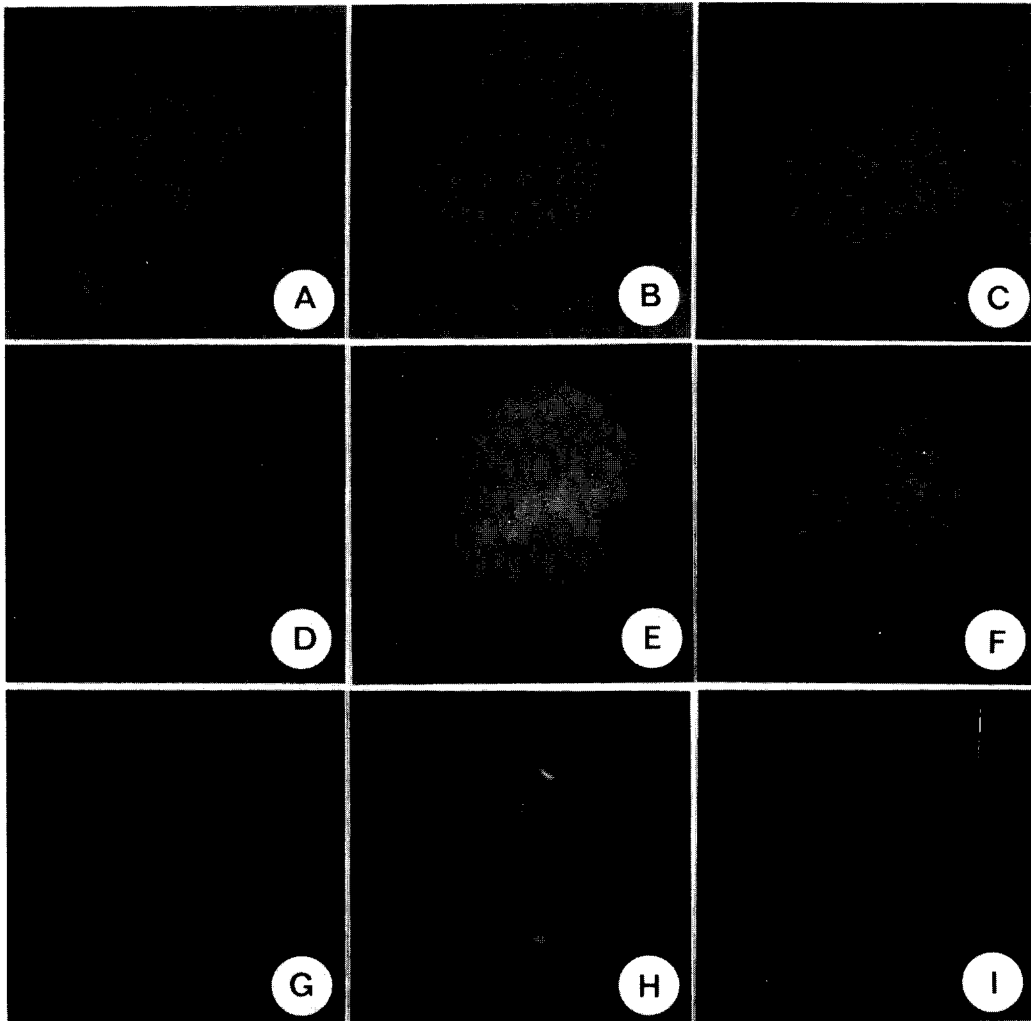


Fig. 2. Phase contrast (A, B, and C), immuno- (D, E, and F) and DAPI-fluorescence (G, H, and I) micrograph of amoebae at interphase (A, D, and G), mitosis (B, E, and H) and cytokinesis (C, F, and I). The immunofluorscence was discrete in the nucleus at interphase (D), but dispersed during mitosis (E) and cytokinesis (F). Formation of cleavage furrow is indicated by arrow (F). DAPI fluorescence was dispersed in the nucleosol at the interphase (G), and condensed during mitosis (H) and cytokinesis (I). In phase contrast microscopy, the nucleus was visible at interphase only (A, arrow head). The bar in A represents 100 μm for all pictures.

bilized in 6 M urea. The 210 KD antigen was mostly left in the pellet after 0.1 M NaOH extraction, and was not dissociated from the NE by 6 M urea. When the pellet of 0.1 M NaOH extraction was washed with buffer, the antigens were completely lost. Thus, the antigens were released from the NE by 0.1 M NaOH, but apparently reassociated with the pellet component. When the NE was extracted with 0.5 M sodium carbonate, pH 10.6, the amount of protein in the upper 210 KD band was significantly reduced and appeared to accumulate at 190 KD band. It could be due to the damaging effect of the elevated pH of the incubation medium containing sodium carbonate.

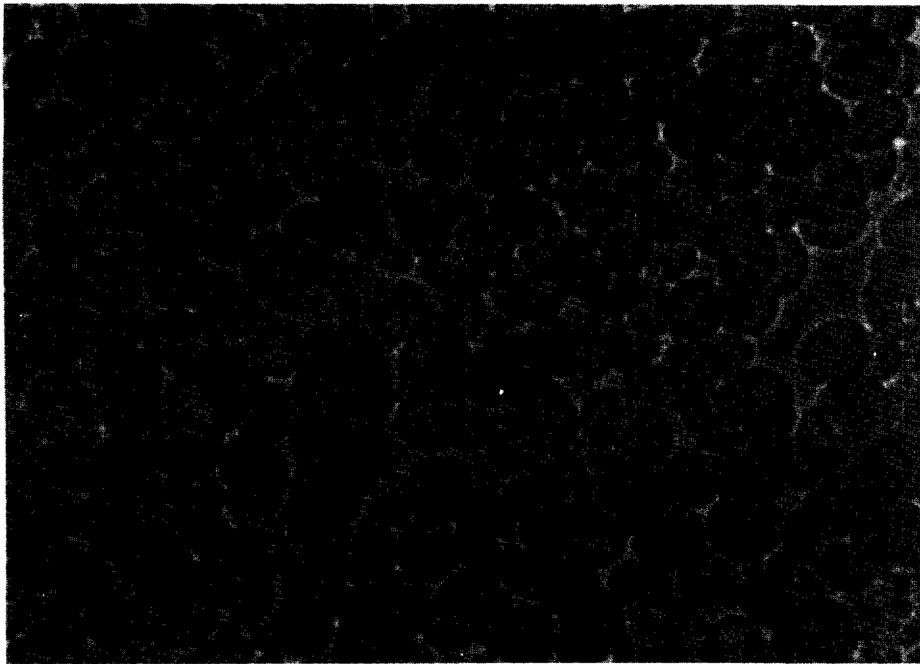


Fig. 3. Phase contrast micrograph of the purified amoebae nuclei. The bar represents 25 μm.

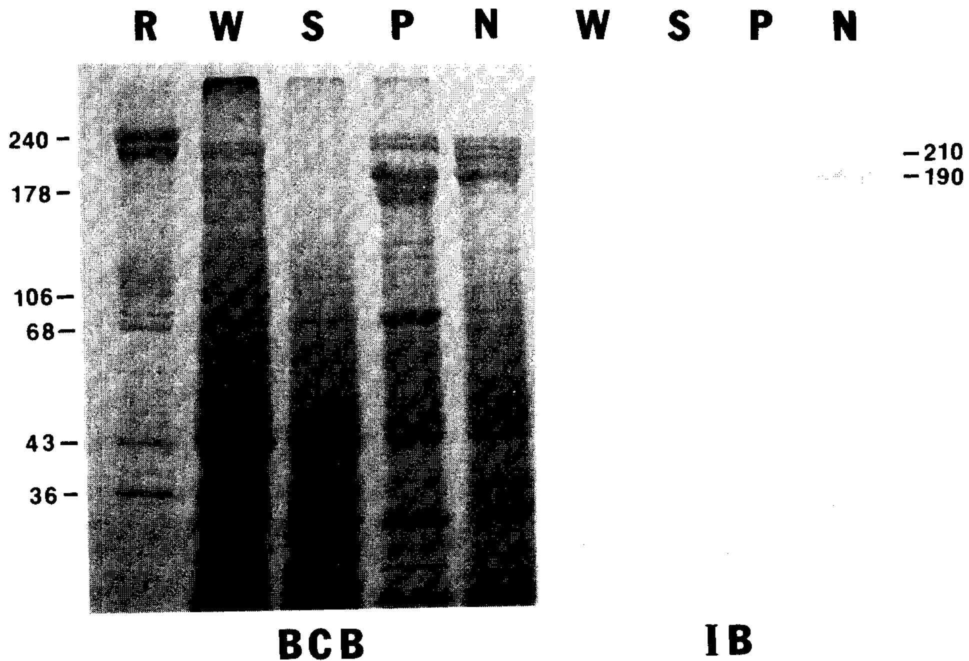
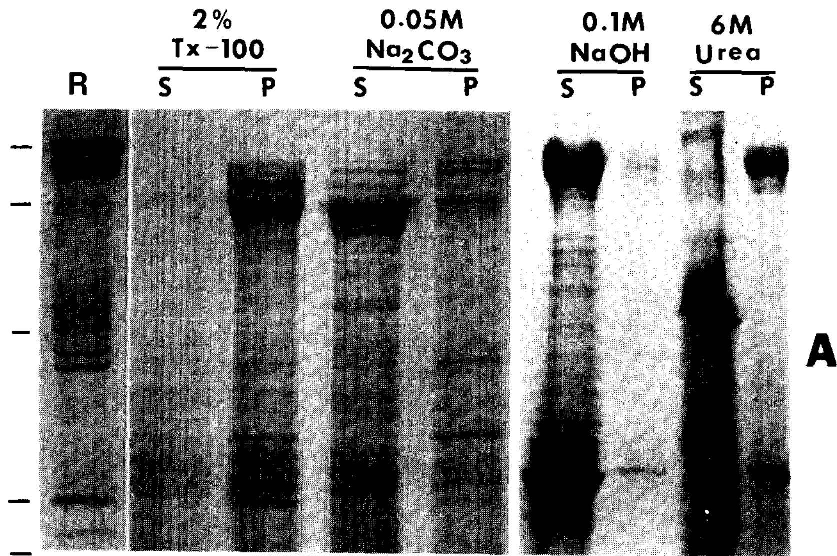


Fig. 4. Brilliant Coomassie Blue (BCB) and corresponding immunoblot (IB) of subcellular fractions of amoebae after SDS-PAGE. R : Membrane proteins of human RBC as molecular size marker. The numbers on the left denotes molecular weight in KD. The same notation and marker were used in following figures. W : Whole homogenate of amoebae, S : Water soluble proteins of amoebae, P : Purified plasmalemma, N : Purified nuclei. Note the two prominent bands (210 KD and 190 KD) of the nucleus fraction were immunostained by the mAb.



B

Fig. 5. BCB stained (A) and immunoblotted (B) patterns of the chemical tractions of amoeba nucleus envelope after SDS-PAGE. Purified nucleus envelopes were processed for chemical fractionation as described in materials and methods. 'S' and 'P' denotes supernatant and pellet fraction of the chemical fractionation, respectively. Note the immunostained bands (B).

For sequential extraction of the antigen, the purified nuclei were incubated in 1% NP-40, 2 M NaCl, then in 8 M urea. After each incubation the reaction was spun for 10 min at 12,000 × g. Then the supernatant was collected and the pellet was processed for next incubation. In this sequential extraction, both of the antigens were not released from the NE by 1% NP-40 or by the followed washing with 2 M NaCl, but solubilized by 8 M urea (Fig. 6).

The pI of the antigen was determined by 2-D EP and transfer blotting. Both antigenic polypeptides were resolved near the top end and were two of the most basic protein (Fig. 7). The apparent pI of the region was 7.64.

Discussion

In immunofluorescence microscopy (Fig. 1, 2)

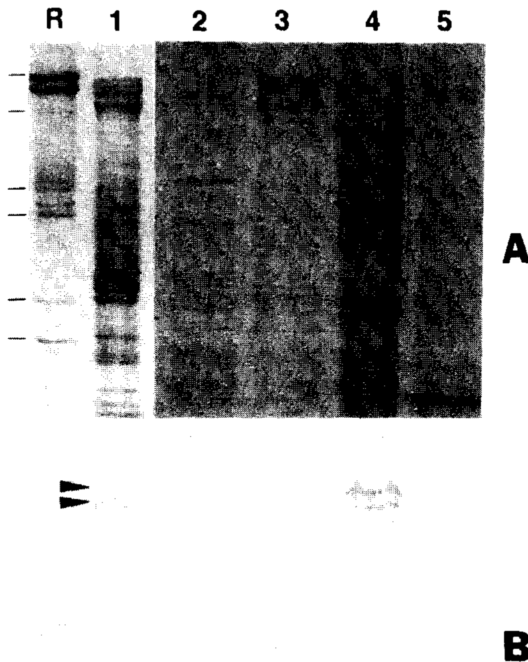


Fig. 6. BCB-(A) and corresponding immuno-stained (B) pattern of the sequential extractions of the nucleus envelope. Lane 1 : Proteins of purified nuclei; Lane 2 : 1% NP-40 soluble proteins of nuclei; Lane 3 : 2 M NaCl soluble proteins of 1% NP-40 pellet; Lane 4 : 8 M urea soluble proteins of 2 M NaCl-pellet; Lane 5 : Proteins of 8 M urea-pellet. The arrows in B lane 1 indicate the 2 antigens, m. wt. 210 and 190 KD.

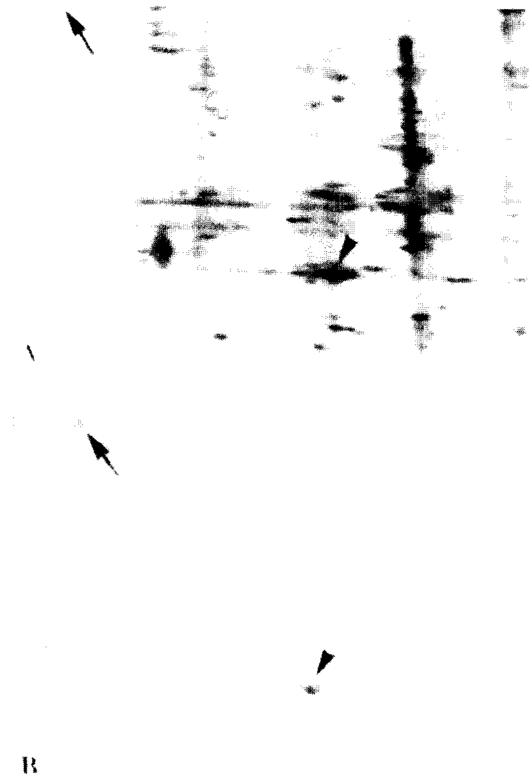


Fig. 7. Two dimensional polyacrylamide gel electrophoresis of the nucleus fractions stained by silver (A) and monoclonal antibodies (B). The arrows in B indicate the nucleus antigen, the arrow head indicates the purified amoebae actin added to the purified nucleus fraction for comparison. Immunostaining was carried out using mAbs to the nucleus and actins of amoebae, simultaneously. The measured pI of the nucleus antigen from the IEF gel was 7.64.

the antigens were localized on the NE during the interphase, dispersed all over the cytoplasm during the cells are in mitosis, and restored on the NE at the end of cytokinesis. The behavior of amoeba NE during mitosis is different from that of higher eukaryotic NE in that it does not break down but persist as large pieces during mitosis as in some other lower eukaryotic cells (Raikov, 1982). The NE of amoebae are known to be continuous by the telophase stage. In electron microscopy, the restoration of the honeycomb lamina occurs some time after completion of cytokinesis (Gromov, 1985; Roth *et al.*, 1960). Thus the results of immunofluorescence microscopy well correspond with the ultrastructural study by electron microscopy. NE breakdown and lamin depolymerization are biochemically distinct and separate process (Newport and Spann, 1987). For discrete localization of the antigens, an ultrastructural study is in progress using immuno-gold as probe.

The antibody used in this study recognized two novel proteins of NE of amoebae. These two antigens appear unique to amoebae, as the antibody did not cross-react with the NE of HeLa or cul-

tured fibroblast (data not shown). In the results we demonstrated the two nucleus antigens, m. wt. 190 KD and 210 KD are the proteins bound to the NE.

Senior and Gerace (1988) characterized an integral protein, gp 190 of NE of rat liver, where they used cytochrome p 450 of the microscome as positive control. GP 190 was left with the pellet after extraction of the NE with 0.1 M NaOH or 6 M urea, but lamins or the nucleolar protein, p 38, of yeast (Aris and Blobel, 1988) were solubilized. In addition, gp 190 was not solubilized in 2% Triton X-100 containing 0.02 M KCl, but was solubilized in Triton X-100 containing 0.15 M KCl. By following this criteria both 190 and 210 KD antigens of amoebae NE are peripheral proteins (ref. Fig. 5, 6). But the 210 KD antigen of amoebae NE was not solubilized in Triton X-100 containing either 0.02 or 0.15 m KCl (Data not shown). This impose the 210 KD antigen as an integral protein.

However, the 210 KD antigen could be solubilized by 8 M urea and 0.05 M sodium carbonate. Thus the 210 KD antigen apparently has a stronger binding on to the NE than 190 KD antigen. By following the operational definition of Findlay (1987), the 210 KD antigen of amoeba NE could be a kind of peripheral protein.

Another interesting aspect of these two antigens is that the isoelectric point of both antigens is verv basic, 7.64 (Fig. 7). Nucleus lamins of mammlian cells are 60-80 KD in m. wt., and have pI between 5.4-7.2 (Shelton, *et al.*, 1980; Krohne and Benavente, 1986). The nucleolar proteins of yeast and rat liver have pI between 6.1-7.0 and 5.8-6.9 (Aris and Blobel, 1988). The cDNA of gp 190 of rat liver NE was cloned and sequenced, but its pI is unknown (Wozniak *et al.*, 1989). By comparisons with some of these characterized uncleus proteins, amoeba NE has exceptionally basic proteins. Futher characterization of these proteins would illucidate the possible role of unusually structured fibrous lamina of amoebae.

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단항체를 이용한 아메바(*Amoeba proteus*)의 2종 핵막 단백질에 대한 면역형광현미경적 및 생화학적 특성 조사

안태인 · 유시욱 · 조양래 (서울대학교 사범대학 생물교육과)

아메바의 세포주기에 따른 핵 항원의 분포를 단항체를 탐침으로 사용하여 면역 형광현미경법에 의해서 추적하였다. 세포간기에 이들 항원은 핵막에 분포하였으나 핵분열 및 세포질 분열기에는 세포질 전면에 분산되었다. 순수분리한 핵의 단백질을 SDS PAGE 및 전이 염색한 결과 이들 항원의 분자량은 210 KD, 190 KD였다. 이들 항원은 비이온성 세탁세에 의해서는 용출되지 않았으며 0.05 M sodium carbonate, pH 10.6, 또는 순차적인 화학적 용해실험에서 8 M urea에 의해서 용해되었으므로 핵막의 수변단백질로 확인되었으며, 2차원 전기영동 및 전이 염색결과 그 등전점은 7.64였다. 이들 항원은 핵막의 주변단백질이면서 세포분열기 동안 세포질에 분산되는 특징을 보면 lamin 유사 단백질일 가능성도 있으나, 분자량이나 염기성인 등전점을 갖는 특징을 보면 이들은 아메바의 핵막에 특이한 섬유상 구조물인 lamina의 조성분일 수도 있다.