Nuclear envelope of the seminal-vesicle epithelium

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The nuclear envelope of seminal-vesicle epithelium was isolated by a procedure involving enzymic digestion with deoxyribonuclease I, sonication in the presence of 0.34 M-sodium citrate, and centrifugation through sucrose density gradients. The mass of envelope DNA was only 0.8% of that of envelope protein, and by transmission electron microscopy the envelope was 98–99% pure. We showed that the envelope possesses a protein kinase activity which is uninfluenced by cyclic nucleotides. Both lysine-rich histone and dephosphophosvitin as substrates gave a greater specific activity than did envelope protein itself. Optimum requirements with respect to Na^+ , Mg^{2+} , pH and ATP were established for each substrate, and the influence of other factors on enzyme activity was investigated. Data, obtained mainly with the use of lysine-rich histone, are presented which indicate that nuclear envelope from intact and 96 h-castrated guinea pigs may have equal protein kinase activities and, in separate experiments, equal phosphoprotein phosphatase activities. Clarification of these initial observations must await identification of the natural substrates for the envelope's phosphorylation–dephosphorylation reactions.

The epithelium of guinea-pig seminal vesicle is an excellent biological preparation for the study of androgen action. It comprises homogeneous cells whose morphology and function are highly dependent on androgen(s). The cells, and therefore their nuclei, can be obtained without contamination from different cell types. The purification of nuclear envelope, the bi-leaflet membrane surrounding the nucleus of eukaryotic cells, had not been described for any tissue comprising a single epithelial-cell type. In the present paper we describe the preparation of nuclear envelope from seminal-vesicle epithelium, show that the organelle possesses protein kinase activity towards endogenous and exogenous phosphoprotein substrates and describe some of the catalytic features of the enzyme, including its response to castration. No previous study has described such an enzyme activity in the nuclear envelope of a sex-steroid-dependent tissue. An investigation of nuclear envelope is strongly indicated for such tissues, because the steroid–receptor complex must interact in some way with this organelle, even if only to traverse the envelope en

route to its final destination(s) within the nuclear chromatin.

Materials and methods

Excision of seminal vesicles and dissection of the epithelium from underlying stroma

This was as described by Venezia *et al.* (1977a).

Preparation of guinea-pig seminal-vesicle nuclei

The vesicular epithelium (5–7 g) was usually isolated from eight animals (700–900 g) at a time. We homogenized it in 4 vol. of Buffer A (0.32 M-sucrose/5 mM-Tris/HCl/1.0 mM-MgCl₂, pH 7.5 at 4°C) in a Potter–Elvehjem homogenizer by 18 up-and-down strokes at 700 rev./min. The crude nuclear pellet was obtained by centrifugation (600 g for 10 min) in a Beckman TJ-6 centrifuge, and was washed once in Buffer A. We suspended the pellet in 32 ml of Buffer B (2.2 M-sucrose/5.0 mM-Tris/HCl (pH 7.5)/1.0 mM-MgCl₂) by drawing it repeatedly through a plastic pipette, and then 16 ml of the suspension was layered over 21 ml of Buffer B in each of two centrifuge tubes. After centrifugation at 55 000 g for 60 min in a SW 27 rotor in a Beckman

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L5-50 centrifuge, the nuclear pellets were combined and washed twice in 12 ml of 0.25 M-sucrose/1.0 mM-MgCl₂ solution by low-speed centrifugation as described above. The pellet of purified nuclei could be stored at -20°C.

Preparation of nuclear envelope

No single published procedure was adequate for this particular tissue, although our method incorporated features employed by others individually in the preparation of liver nuclear membranes (see, e.g., Kasper, 1974). The nuclei were suspended in 10 ml of TKM buffer (50 mM-Tris/HCl/25 mM-KCl/5 mM-MgCl₂, pH 7.5 at 4°C). Deoxyribonuclease I [2100 units (Kunitz, 1950a,b)/mg; Millipore Corp., Freehold, NJ, U.S.A.] dissolved in 1 ml of TKM buffer was added, followed by 2 ml of 60% (w/v) sodium citrate, giving concentrations in a final volume of 13 ml of 50 µg of enzyme and 100 mg of citrate/ml. This somewhat gelatinous mixture was stirred with a Teflon rod and sonicated for 15 s by using a micro-tip at a setting of 30 on a scale of 0-100 (Biosonic III; Bronwill Scientific, Rochester, NY, U.S.A.). It was then allowed to incubate for 1 h at room temperature with occasional stirring. Subsequently, 10-20 µl of the incubation mixture, no longer gelatinous, was examined by phase microscopy. If 5-10% of the nuclei were still intact, we employed additional sonication for 5-10 s at a setting of 20. If 10-20% or more of the nuclei were intact, we added 250 µg of deoxyribonuclease I in 4.2 ml of TKM buffer and 0.8 ml of the concentrated sodium citrate, and incubated for 30 min more.

The procedure resulted in disruption of virtually all nuclei. The suspension was then transferred to a thick-walled centrifuge tube for centrifugation in a SW 27 rotor at 58 500g for 30 min at 3°C. This gave a gelatinous pellet with a brownish tinge. We then added 1.5 ml of TKM buffer and 5 ml of sucrose solution I (2.0 M-sucrose, TKM buffer and 10% sodium citrate). By withdrawing and expressing it repeatedly through a plastic Pasteur pipette, we made the suspension relatively homogeneous before sonication for 10 s at a setting of 20. The suspension was transferred to a 37.5 ml cellulose nitrate tube together with an additional 2.5 ml of sucrose solution I which had been used to wash out the original thick-walled vessel. The resulting suspension was mixed well; its volume was approx. 9 ml and its sucrose concentration 1.7 M. We then layered on successively 18.2 ml of sucrose solution II (1.08 M-sucrose, TKM buffer and 10% sodium citrate) and 9.0 ml of sucrose solution III (0.56 M-sucrose, TKM buffer and 10% sodium citrate) to create a discontinuous sucrose/TKM/citrate gradient comprising densities of 1.12, 1.20 and 1.22 g/ml from top to bottom, based on refractive-index measurements.

The tubes were centrifuged at 120 000g in a SW 27 rotor for 6 h at 3°C. Purified nuclear envelope floated to the top of the 1.20 g of sucrose/ml layer. We established by centrifugation studies that the envelope particles have a density of 1.16-1.18 g/ml. The envelope layer was removed with a plastic Pasteur pipette and was transferred to a thick-walled SW 27 centrifuge tube. This material was suspended in 3 vol. of water and centrifuged at 125 000g for 30 min. The supernatant was discarded, and the pellet of pure envelope was dispersed by gentle homogenization in a loose-fitting Dounce homogenizer in 1.0 ml of 10% (v/v) glycerol and was stored at -20°C in small batches.

Transmission electron microscopy

Freshly pelleted nuclear envelope was fixed in phosphate-buffered glutaraldehyde solution and prepared by conventional techniques as described elsewhere (Barham *et al.*, 1980) for examination with a Philips EM 400 transmission electron microscope operated at 60 kV.

Other methods

Protein was assayed by the method of Hartree (1972), DNA by a modification (Spelsberg *et al.*, 1971) of the Burton (1956) method, and RNA by the orcinol method (Ceriotti, 1955). Envelope was suspended in 1% sodium dodecyl sulphate/8 M-urea/1 mM-β-mercaptoethanol and maintained at the temperature of boiling water for 5 min in preparation for disc gel electrophoresis in 5.6% polyacrylamide containing 1% sodium dodecyl sulphate.

Experiments to determine which endogenous envelope-associated proteins, if any, could serve as preferential substrate for envelope protein kinase were conducted as follows: 44 µg of nuclear-envelope protein in a final volume of 70 µl was mixed with dithiothreitol (1 mM), MgCl₂ (11.6 mM), NaF (17 mM), Tris/HCl (103 mM, pH 7.4) and [γ -³²P]-ATP (5 µM; approx. 10⁶ c.p.m.). Control tubes contained unlabelled ATP instead of [γ -³²P]ATP. The reaction was carried out for 15 min at 37°C and then was stopped by addition of 70 µl of urea/sodium dodecyl sulphate/mercaptoethanol solution, heated as described above, and submitted to sodium dodecyl sulphate/polyacrylamide-disc-gel (4 mm diameter, 100 mm length) electrophoresis. Control gels were stained for protein with Coomassie Blue. Gels containing ³²P were frozen and sliced with an automatic slicer to give 75-80 sections, each of which was placed in 10 ml of Aquasol (New England Nuclear, Boston, MA, U.S.A.) for measurements of radioactivity in a Beckman LS 7500 liquid-scintillation spectrometer.

Preparation of chromatin

Purified nuclei were used as the source of

water-soluble chromatin, which was prepared by a previously described procedure (Ueda *et al.*, 1971).

Protein kinase assays

Activity of the nuclear-envelope-associated enzyme was initially determined as described for endogenous and exogenous protein substrates with rat liver envelope (Steer *et al.*, 1979a). Because of tissue differences, it became necessary to establish $V_{\max}$ conditions for the enzyme of seminal-vesicle envelope (see the Results section). 'Endogenous protein' substrate refers to envelope protein itself as substrate for phosphorylation by envelope-associated protein kinase. The exogenous substrate dephosphophosvitin was prepared as described by Ahmed *et al.* (1975). Lysine-rich histone from calf thymus was obtained from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Preparation of [γ - 32 P]ATP was as described elsewhere (Ahmed *et al.*, 1975). The conditions for a $V_{\max}$ assay for nuclear-envelope protein kinase at 37°C with various substrates were as follows: for endogenous protein as substrate, 12 mM-MgCl₂, 5 μ M-[γ - 32 P]-ATP, 10 mM-NaF, pH 7.45; for dephosphophosvitin as substrate, 12 mM-MgCl₂, 5 μ M-[γ - 32 P]ATP + 58 μ M-ATP, 50 mM-NaCl, 1 mg of dephosphophosvitin, pH 6.41; for lysine-rich histone as substrate, 8 mM-MgCl₂, 5 μ M-[γ - 32 P]ATP + 45 μ M-ATP, 1.5 mg of lysine-rich histone, pH 7.4. The final reaction volume was 0.5 ml, and all contained approx. 12 μ g of envelope protein which was added to start the reaction. All other experimental conditions are given in the legends to appropriate Figures or have been described elsewhere (Steer *et al.*, 1979a).

For measuring chromatin-associated protein kinase activity we used the same procedure described for studies on rat ventral-prostate chromatin (Ahmed & Wilson, 1975; Wilson & Ahmed, 1977). Six intact animals provided 5.1 g of epithelium, from whose nuclei we extracted 15.3 mg of chromatin protein. Briefly, for chromatin-associated protein as substrate, the reaction medium contained 6 mM-MgCl₂, 5 μ M-[γ - 32 P]ATP, 5 mM-NaCl, 30 mM-Tris/HCl, pH 7.4, and 34–37 μ g of protein in a final volume of 0.5 ml. Reaction time was 1 min at 37°C. For dephosphophosvitin as substrate, we used 3 mM-ATP containing trace amounts of [γ - 32 P]ATP, 5 mM-MgCl₂, 200 mM-NaCl, 30 mM-Tris/HCl, pH 7.4, 1 mg of dephosphophosvitin and 1 mM-dithiothreitol, and the reaction was carried out for 20 min. For lysine-rich histone as substrate, 100 μ M-ATP containing trace amounts of [γ - 32 P]ATP, 5 mM-MgCl₂, 100 mM-NaCl, 30 mM-Tris/HCl, pH 7.4, and 1.5 mg of lysine-rich histone were included in a 20 min incubation.

Phosphoprotein phosphatase activity

This was assayed with 32 P-labelled lysine-rich

histone as described (Wilson & Ahmed, 1978; Steer *et al.*, 1979b). Rabbit skeletal-muscle protein kinase inhibitor was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

Results

Purification of nuclear envelope

After high-speed centrifugation in a discontinuous sucrose gradient, which was used during the final step of the purification procedure, the membranes float to the least-dense gradient fraction because they have densities of 1.16–1.18 g/ml. This purification yielded envelope which was at least 98–99% pure as determined by transmission electron microscopy (see Plate 1). At low magnification occasional opaque areas were visible. These might represent minor amounts of contaminating chromatin or condensed membrane fragments. Discrete pore complexes could not be readily identified. However, the bileaflet character of the envelope was evident at both intermediate and high magnifications.

Chemical composition of envelope

The data of Table 1 show that the DNA:protein ratio (w/w) was 0.395 for nuclei, 0.432 for chromatin and only 0.008 for nuclear envelope.

Sodium dodecyl sulphate/polyacrylamide-disc-gel electrophoresis of envelope proteins (Fig. 1) revealed three prominent bands of protein ranging from approx. 65 000 to 75 000 daltons, which were similar to the 'triplet' described for liver envelope (Lam & Kasper, 1979). Relatively major bands in most molecular-weight ranges were noted, including the 14 000–25 000 range. When envelope protein (used as both enzyme source and protein substrate in the presence of [γ - 32 P]ATP) was electrophoresed and radioactivity in 75 gel slices was measured, we found that: (1) slices throughout the gel up to R_F values of 0.9 uniformly contained the same low amount of radioactivity; and (2) gel slices corresponding to R_F values of 0.9–1.0 (but running behind the dye) had slightly greater radioactivity. Most likely, the radioactivity near the dye front was due partly to traces of [γ - 32 P]ATP. In any case, no single protein band was preferentially phosphorylated.

Properties of the protein kinase reactions

The requirement for Na⁺ depended on the exogenous substrate used, whereas phosphorylation of endogenous envelope protein was independent of the Na⁺ concentration. Na⁺ was mainly inhibitory when lysine-rich histone was substrate and was needed at 50 mM to give maximum reaction rates when dephosphophosvitin was acceptor. Mg²⁺ was essential for the reactions with all three substrates, and its optimal concentration varied from 7 to

Table 1. *Composition of nuclear envelope, chromatin and intact nuclei*

All animals were intact. The envelope data are for four groups of eight animals plus one group of nine. Mean epithelium weight ($\pm$ s.d.) for the five groups was 6.1 ± 1.0 g; mean total envelope protein for each of the five groups was 1109 ± 237 μ g.

	No. of guinea pigs	Wet wt. of epithelium (g)	Content (μ g/g wet wt. of epithelium)		
			Protein	DNA	RNA
Nuclei	7	5.8	4380	1730	315
Chromatin	12	10.4	2730	1180	119
Nuclear envelope	41	30.6	181	1.4	5.0

Fig. 1. Sodium dodecyl sulphate/polyacrylamide-disc-gel electrophoresis of: (a) 13 μ g of nuclear-envelope protein from seminal-vesicle epithelium; (b) 14 μ g of standard proteins

From top to bottom, standards were phosphorylase b (mol.wt. 94 000), bovine serum albumin (68 000), ovalbumin (43 000), carbonic anhydrase (30 000), soya-bean trypsin inhibitor (21 000) and lysozyme (14 300). See the Materials and methods section for additional information.

12 mM. The pH requirement of the reactions toward various substrates also varied. For lysine-rich histone there was a broad pH optimum (6.5–9.0), which was not shared by dephosphophosvitin

(6.0–6.5). With respect to ATP, concentrations of 50–60 μ M allowed $V_{\max}$ rates to be achieved towards both lysine-rich histone and dephosphophosvitin (results not shown).

Effects of various agents on protein kinase activities

When CaCl_2 or ZnCl_2 was substituted for MgCl_2 in the assays, a 99% inhibition of activity towards lysine-rich histone and a 65% inhibition of that towards dephosphophosvitin were noted. MnCl_2 was a satisfactory substitute for MgCl_2 when either protein substrate was employed. The cyclic nucleotides, cyclic AMP and cyclic GMP, at concentrations of 1 or 10 μ M were without influence. The cyclic AMP-dependent protein kinase inhibitor caused a decline in histone kinase activity of 31% and 51% of control values when this agent was added at 10 and 50 μ g respectively. Calmodulin (1 μ M) had no effect on enzyme activity towards any substrate.

Effects of castration on protein kinase activities

For these studies we relied mainly on the results obtained with lysine-rich histone as substrate. The activity of envelope-associated enzyme appeared undiminished (2.51 versus 2.38 nmol/h per mg) 96 h after castration (Table 2), although multiple functional and structural features of the tissue undergo involution during the 4 days after castration (Veneziale *et al.*, 1977b; Burns *et al.*, 1979).

Phosphoprotein phosphatase activity

The assayable activity of four envelope preparations (each preparation had been derived from eight intact animals) was 0.36 ± 0.12 (s.d.) nmol of P_i released/h per mg of protein when a ^{32}P -labelled phosphoprotein was used as substrate. Values for envelope from animals castrated 24 h (eight), 48 h (seven) or 96 h (seven) previously were 0.40, 0.65 and 0.42 nmol/h per mg respectively. Thus phosphoprotein phosphatase activity of nuclear envelope was also resistant to androgen depletion.

EXPLANATION OF PLATE 1

Transmission electron micrograph of purified nuclear envelope from seminal-vesicle epithelium
Magnification: (a) $\times 17\,500$; (b) $\times 82\,500$; (c) $\times 175\,000$. See the Materials and methods section for additional information.

Table 2. *Protein kinase activity of envelope*

The numbers of envelope preparations studied are shown in parentheses. Each envelope preparation came from a group of animals. There were usually eight animals in a group, no less than six and as many as 16; 99 animals in all were used; 23 animals comprised the three groups of 96 h-castrated and 56 animals the six groups of intact animals. Statistical analyses by Student's *t* test showed no significant differences between data for the castrated groups and the corresponding intact animal groups. With chromatin as enzyme source, the protein kinase activities obtained towards endogenous proteins, dephosphophosvitin and lysine-rich histone as substrates were 2.9, 115.6 and 1.1 nmol/h per mg respectively. The data for protein kinase activity are presented as mean values $\pm$ S.D.

Guinea pigs	Substrate ...	Activity (nmol of P _i /h per mg of protein)		
		Endogenous	Dephosphophosvitin	Lysine-rich histone
Intact		0.46 $\pm$ 0.23 (6)	3.00 $\pm$ 1.31 (6)	2.51 $\pm$ 0.41 (3)
24 h-castrated		0.21 (1)	1.64 (1)	3.38 (1)
48 h-castrated		0.32 (2)	2.84 (1)	3.35 (2)
96 h-castrated		0.26 $\pm$ 0.06 (3)	3.75 $\pm$ 1.32 (3)	2.38 $\pm$ 0.53 (3)

Discussion

Little is known about the nuclear envelope in comparison with other cellular organelles. Most studies on animal tissues have involved rat liver or avian erythrocytes. We are aware of only a few studies on nuclear envelope from an androgen-dependent tissue (Moore & Wilson, 1972; Shimazaki *et al.*, 1973; Lefebvre & Novosad, 1980). Moore & Wilson (1972) described a role for this organelle in the transformation of testosterone to its reduced metabolite dihydrotestosterone, by 5 α -reductase. That study, however, involved nuclear envelopes derived from prostate, which contains multiple cell types, and as such would not allow a precise determination of the relative contribution of any given cell type to the total enzyme activity. This limitation has been overcome by the selection of an androgen-dependent portion of the male accessory glands which comprises only one cell type, i.e. the epithelium of the guinea-pig seminal vesicle (Veneziale *et al.*, 1977a). Accordingly, the present paper provides the first report on the isolation and partial characterization of the seminal-vesicle epithelium nuclear envelope and its associated protein kinase activity. The presence of protein kinase activity in a nuclear-envelope fraction has been previously described only for rat liver (Steer *et al.*, 1979a, 1980; Lam & Kasper, 1979; Clawson *et al.*, 1980).

We could not rely on marker enzyme activities as proof of envelope purity; no suitable enzyme marker for seminal-vesicle envelope has been disclosed. To address the consideration of envelope purity, in addition to electron microscopy, we relied on two important facts: (i) we have established (Veneziale *et al.*, 1977a) that the nuclei were pure and free of extra-nuclear contamination; and (ii) chemical composition studies (Table 1) demonstrated a 320-fold purification, on the basis of nucleic acids remaining [i.e. (1730 + 315)/(1.4 + 5.0)].

Phosphoprotein metabolism in the male accessory sex glands is strongly influenced by the androgenic status of the animal (Ahmed & Wilson, 1978; Ahmed *et al.*, 1979). For example, activity of the rat ventral-prostate chromatin protein kinase declines rapidly after castration and is maintained at the value in intact controls by administration of androgen. Our studies on seminal-vesicle epithelium chromatin also demonstrate a marked decline in kinase activity toward multiple substrates after castration (R. C. Steer, K. Ahmed & C. M. Veneziale, unpublished work). The apparent resistance of the envelope histone kinase activity to castration suggests that the enzyme(s) is not regulated *in vivo* by testicular androgen. The results of our work with several phosphoprotein substrates suggest multiple protein kinases in the envelope. Therefore it is possible that various envelope kinases exist and may be affected differentially by castration. Our measurement of enzyme activity may reflect only average values. From the above, it also seems conceivable that nuclear-envelope-associated enzymes might provide a 'defence' against the deleterious effects of a decreased androgen supply by supporting the continuation of some key process during the involutional period. It must, however, be emphasized that the above studies do not consider the behaviour of the envelope kinases at any period longer than 96 h after castration. It is possible that different results would be obtained in the very-long-term-castrated animal. However, isolation of epithelium from animals castrated longer than 96 h is extremely difficult, owing to atrophy.

The present paper is the first report on the presence of protein kinase activity in the nuclear envelope of seminal-vesicle epithelium. Only two other reports have discussed enzyme activity associated with this organelle derived from a male accessory sex organ (Moore & Wilson, 1972; Shimazaki *et al.*, 1973). They showed an en-

richment of 5 α -reductase activity in rat ventral-prostate nuclear envelope. The importance of that enzyme for the formation of dihydrotestosterone is well known, but the reason for its relative abundance at the nuclear envelope is uncertain. For such an enzyme, one could speculate that the perinuclear localization is intended to ensure sufficient concentration of dihydrotestosterone to interact with receptor molecules which might also be compartmentalized in the same vicinity. Such proximity would increase the likelihood of steroid reacting with its receptor, and the net overall effect might be a hastening of delivery of steroid-receptor complex to chromatin acceptor sites. What then might be the role of the envelope-associated protein kinase? Is it involved in influencing the rate of steroid-receptor migration into the nucleus? Does it preferentially phosphorylate another enzyme within the nuclear envelope or pore complex, or does it act on some other subnuclear structure such as the matrix or nucleosome? The answers to these questions will improve our general understanding of the nuclear envelope and of the molecular mechanism of androgen action.

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