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INDEPENDENT RESPONSES OF NUCLEOSIDE TRIPHOSPHATASE AND PROTEIN KINASE ACTIVITIES IN NUCLEAR ENVELOPE FOLLOWING THIOACETAMIDE TREATMENT

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SUMMARY

Two methods were used to prepare rat-liver nuclear envelope from control and thioacetamide-treated animals. The method employing DNase-digestion and Tris-EDTA dissociation of ribosomes resulted in much lower specific NTPase and protein kinase activities. Monneron's preparative technique yielded NE preparations with high specific activities. Following thioacetamide treatment, the NE NTPase increased to nearly 300% of the control activity, with no change in the protein kinase activity. It appears that the NE protein kinase activity is not related to NE NTPase and is not involved in RNA transport.

INTRODUCTION

Evidence is accumulating which distinguishes the nuclear membrane from the endoplasmic reticulum (1,2) and which suggests a regulatory role for nuclear envelope (NE)[1] constituents. For instance, many hormone receptors are located on the NE (3,4) and the enzymatic response of the NE can often be differentiated from that of the ER, as is the case following treatment of rats with 3-methylcholanthrene (5) or thioacetamide (2). A protein kinase has been identified on the NE (6,7) which selectively phosphorylates a 68,000 dalton polypeptide (7), and which may be involved in regulatory processes. A NE nucleoside triphosphatase (NTPase) appears to be a key regulatory element in nucleocytoplasmic RNA translocation (8-10). Following treatment of rats with the hepatocarcinogen thioacetamide (TIAA), large increases in the NE NTPase activity occur (8,10) which relate to increased RNA transport observed *in vitro* (8) and *in vivo* (10), and which may relate to altered restriction of nuclear RNA during carcinogenesis (11). The

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[1]Abbreviations: NE, nuclear envelopes; ER, endoplasmic reticulum; NTPase, nucleoside triphosphatase (EC 3.6.1.4); TIAA, thioacetamide; TKM buffer, 50 mM Tris-HCl (pH 7.6), 25 mM KCl, 5 mM $MgCl_2$; EDTA, ethylenediamine tetraacetic acid; TM buffer, 50 mM Tris-HCl (pH 7.6), 500 mM $MgCl_2$; TCA, trichloroacetic acid.

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NE NTPase and protein kinase activities show some similarities, such as maximal activity with Mg and inhibition by NaF (7,10). Since these activities may be related, protein kinase activity was examined at 48 hr following TIAA administration, the period at which the NE NTPase is maximal (10).

MATERIALS AND METHODS

Male, Sprague-Dawley rats were used with body weights of approximately 400 g. Rats were given 5 mg TIAA/100 g body weight via stomach tube, and controls were given water. Rat-liver nuclei were prepared as described (10) by sedimentation through 2.3 M sucrose-TKM buffer (Tris-HCl, pH 7.6; 25 mM KCl; 5 mM $MgCl_2$) plus 5 mM 2-mercaptoethanol.

Nuclear envelopes were prepared using two methods. In method I, purified nuclei were swollen in 2 mM $NaHCO_3$ for 10 min at 0 C (12). DNase I (RNase-free, from Worthington Biochemical, Freehold, NJ) was added to 50 µg/ml, $MgCl_2$ was added to 0.1 mM, phenylmethylsulfonyl fluoride was added to 0.5 mM, and the suspension was incubated for 25 min at 23 C. The nuclear envelopes were then collected by centrifugation at 17,500 rpm at 4 C in an SS-34 rotor and Sorval centrifuge. Approximately 4.7± 0.7% of nuclear DNA was recovered in the crude NE from control animals, while 3.8± 0.3% was recovered in crude NE from TIAA-treated animals.

The crude envelopes were resuspended in and rinsed twice with TKM buffer. The envelopes were then resuspended in Tris-EDTA buffer (0.25 M sucrose, 20 mM Tris-EDTA, pH 7.6) by homogenization (6). This suspension was layered over a 14 ml layer of 1.5 M sucrose-TKM buffer and a 14 ml layer of 2.2 M sucrose-TKM buffer; centrifugation was for 18 hr at 21,000 rpm at 4 C in an SW27 rotor and Beckman ultracentrifuge. The envelopes, which banded at the 1.5-2.2 M interface, were removed with a pasteur pipette, diluted with TKM buffer, and collected by centrifugation at 17,500 rpm at 4 C for 15 min in an SS-34 rotor.

Method II was modified from that described by Monneron (13). Purified nuclei were thoroughly homogenized in 5 ml of 50% sucrose-TM buffer (50 mM Tris-HCl, pH 7.6; 500 mM $MgCl_2$). This viscous suspension was overlayered with a 10 ml layer of 40% sucrose-TM buffer, and a linear gradient of 40-20% sucrose-TM buffer. The gradient was centrifuged at 52,000 rpm at 4 C for 2 hr in a 60Ti rotor and Beckman ultracentrifuge. The fractions of the gradient containing the envelope were removed, diluted with TKM buffer, and the envelopes were harvested by centrifugation at 58,000 rpm at 4 C for 1 hr in a 60Ti rotor. Pelleted envelopes were resuspended in TKM buffer by homogenization. This method offers two distinct advantages; it is rapid (enzymatic experiments can be completed within 8 hr), and it does not employ degradative enzymes, whose extent of action is often difficult to control.

For composition analyses, protein content was determined by the Lowry protein assay (14) or the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA; see ref. 15), and are expressed on the basis of Lowry values with 3X-recrystallized bovine serum albumin as a standard. An aliquot of the envelope suspension was precipitated in 5% trichloroacetic acid (TCA) on ice for 20 min, and TCA-precipitable material was harvested by centrifugation. Precipitated envelopes were then resuspended in 5% TCA, repelleted, and resuspended in a small volume of 5% TCA. DNA and RNA were solubilized from TCA-precipitable material by heating at 90 C for 30 min. RNA was then measured by the orcinol technique (16) and DNA was measured by the diphenylamine reaction (17). Phospholipid phosphorus was determined by the Bartlett procedure (18) after extraction of an aliquot of NE suspension with chloroform: methanol (19).

NTPase assays were conducted as previously described (10). Protein kinase activity was measured in reaction mixtures containing NE (generally 0.1-1 mg protein) and 1 mM γ-^{32}P-ATP (Amersham, Arlington Hts., IL; reac-

tions contained on the order of 10^7 cpm/mg protein) in TKM buffer. Reactions were for 20 or 30 min at 37 C, and were terminated by addition of TCA to 5%. After incubation on ice for 20 min, the TCA-precipitable material was either collected by centrifugation, or was collected on filters. Pelleted material was resuspended in 5% TCA and recentrifuged 3 times, and subsequently resuspended in sample buffer (62.5 mM Tris-HCl, pH 6.8; 2.3% sodium dodecyl sulfate; 10% glycerol; 5% 2-mercaptoethanol), and an aliquot was removed for determination of Cerenkov radiation in water. TCA-precipitable material which was collected on filters was washed with 10 volumes of 5% TCA, and the radioactivity was assessed in water. Zero-time controls were run for both procedures to assess nonspecific adsorption to membranes during handling, and the values (about 10% of total radioactivity recovered) were subtracted to obtain figures for incorporated radioactivity.

Nuclear envelope proteins were examined by polyacrylamide gel electrophoresis on 7% gels according to Laemmli (20).

RESULTS AND DISCUSSION

In this study, we employed two procedures for isolation of rat-liver nuclear envelopes. Widely differing specific activities were found (Table 1). Method I yields NE preparations which do not contain histone or ribosomes attached to the outer nuclear envelope; both the NTPase and protein kinase activities were unchanged in NE isolated via this procedure following thioacetamide treatment. However, the specific NTPase values obtained were only about 10% of the values obtained using Method II (see also legend to Table 1). We therefore used Method II to prepare NE, and observed a large increase in NTPase at 48 hr following TIAA treatment, and the values obtained were in good agreement with those previously reported (8,10). When protein kinase activity was measured in the same preparations used for the NTPase assays, there was no difference in activities (Table 1). These data indicate independent responses of NE NTPase and protein kinase activities, and suggest that the Monneron procedure is superior to other procedures for measurement of enzymatic activities. The specific activity for NE protein kinase reported by Steer et al. (6) is intermediate between those of NE prepared with Methods I or II, suggesting that the preparative procedure they employed may also have resulted in substantially reduced enzymatic activity compared with Method II. Two arguments suggest that the higher specific activity we measured with Method II depends upon the preparative technique and not upon the presence of histone or chromatin. First, although some small molecular weight proteins (ostensibly histone) can be identified on polyacrylamide gels, the specific activity obtained with Method II preparations is higher than that Steer et al. (6) measured with exogenous purified histone. Second, previous studies have demonstrated that phosphorylation of histones correlates with DNA synthesis (21): Since DNA synthesis is increased to about 240% of control synthesis at this interval following TIAA administration (2), and since there was no increase in protein kinase activity, it appears that phosphorylation of histones cannot explain our data.

Table 1. Protein Kinase and NTPase Activities of NE from livers of Control and TIAA-treated Rats.

Sample	Specific Activity of NE Preparations (cpm/h-mg protein)	Protein Kinase Activity (nmol γ-P/h-mg protein)	NTPase Activity (nmol γ-P released/ 20 min-mg protein)
	Envelopes Prepared with Method I[a,b]		
Control	21500± 3060	4.49± 0.90	63± 29[b]
TIAA	22820± 1200	4.76± 0.25	64± 51
	Envelopes Prepared with Method II[c]		
Control	231480± 121280	53.4± 28.0	591± 240
TIAA	218660± 30660	50.4± 7.1	1522± 320

Values are means ± S.D. of three measurements.

[a]Control envelopes prepared with this method contained (per mg protein) 0.26± .02 mg phospholipid, 0.195± .012 mg RNA, and 0.095± .013 mg DNA. NE from TIAA-treated animals contained 0.26± .01 mg phospholipid, 0.145± .010 mg RNA, and 0.095± .002 mg DNA. Protein recovery was higher in TIAA preparations, giving 0.145± .037 mg/ g liver as opposed to 0.085 ± .036 mg/ g liver in control preparations.

[b]Method I appears to be destructive to a number of NE components. For instance, Method II yields preparations with cytochrome b_5 content of 0.262 nmol/mg protein and NADH-ferricyanide reductase activity of 4.42 Units/mg protein, while Method I yields preparations with values of 0.052 and 0.472 (respectively). These decreases are similar in magnitude to the decrease observed in NTPase. Subjecting microsomes to hypotonic swelling and DNase treatments used in Method I also causes major decreases in cytochrome b_5 and NADH-ferricyanide reductase (cytochrome P_{450} is unaffected). Addition of phenylmethylsulfonyl fluoride did not inhibit the decreases. Also, others (23) have shown that repeated washing of nuclei removes the myokinase activity we originally reported (10); and removal of RNA, which stimulates NE NTPase (10), may be partially responsible for the lower specific NTPase activity.

[c]The composition of NE prepared with this method was as previously reported (8,10). There was no difference noted in protein recovery.

Ishikawa et al. (22) have suggested that a phosphorylated intermediate may be involved in RNA transport, and others have modified this basic premise and suggested that the phosphorylated NE protein represents such an intermediate (23). The results presented here argue against this latter interpretation. Although the NE protein kinase may indeed represent an important regulatory mechanism, it does not appear to be involved in regulation of nucleocytoplasmic RNA translocation.

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