Inhibition of protein A24 lyase by nitrosoureas

Gunnar Markussen, Ingrid Smith-Kielland and John M. Dornish+*

Department of Biochemistry, University of Oslo, Blindern, Oslo 3, and *+Department of Tissue Culture, Norsk Hydro's Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, Oslo 3, Norway*

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The protein A24 content of Ehrlich ascites tumor cells increased several-fold following treatment of cell cultures with nitrosoureas, but did not increase when other alkylating agents not containing carbamoyl moieties were tested. The same nitrosoureas and, in addition, 2-chloroethyl isocyanate inhibited an A24 lyase-containing cytoplasmic extract in cleaving protein A24 into histone H2A and ubiquitin. It appears that carbamoylation of A24 lyase by nitrosoureas inhibits the enzyme and is responsible for the measured increases in cellular protein A24 content due to reduced turnover of this protein.

A24 (uH2A) *Alkylating agent* *Histone H2A* *Nitrosoureas* *Carbamoylation* *A24 lyase (Ehrlich ascites tumor cell)*

1. INTRODUCTION

We have previously reported that the protein A24 (a conjugate of histone H2A and ubiquitin) content of Ehrlich ascites tumor cells increased several-fold following 1-h treatments with either 1-methyl-1-nitrosourea or 1,3-bis-(2-chloroethyl)-1-nitrosourea [1]. In that report we discussed the possibility that A24 lyase, the enzyme responsible for cleavage of protein A24, may be inhibited in nitrosourea-treated cells. We suggested that carbamoylation of the enzyme by nitrosourea degradation products, notably isocyanates, would inactivate the enzyme and lead to increases in protein A24 due to the rapid formation of this protein [2].

The enzyme responsible for cleavage of protein A24 has been isolated and investigated by several groups [3–5]. The enzyme cleaves the isopeptide linkage in protein A24 to release histone H2A and ubiquitin [3,5], and is found mostly in the cytoplasm of eukaryotic cells [3]. A24 lyase activity appears to be responsible for the disappearance of protein A24 from chromatin of mitotic cells [2,6]. Reduced amounts of A24 have also been observed following partial hepatectomy [7], following thioacetamide treatment [4,8], and in transcriptionally active rat liver chromatin [9].

In [1] we inferred that alkylating agents may be used as probes to study the function of A24 lyase. Here we report that only those alkylating agents containing a carbamoyl moiety affected A24 lyase activity measured either indirectly as increases in A24 content of drug-treated cells, or directly in cytoplasmic enzyme extracts.

2. MATERIALS AND METHODS

2.1. *Cell culture and drug treatment*

Ehrlich ascites tumor cells adapted to suspension culture in Eagle's minimum essential medium supplemented with 10% fetal calf serum were cultured as in [1]. 1-Ethyl-1-nitrosourea (ENU) and 1-methyl-1-nitrosourea (MNU) were purchased from Sigma, St. Louis, MO. 1,3-Bis-(2-chloroethyl)-1-nitrosourea (BCNU) was a gift from Dr Engle, Drug Research and Development Program, National Cancer Institute, National Institutes of Health, Bethesda, MD. Ethylmethanesulfonate (EMS) was a product of Koch-Light, Colnbrook,

* To whom correspondence should be addressed

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England. Methylmethane-sulfonate (MMS) and 2-chloroethyl isocyanate were from Eastman Kodak, Rochester, NY. Busulfan (1,4-dimethylsulfonyloxybutane) was a gift from the Wellcome Foundation, Burroughs Wellcome & Co., London, England. Drugs were dissolved in ethanol and stock solutions made in 0.9% NaCl. Further dilutions to appropriate drug concentrations were made either in culture medium or in A24 lyase incubation buffer. Drug treatment was for 1 h, 37°C for cells in culture. Nuclei from control or drug-treated cells were isolated by homogenization in hypotonic buffer and the remaining cytoplasm was removed with Triton X-100 [1]. Nuclear proteins were extracted in 0.4 N H_2SO_4 and recovered by acetone precipitation.

2.2. *A24 lyase and reductive methylation of protein A24*

A cytoplasmic extract containing A24 lyase was prepared from Ehrlich ascites cells according to [3]. Protein A24 was isolated from calf thymus [10] and purified by preparative polyacrylamide gel electrophoresis (PAGE) on acetic acid/urea gels. Reductive methylation of protein A24 was performed as in [11,12] using formaldehyde and $NaB[^3H]H_4$ (Amersham). The radioactivity of A24 was ~130000 cpm/μg protein. The effect of alkylating agents on A24 lyase activity was determined by preincubating the enzyme extract 25 min at 37°C with each drug before [^{3}H]A24 was added. Incubation was then continued for 60 min, 37°C. The incubation buffer was 50 mM Tris–HCl, pH 8.5, 2 mM mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride [3]. The reaction was stopped by addition of SDS–PAGE sample buffer. Reaction products were analyzed on 15% SDS–PAGE as described below.

2.3. *Polyacrylamide gel electrophoresis*

Acid-extracted proteins were run on acetic acid/urea gels [13]. The gels were subsequently stained with Coomassie blue and scanned at 600 nm. The relative amounts of H2A, H2B, H3, H4 and protein A24 were determined by weighing the areas under each peak. To determine the effect of alkylating agents on A24 lyase activity, incubation mixtures were separated on polyacrylamide–SDS gels in the Laemmli system [14].

3. RESULTS

3.1. *Protein A24 content of Ehrlich ascites cells treated with alkylating agents*

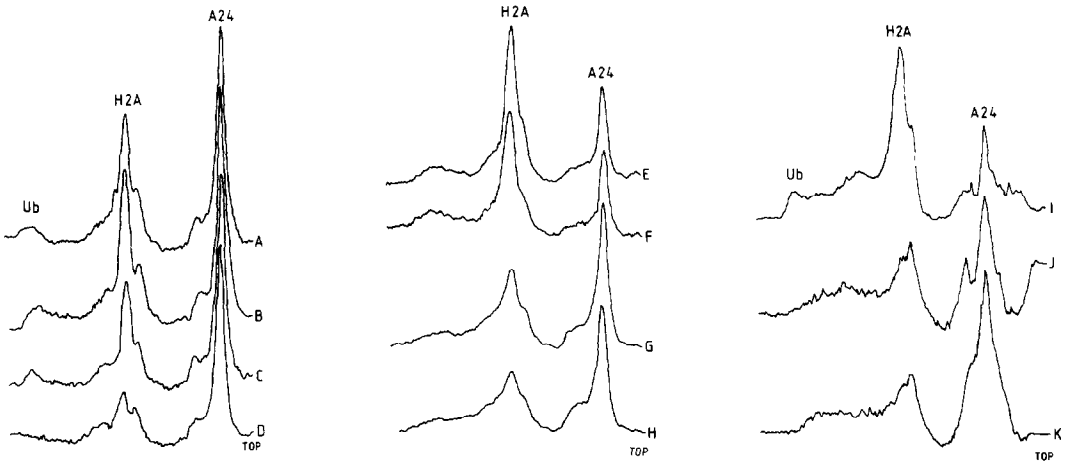
Table 1 represents data on the effect of several different alkylating agents on the protein A24 content of Ehrlich ascites cells following a 1-h drug treatment. Drug concentrations used in these experiments were based on [1] and unpublished observations. As can be seen, increases in protein A24 appeared only in cells treated with nitrosoureas (MNU, ENU and BCNU) and with 2-chloroethyl isocyanate (a BCNU decomposition product). 2-Chloroethyl isocyanate was chosen as a representative of nitrosourea decomposition products possessing carbamoylating activity. Other alkylating agents (EMS, MMS and Busulfan) did not cause any change in the A24 content from that of control cells. In addition, nitrogen mustard (100 μg/ml) did not cause any increase in cellular A24 content [1]. As the acid-soluble proteins were

Table 1

Protein A24 content of Ehrlich ascites tumor cells following treatment with alkylating agents

Alkylating agent	Concentration (μg/ml) during treatment (1 h, 37°C)	% of control ± SD
None	–	100
MNU	200	250 ± 20
ENU	200	200 ± 15
BCNU	50	230 ± 12
EMS	200	100 ± 10
MMS	200	100 ± 10
Busulfan	200	100 ± 10
2-Chloroethyl isocyanate	100	190 (1 experiment)

Suspension cultures of Ehrlich ascites tumor cells were treated for 1 h at 37°C with each of the indicated alkylating agents. Nuclei from control and drug-treated cells were isolated and the acid-soluble proteins extracted with 0.4 N H_2SO_4. Proteins were separated in an acetic acid/urea PAGE system (10% gels [13]). Densitometric scans were made of Coomassie blue-stained gels and the ratio of protein A24 to core histones (H2A, H2B, H3, H4) was determined by weighing the areas under the protein peaks



TRACING	TREATMENT*	% OF CONTROL
A	None	100
B	0.94 μg MNU	91.5
C	1.88 μg MNU	71.9
D	3.13 μg MNU	37.7

TRACING	TREATMENT*	% OF CONTROL
E	None	100
F	1.43 μg BCNU	89.7
G	2.86 μg BCNU	49.0
H	4.29 μg BCNU	40.7
NOT SHOWN	7.14 μg BCNU	29.0

TRACING	TREATMENT*	% OF CONTROL
I	None	100
J	2.50 μg 2-CNCO	26.0
K	5.00 μg 2-CNCO	16.7

Fig.1. Densitometric scans of fluorograms from separation of A24 lyase reaction products on polyacrylamide–SDS gels. Cytoplasmic A24 lyase enzyme extract (100–150 μg total protein) was preincubated with nitrosoureas or 2-chloroethyl isocyanate (2-CNCO) for 25 min at 37°C in a total volume of 100–200 μl. [^{3}H]A24 (1 μg) was then added and incubation for cleavage of A24 to H2A and ubiquitin (Ub) was continued for 60 min at 37°C. The reaction was stopped by addition of polyacrylamide–SDS sample buffer. Samples were heated to 100°C for 2 min and the entire incubation mixture was applied onto polyacrylamide–SDS gels [14]. The gels were subsequently treated with a fluorographic enhancer (EN^{3}HANCE, New England Nuclear, Dreieich, FRG), dried under vacuum, and overlayed with Kodak X-Omat film. Exposure at −70°C was for 2 to 7 days. The developed films were scanned and the ratios of H2A/A24 were determined by weighing the areas under each peak. % of control refers to comparison of H2A/A24 ratios for drug-treated samples relative to control values. TOP refers to the top of each gel. *TREATMENT is indicated as μg nitrosoureas or isocyanate/μg total protein in incubation mixtures.

extracted from nuclei immediately isolated from both control and drug-treated cells, the effect induced by 1-h treatments with nitrosoureas represents quite rapid increases in cellular A24 content. The results with nitrosoureas indicate that inhibition of an A24-degrading enzyme may be responsible for the increases in cellular A24.

3.2. *Effect of alkylating agents on A24 lyase activity*

A cytoplasmic extract containing A24 lyase activity which cleaved exogenous [^{3}H]A24 into radioactive H2A and ubiquitin was obtained from Ehrlich ascites cells. When the enzyme extract was preincubated with nitrosoureas before addition of [^{3}H]A24, a dose-dependent inhibition of A24 lyase activity was found (fig.1). Protein A24 is stoichiometrically cleaved by A24 lyase into H2A and ubiquitin [3], thus H2A + A24 content would be constant in each incubation mixture. Inhibition of A24 lyase would lead to a greater amount of A24 detected in the incubation mixture and thus lower H2A/A24 ratios. When compared to control values from the same experiment, the H2A/A24 ratios would be a relative measure of A24 lyase activity. From fig.1, both MNU and BCNU are effective inhibitors of A24 lyase. Treatment of an A24 lyase-containing cytoplasmic extract with either 3.1 μg MNU or 2.8 μg BCNU/μg total protein for 60 min prior to addition of radioactive A24 resulted in 62 and 51% inhibition in cleavage of A24 to H2A + ubiquitin, respectively. Further increases in the BCNU concentration appeared to maximally inhibit A24 lyase activity, reaching a lower limit of approx. 30% of control values. The data in fig.1 also show that 2-chloroethyl iso-

cyanate is a strong inhibitor of A24 lyase. However, doubling the amount of 2-chloroethyl isocyanate from 2.5 to 5 $\mu g/\mu g$ total protein did not further reduce A24 lyase activity to any extent. Different preparations of cytoplasmic A24 lyase extract yielded similar results within 10–25% of the values shown in fig.1.

4. DISCUSSION

Increases in protein A24 content of Ehrlich ascites tumor cells were observed following treatment with nitrosoureas or 2-chloroethyl isocyanate, but not following EMS, MMS or Busulfan treatment (table 1). As the drug treatment period was short (1 h), these results indicate a rapid formation of protein A24 in cells. The protein A24 degrading enzyme, A24 lyase, appears to be inhibited in nitrosourea- and isocyanate-treated cell cultures. However, it is interesting to note that, although not presently isolated, a suspected A24 ligase [3] responsible for forming A24 is not inhibited by these drugs. Since the amino groups of lysine and arginine are primary sites of carbamoylation by nitrosourea-derived isocyanates [15], A24 ligase may have few of these amino acids.

Inhibition by nitrosoureas and isocyanates of A24 lyase activity was determined directly by a reduction in the formation of H2A (fig.1). Cytoplasmic enzyme extracts preincubated with MNU, BCNU or 2-chloroethyl isocyanate before addition of exogenous [^{3}H]A24 were inhibited in degrading protein A24 in a dose-dependent manner.

In order to exclude the possibility that nitrosoureas may activate specific protease activity which in itself could degrade A24 lyase and lead to reduced A24 cleavage, the protease inhibitor phenylmethylsulfonyl fluoride was included in the preparation of A24 lyase extract [3] and in the incubation mixtures containing A24 lyase extract and alkylating agents. The presence of a protease inhibitor did not alter the data presented in fig.1.

Structure-activity relationships for the nitrosoureas indicate that alkylation reactions may be responsible for the therapeutic activity of these drugs while carbamoylation may be responsible for a portion of the toxic effect [15–17]. Inhibition of essential enzymes such as DNA polymerase by carbamoylating products of nitrosourea decomposition is known [18,19].

Protein A24 is normally absent from cells in mitosis [2,6]. Condensation of chromatin would therefore be quite different with A24 residues present in nucleosomes of cells in which A24 lyase was inhibited. Even low doses of the nitrosoureas could inhibit A24 lyase to an extent such that local areas of chromatin contain the H2A–ubiquitin conjugate. The effect this would have on both transcription and the mitotic process is unknown but may represent an additional toxic effect of nitrosoureas.

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