Protein phosphorylation of lysosomal arylsulfatase B in normal and leukemic leukocytes

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An acidic variant form of arylsulfatase B from normal leukocytes and chronic myelogenous leukemia (CML) leukocytes was found to be phosphorylated at its serine and threonine residues through in vivo phosphorylation with $^{32}P_i$. However, the predominant phosphorylation site was serine in normal cells, in contrast to threonine in CML cells. A cyclic AMP-dependent protein kinase was responsible for phosphorylation of the sulfatase of CML cells.

Arylsulfatase B *(Human leukocyte)* *Chronic myelogenous leukemia* *Phosphorylation* *cyclic AMP dependence* *Protein kinase* *Lysosome*

1. INTRODUCTION

A lysosomal arylsulfatase B enzyme catalyzes hydrolysis of the sulfate ester bond of *N*-acetylgalactosamine 4-sulfate at the non-reducing termini of glycosaminoglycans [1] and is a basic glycoprotein in itself [2]. We have reported that an acidic variant form (named B_1) of arylsulfatase B, which was not detected in many normal tissues, was detected by anion-exchange chromatography in leukocytes from normal subjects and chronic myelogenous leukemia (CML) patients [3]. The amount of the variant was considerably greater in CML cells than in normal leukocytes. The anionic property of the B_1 variant was mainly due to phosphate groups bound to the carbohydrate moiety of arylsulfatase B [3]. Through in vivo ^{32}P-labeling experiments, the B_1 enzyme of transplantable human lung tumor was shown to be phosphorylated on its protein moiety in addition to phosphorylation on the carbohydrate chains [4]. These observations indicate that the B_1 enzyme is a variant form of arylsulfatase B modified by phosphorylation at both carbohydrate and protein moieties and suggest that arylsulfatase B can be a substrate for a protein kinase. Protein phosphorylation of lysosomal enzymes from normal cells is not known. This study was undertaken to define whether the arylsulfatase B variant from normal as well as CML leukocytes is phosphorylated on its protein moiety.

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2. MATERIALS AND METHODS

2.1. *Materials*

Leukocytes from heparinized venous blood of healthy volunteers and CML patients were prepared separately by centrifugation using dextran [5]. Antiserum against human arylsulfatase B was prepared in rabbits immunized with the highly purified arylsulfatase B from normal human liver. The antiserum reacted with both human arylsulfatase B and B_1 enzymes from either normal or tumor tissues and cells, but not with arylsulfatase A [4]. The activity of arylsulfatase B was assayed using *p*-nitrocatechol sulfate as substrate, by the method of Baum et al. [6] with a slight modification. One unit of enzyme activity is

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the amount of enzyme required to liberate 1 nmol *p*-nitrocatechol per h. $^{32}P_i$ and [γ-^{32}P]ATP were obtained from New England Nuclear; cAMP-dependent protein kinase (from bovine heart) and alkaline phosphatase (from *Escherichia coli*) from Sigma; DEAE-cellulose (DE-52) and cellulose-coated thin-layer plates (10 × 10 cm) from Whatman. All chemicals used were of reagent grade.

2.2. *In vivo isotopic labeling of leukocyte arylsulfatase*

Cells ($5.7 \times 10^8 - 2.5 \times 10^9$) were preincubated in phosphate-free RPMI 1640 medium without antibiotics and calf serum at 37°C for 30 min in a 5% CO_2 chamber. 1 mCi $^{32}P_i$ was then added and incubation pursued at 37°C for 20–24 h. Viability of cells after incubation was almost 100% as examined by the standard trypan blue exclusion method. The ^{32}P-labeled cells were homogenized in the absence of phosphatase inhibitor and centrifuged at 10000 × *g* for 20 min. The supernatant was applied onto a PD-10 column (Pharmacia) equilibrated with 10 mM Tris-HCl, pH 7.5. The excluded fraction was applied onto a DE-52 column (1.5 × 3.5 cm) equilibrated with the same buffer. B enzyme recovered in the effluent and B_1 enzyme eluted with 0.3 M NaCl in the same buffer were concentrated separately over an Amicon PM-10, and immunoprecipitated as described [4]. The immuno complex was solubilized in 0.125 M Tris-HCl, pH 6.8, containing 1% SDS and 10 mM dithiothreitol, and an aliquot was assayed for radioactivity.

2.3. *Characterization of phosphorylated amino acids*

The ^{32}P-labeled enzyme recovered in the immuno complex was hydrolyzed with 6 M HCl at 100°C for 4 h and subjected to two-dimensional separation on a thin-layer plate as in [4]. The labeled phosphoamino acids were identified by co-separation using standard non-labeled phosphoamino acids detected by ninhydrin.

2.4. *In vitro phosphorylation of leukocyte arylsulfatase B by endogenous protein kinase*

Leukocytes were homogenized with 50 mM Hepes buffer, pH 6.8, and centrifuged at 500 × *g* for 10 min. The supernatant (0.3 ml, about 600–2000 U arylsulfatase B activity) from either normal granulocytes or CML cells was supplemented with 10 mM NaF, 10 mM $Mg(CH_3COO)_2$, 0.25 mM EGTA, 0.1 mM isobutylmethylxanthine (BMX) and 20 μM [γ-^{32}P]ATP (2×10^8 cpm/nmol) in the presence or absence of 5 μM cAMP to a final volume of 1 ml. After 20 min at 30°C, the reaction was terminated by 2 mM ATP, and the mixture was applied onto a PD-10 column equilibrated with 10 mM Tris-HCl, pH 7.5, and eluted with the same buffer. Further treatments were performed as described in section 2.2.

2.5. *In vitro phosphorylation of arylsulfatase B by cAMP-dependent protein kinase*

Arylsulfatase B purified (spec. act. 48667 U/mg protein) from leukocytes was used as substrate for the protein kinase reaction. The incubation mixture (200 μl) contained 876 U B enzyme, 20 μM [γ-^{32}P]ATP (6.3×10^5 cpm/nmol), 5 μg cAMP-dependent protein kinase, 0 or 5 μM cAMP, 10 mM NaF, 10 mM Mg $(CH_3COO)_2$, 100 μM BMX, 0.25 mM EGTA and 50 mM Hepes buffer, pH 6.8. Incubation was started by the addition of [γ-^{32}P]ATP and continued for 25 min at 30°C, terminated and immunoprecipitated as described above. The radioactivity contained in the solubilized immunoprecipitate was determined and the product examined by 10% polyacrylamide gel electrophoresis (PAGE). In each phosphorylation experiment, duplicate determinations were made on two samples, and the average values are represented.

2.6. *Dephosphorylation of arylsulfatase B*

For this, the purified leukocyte arylsulfatase B (2900 U) was treated with 0.2 U alkaline phosphatase in 0.15 M Tris-HCl, pH 8.2, in 200 μl for 16 h at 37°C.

3. RESULTS

3.1. *Protein phosphorylation of arylsulfatase B_1 from normal and CML leukocytes*

Normal and CML leukocytes were incubated in the presence of [^{32}P]orthophosphate. Arylsulfatase B and its acidic variant form, B_1, were separated on a DE-52 column, and purified using anti-arylsulfatase B antiserum. As shown in table 1, arylsulfatase B_1 isolated from normal and CML

Table 1

Phosphorylation of arylsulfatase B_1 in normal and CML leukocytes

	Incorporation of ^{32}P into B_1 (cpm/10^8 cells)
Normal leukocytes	2844
CML leukocytes	8544

leukocytes was significantly phosphorylated. Phosphorylation of the B_1 enzyme was higher in CML cells than in normal leukocytes under the conditions employed here. Phosphorylation of arylsulfatase B was low in normal and CML cells (not shown). Although the anionic property of the B_1 enzyme from CML cells [3] and lung tumor [7] was largely due to the highly phosphorylated carbohydrate chains bound to the enzyme, the B_1 enzyme from the tumor tissue was also phosphorylated on its protein [4]. Protein phosphorylation of the B_1 enzyme from leukocytes was examined through detection of phosphoamino acids from the ^{32}P-labeled enzyme. As shown in fig.1, the enzyme protein of the B_1 enzyme from normal as well as CML leukocytes was phosphorylated at serine and threonine residues. However, the predominant phosphoamino acids were clearly different between normal and leukemic cells. Quantitation of the radioactivity in each spot revealed that phosphoserine represented 60% of the total phosphoamino acids in the normal leukocytes (fig.1a), while threonine was predominantly (87%) phosphorylated in the CML leukocytes (fig.1b).

3.2. *Phosphorylation of arylsulfatase B and B_1 by endogenous protein kinase in leukocytes*

To examine protein phosphorylation of the B_1 enzyme by endogenous protein kinase, leukocyte homogenates were incubated with [γ-^{32}P]ATP in the presence or absence of cAMP. Significant incorporation of phosphate was observed in the B_1 enzyme from either normal cells or CML cells, while incorporation into the B enzyme from two sources was almost negligible (table 2). Phosphorylation of the B_1 enzyme in CML leukocytes was increased in the presence of cAMP. On the other hand, phosphorylation of the enzyme in normal leukocytes was essentially unaffected by

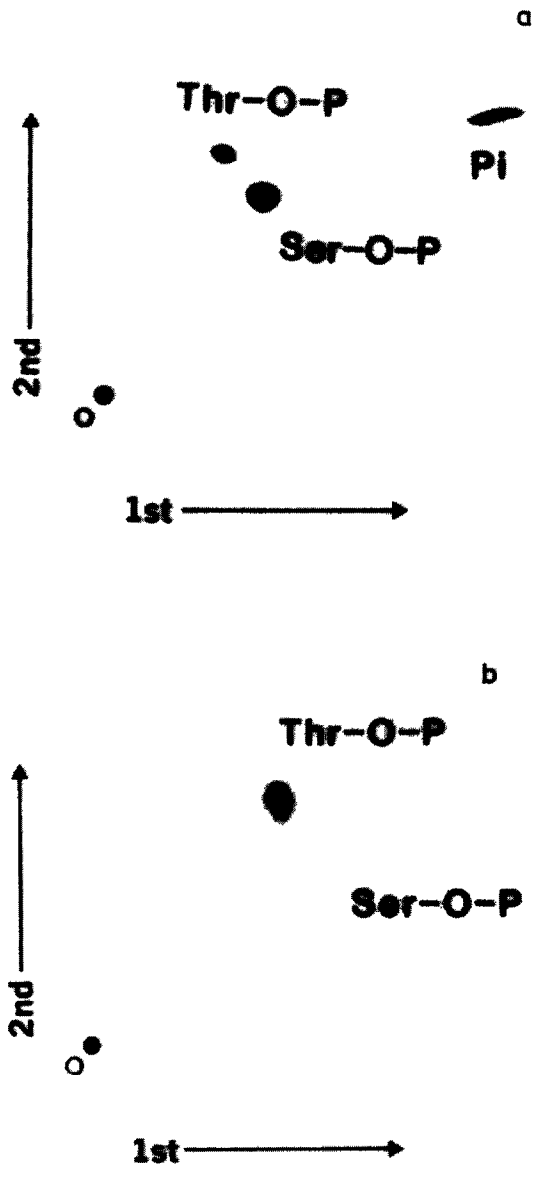

Fig.1. Two-dimensional separation of the acid hydrolysates of radioactive arylsulfatase B_1 from leukocytes. ^{32}P-labeled arylsulfatase B_1 was acid-hydrolyzed and subjected to two-dimensional separation on a thin-layer plate. 1st, electrophoresis for 1000 V for 1 h in $CH_3CO_2H/HCO_2H/H_2O$ (15:5:80, v/v, pH 1.9); 2nd, chromatography in isobutyric acid/1 M pyridine (5:3, v/v). The plate was fluorographed for 5–7 days at −80°C. (a) Normal leukocytes, (b), CML leukocytes. Ser-O-P, phosphoserine; Thr-O-P, phosphothreonine; O, origin; Pi, inorganic phosphate.

Table 2

Phosphorylation of arylsulfatases B and B_1 enzymes in normal and CML leukocytes by endogenous protein kinase

	Incorporation of ^{32}P (fmol/100 U sulfatase)			
	B enzyme		B_1 enzyme	
	cAMP (+)	cAMP (−)	cAMP (+)	cAMP (−)
Normal leukocytes	ND	ND	0.26	0.25
CML leukocytes	ND	ND	0.21	0.11

ND, not detected (<0.01 fmol/100 U sulfatase)

Table 3

Phosphorylation of arylsulfatase B from CML leukocytes by exogenous cyclic AMP-dependent protein kinase

	Incorporation of ^{32}P into ASB (pmol/100 U sulfatase)
Native ASB, cyclic AMP (+)	4.2
Dephosphorylated ASB, cyclic AMP (+)	6.5
Native ASB, cyclic AMP (−)	0.65

*ASB, arylsulfatase B

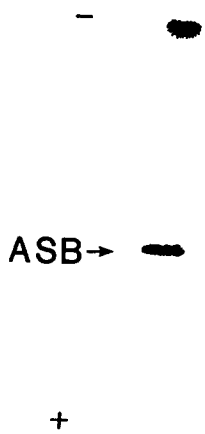

Fig.2. SDS-PAGE of the ^{32}P-labeled arylsulfatase B_1. Arylsulfatase B_1 phosphorylated by cAMP-dependent protein kinase was precipitated with anti-arylsulfatase B antiserum. The precipitate was electrophoresed according to Laemmli [10]. ASB, migration position of purified arylsulfatase B; −, cathodic origin where the labeled materials were applied. ^{32}P-labeled compound in the origin is considered to be an aggregate of ^{32}P-ASB.

the addition of cAMP, and the phosphorylation level was somewhat higher in normal leukocytes than in CML leukocytes.

3.3. *Phosphorylation of native and dephosphorylated arylsulfatase B by exogenous cAMP-dependent protein kinase*

Since the addition of cAMP enhanced phosphorylation of the sulfatase from CML cells, phosphorylation can probably be attributed to a cAMP-dependent protein kinase. The purified arylsulfatase B served as a substrate for phosphorylation by the cAMP-dependent protein kinase of bovine heart. Maximum incorporation of ^{32}P from [γ-^{32}P]ATP into the sulfatase was attained within 10 min. As shown in table 3, phosphorylation of the sulfatase was much enhanced by cAMP. On the other hand, the sulfatase which had been treated with alkaline phosphatase prior to the kinase reaction incorporated 1.5-times more ^{32}P than the native arylsulfatase B. When the B_1 enzyme served as a

substrate, the phosphorylated sulfatase was hardly detected. The phosphorylated arylsulfatase B behaved similarly to the native arylsulfatase B on SDS-PAGE (fig.2). The native B and B_I enzymes from lung tumor could not be distinguished by SDS-PAGE [4].

These results provide evidence that arylsulfatase B from leukocytes is a substrate for the cAMP-dependent protein kinase.

4. DISCUSSION

In this study we demonstrated that the acidic variant form (B_I) of arylsulfatase B from normal as well as CML leukocytes is phosphorylated in the protein moiety. The in vitro phosphorylation was stoichiometric, although stoichiometric analysis of in vivo phosphorylation of the sulfatase was difficult due to the low amount of sulfatase available from the cultured cells. This is the first demonstration that a lysosomal enzyme from normal cells is a phosphoprotein. Protein phosphorylation of lysosomal enzymes has been shown in arylsulfatase B_I [4] and β-glucuronidase [8] from human tumors. However, the predominant sites of phosphorylation in leukocyte sulfatase by in vivo phosphate incorporation differ between normal and CML cells. This difference may relate to the observation that the sulfatase from normal cells was not affected by added cAMP in contrast to the significant effect of this nucleotide in CML cells by in vitro phosphorylation with endogenous protein kinase(s) (table 2). In addition to the difference in conditions between the in vivo and in vitro experiments, other explanations are possible for the above observations: (i) the concentration of cAMP is deficient in CML cells, (ii) there are differences in the level of adenylate cyclase activity or in the mode of action of cyclase modulation [9] in normal and CML cells or, (iii) a cAMP-dependent protein kinase is responsible for phosphorylation of the sulfatase in CML cells whereas cAMP-independent kinase(s) may play a role in normal cells.

Enzyme phosphorylation and dephosphorylation play a major role in metabolic regulation. Since a fair amount of purified enzyme is required to examine this possibility, we studied this on the purified sulfatase from normal human liver, a tissue rich in lysosomal hydrolases. The liver sulfatase was found to possess a phosphorylation site for a cAMP-dependent protein kinase, and the sulfatase activity was enhanced about 50% upon phosphorylation (to appear elsewhere).

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