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ALTERATIONS OF PROTEIN KINASE ISOZYMES IN TRANSPLANTABLE HUMAN LUNG CANCER WITH SPECIAL REFERENCE TO THE PHOSPHORYLATION OF ARYLSULFATASE B

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SUMMARY: Since a lysosomal arylsulfatase B has been shown to be phosphorylated by a cAMP-dependent protein kinase (cAMP-PK) in transplantable human lung tumor, protein kinase isozymes were investigated in the tumor. Although the kinase of normal human lung comprised both type I and II isozymes at lower level, the tumor kinase was elevated in the activity and occupied almost exclusively by the type II which was responsible for the phosphorylation of arylsulfatase B. The isozyme deviation was also observed in the casein kinase of the tumor with predominance of type II in the tumor in contrast to the coexistence of both types I and II in normal lung. © 1988 Academic Press, Inc.

A lysosomal arylsulfatase B (ASB . EC 3.1.6.12) catalyzes desulfation of a physiological substrate, N-acetylgalactosamine-4-sulfate, resided in non-reducing termini of glycosaminoglycans of which accumulation (Maroteaux-Lamy syndrome) is caused by deficient activity of the hydrolase (reviewed in ref. 1). Previous studies from this laboratory have demonstrated that an increased ASB activity in transplantable human lung tumors was largely occupied by an acidic variant form of the hydrolase (2), which was highly phosphorylated both on its carbohydrate and protein moieties (3). ASB was found to be phosphorylated *in vitro* by a cAMP-PK of which activity toward ASB was considerably enhanced in the transplantable tumor (4). Moreover, ASB from the tumor and normal lung was differently phosphorylated *in vivo* and *in vitro*, respectively (4). These observations prompted us to investigate protein kinases, especially the kinase toward ASB, in the lung tumor.

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Abbreviations: ASB, arylsulfatase B; cAMP-PK, cAMP- dependent protein kinase; CK, casein kinase; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

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EXPERIMENTAL PROCEDURES

Materials---Purified human liver ASB and rabbit anti-ASB IgG were prepared as in (4). Transplantable human lung cancer of squamous cell carcinoma type was maintained in nude mice (4) and human lung tissues obtained at surgery were stored at -80°C until use. Casein kinase isozymes were prepared from rat ascites hepatoma cells (5). Protein A-Sepharose CL-4B was purchased from Sigma; [γ-^{32}P]ATP (3,000 Ci/mmol) and 8-N_3-[^{32}P]cAMP (50.2 Ci/mmol) from Amersham and ICN Radiochemicals, respectively. All reagents were of analytical grade.
Subcellular Fractionation of Tissue---Normal human lung (1.5 g) and transplanted human lung tumor (7 g) were separately homogenized in a Potter-Elvehjem's teflon homogenizer with 7 volumes of 10 mM HEPES buffer, pH 6.8, containing 0.2 mM EDTA and 0.1 mM dithiothreitol (DTT) (buffer A). The homogenate was centrifuged for 15 min at 2,000 x g to obtain a particulate fraction (designated P_1). The supernatant was centrifuged for 1 h at 105,000 x g to prepare a second particulate fraction (P_2) and cytosol. P_1 and P_2 fractions were homogenized with buffer A containing 0.15% Triton X-100 and centrifuged. The detergent-solubilized proteins were employed for protein kinase experiments.
Protein Kinase Assay and Phosphorylation of ASB---cAMP-PK activity was assayed as described previously (4) in a final volume of 0.2 ml. The reaction mixture contained 50 mM HEPES buffer, pH 6.8, 10 mM $Mg(CH_3COO)_2$, 1 μM cAMP, 20 μM [γ-^{32}P]ATP (5 x 10^5 cpm), substrate and protein kinase. Human liver ASB (2,000 units) as a substrate was phosphorylated at 30°C for 15 min, immunoprecipitated with 50 μg of anti-ASB IgG and adsorbed onto 60 μl of Protein A-Sepharose beads and subjected to SDS-PAGE using a gradient (10 to 20%) slab gel followed by fluorography as in (6). Histone II AS (100 μg) was phosphorylated as above, precipitated with trichloroacetic acid (TCA) following the addition of 500 μg of bovine serum albumin and processed as in (4). cAMP-independent protein kinase (casein kinase) activity was assayed as described before (5) in a reaction mixture containing 50 mM Tris-HCl buffer, pH 7.5, 0.1 M NaCl, 5mM $MgCl_2$, 0.1 mM [γ-^{32}P]ATP (12,000 cpm/nmol), protein kinase and 400 μg of casein which had been dephosphorylated, in a final volume of 0.1 ml. The reaction was carried out at 37°C for 5 min, stopped with TCA and assayed for ^{32}P as described above. All assays were done in triplicate determinations. Other methods employed were those described previously (4).
DE52 Chromatography of Protein Kinases---Cytosol fraction from normal lung or transplanted tumor was applied onto a DE52 column equilibrated with buffer A without DTT. After washing the column with the same buffer, protein kinases were eluted with a gradient of 0 to 0.4 M NaCl in the same buffer.
Photoaffinity Labeling of Regulatory Subunits of cAMP-PK--- This was done as described previously (7). The reaction mixture (50 μl) contained cAMP-PK, 1 mM $MgCl_2$, 0.1 mM ATP, and 0.7 μM 8-N_3-[^{32}P]cAMP in the presence or absence of 0.1 mM cAMP (see figure legend). The labeled materials were separated on SDS-PAGE followed by autoradiography.

RESULTS

Activities and Subcellular Distribution of Lung Tumor Protein Kinases---Transplantable human lung tumor has markedly enhanced activities of cAMP-PK toward histone and CK measuring 51.9 and 5.0 pmol of phosphate/min/mg protein, respectively, as compared to those

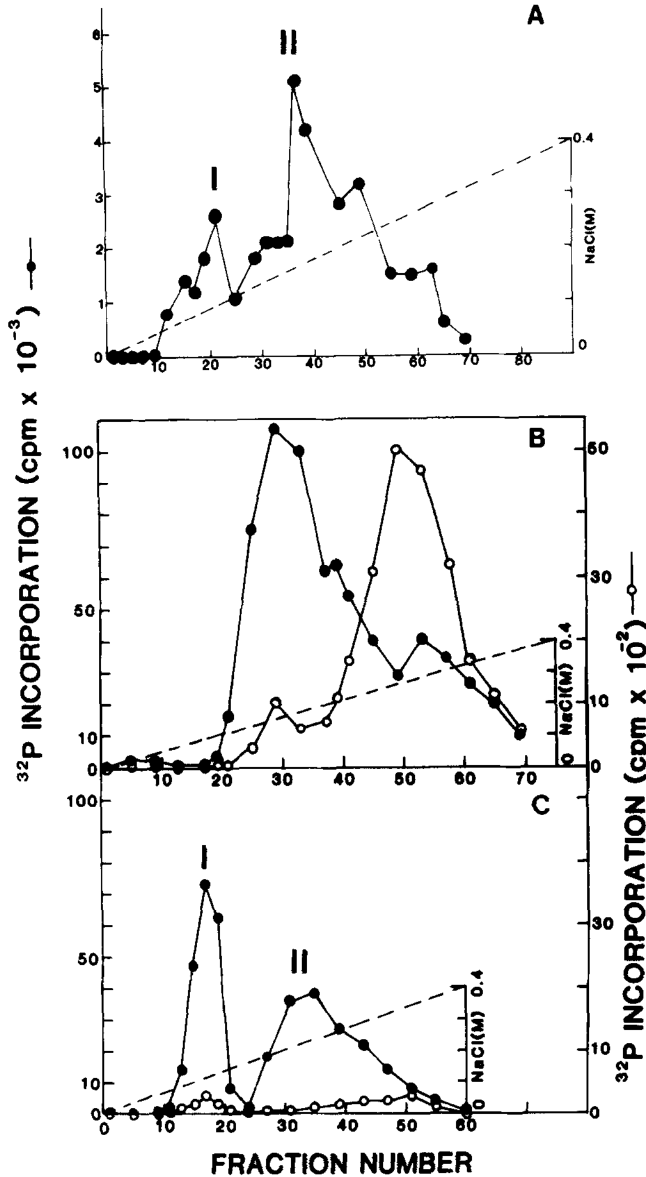

Fig. 1. Fractionation of protein kinases in cytosols of normal human lung and the tumor. A, Tumor cytosol (38 mg protein) was chromatographed on a DE52 column (2.2 x 6.5 cm). A 50 µl of each fraction (1.5 ml) was assayed for cAMP-PK activity toward ASB. B, The same fractions as A were assayed for cAMP-PK activity toward histone (●) and for casein kinase activity (o). C, Cytosol (10 mg protein) of normal lung was fractionated on a DE52 column (1.2 x 2 cm). A 50 µl of each fraction (0.5 ml) was assayed for cAMP-PK toward histone (●) and for casein kinase (o).

(respective 6.2 and 0.04 pmol/min/mg) of normal lung, similarly to the increased activity of cAMP-PK toward ASB (4). Eighty three % of the total activity of cAMP-PK toward ASB is located in the tumor cytosol, 17% in P_1 fraction and no activity in P_2 fraction.

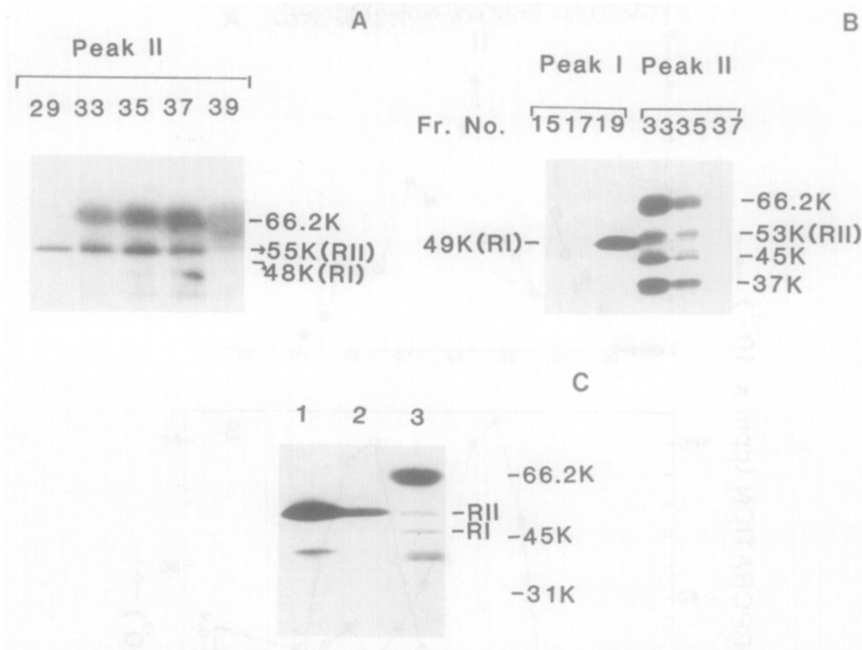

Fig. 2. Autoradiograms of photoaffinity-labeled regulatory subunits of cAMP-PK on SDS-PAGE. A, Aliquot (40 μl) from fractions in Fig. 1A was labeled using 0.7 μM of 8-N_3-[^{32}P]cAMP followed by SDS-PAGE (12.5% gel). B, A 40 μl of fractions in Fig. 1C was used for the labeling by 0.7 μM of 8-N_3-[^{32}P]cAMP (12.5% gel). C, 200 μg protein (lane 1) and 100 μg protein (lane 2) of whole cytosol from the lung tumor and 90 μg protein (lane 3) of whole cytosol from norml lung were labeled with 0.7 μM of 8-N_3-[^{32}P]cAMP followed by SDS-PAGE (5 to 20% gradient gel). In all experiments, parallel labelings were performed in the presence of cAMP for the confirmation of specific detection of RI and RII, and autoradiography was done for 2 days.

Characterization of Protein Kinases in Lung Tumor--- As shown in Fig. 1, the elution profiles of protein kinases from lung tumor was cosiderably different from that of normal lung. The same elution profiles of protein kinases were obtained in the other experiments on another tumor and normal lung, respectively. cAMP-PK from the tumor toward ASB showed two enzyme-active peaks (peak I including fraction Nos. 11 to 21 and peak II from Nos. 27 to 65), (Fig. 1A). cAMP-PK

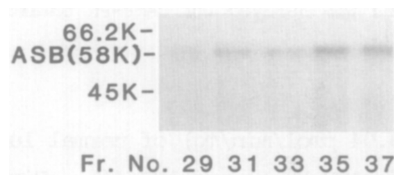

Fig. 3. Fluorogram of ASB phosphorylated by cAMP-PK from the lung tumor. Purified ASB was phosphorylated by cAMP-PK in eluates (50 μl of each) at fraction Nos. 29 to 37 in Fig. 1A, immunoprecipitated and subjected to SDS-PAGE and fluorography for one week.

toward histone (Fig. 1B) appeared to be almost occupied by type II isozyme which showed some activity shoulders at higher NaCl concentrations. Somewhat different chromatographic profiles between ASB and histone as substrates are most probably ascribed to that protein kinases other than cAMP-PK also act on histone. To confirm cAMP-PK type I and II isozymes, 8-N_3-[^{32}P]cAMP labeling of two regulatory subunits (RI and RII, respectively) was carried out on the chromatographic fractions. As shown in Fig. 2A, most of the fractions of cAMP-PK at peak II comprised exclusively RII (Mr 55,000). RI (Mr 48,000) was slightly detected in some fractions of peak I (data not shown). On the other hand, cAMP-PK of normal lung showed the coexistence of both types I and II (Fig. 2B). These observations were also verified in whole cytosols from the two sources (Fig. 2C). Data in Fig. 3 confirm that fractions of type II cAMP-PK from the tumor phosphorylate ASB.

Lung cancer had also considerably elevated activity of CK compared to normal lung (see above). Refering to the elution profile of CK on DE52 chromatography (5), the activities eluted at lower and higher NaCl concentrations will be type I and type II, respectively, in normal and tumor tissues (Fig. 1B and 1C). Both type I and type II CK were at very low level in normal lung tissue (Fig. 1C), whereas CK in the lung tumor had markedly enhanced type II CK (Fig. 1B). Thus, the elevated activity of CK in the lung tumor can be ascribed largely to selective increase of type II CK.

ASB was phosphorylated at two sites of the hydrolase in the lung tumor, and one of them was phosphorylated by cAMP-PK (4). It was examined whether or not another site is phosphorylated by CK. However, two CK isozymes did not act on ASB of both the native and alkaline phosphatase-treated forms.

DISCUSSION

The present study showed that cAMP-PK of normal human lung tissue consisted of type I and II isozymes, similarly to the previous observations on normal human lung fibroblasts (8) as well as normal mouse lung tissue (9). In contrast, cAMP-PK in the transplanted lung tumor was almost exclusively type II, which is most probably responsible for the occurrence of a highly phosphorylated ASB form (2,3). Altered cAMP-PK isozyme composition has been noted in some human tumors such as renal cell carcinoma (10) and gastric adenocarcinoma (11) in which type I isozyme of cAMP-PK was elevated

compared to the respective normal controls. On the other hand, human glioblastoma was predominant in type II isozyme (12) similar to the observation in the present work. These inconsistent observations will explain that selective increase either of typr I or type II isozyme arises from difference of the tissues from which tumors are derived.

The present study also demonstrated enhanced CK activity and the isozyme deviation in the lung tumor. Although CK was not responsible for the phosphorylation of ASB and the role of CK in cancer is not well understood at present, implication of this kinase in cancer has been noted. A hepatoma ascites cell line, compared to normal rat liver, had several cytosolic proteins heavily phosphorylated by CK in which type II was predominant (13). Also, stimulation of type II was reported in human glioblastoma cell cultures (14). Since immortalization of tumor cells cultured from human brain tumors was noted to accompany increased activity of CK II (14), the marked enhancement of this isozyme may associate with established transplantability of human lung tumor.

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REFERENCES

1. McKusick, V. A., and Neufeld, E. F. (1983) in The Metabolic Basis of Inherited Disease (Stanbury, J. B., Wyngaarden, J. B., Fredrickson, D. S., Goldstein, J. L., and Brown, M. S., eds.) pp. 751-777, McGraw Hill, New York.
2. Gasa, S., Makita, A., Kameya, T., Kodama, T., Koide, T., Tsumuraya, M., and Komai, T. (1981) Eur. J. Biochem. 116, 497-503.
3. Gasa, S., and Makita, A. (1983) J. Biol. Chem. 258, 5034-5039.
4. Gasa, S., Balbaa, M., Nakamura, M., Yonemori, H., and Makita, A. (1987) J. Biol. Chem. 262, 1230-1238.
5. Nakajo, S., Shinkawa, K., Shimizu, T., Nakaya, K., and Nakamura, Y. (1984) J. Biochem. 96, 1575-1585.
6. Gasa, S., and Kornfeld, S. (1987) Arch. Biochem. Biophys. 257, 170-176.
7. Hunzicker-Dunn, M. (1981) J. Biol. Chem. 256, 12185-12193.
8. Liu, A. Y.-C., Chang, Z.-F., and Chen, K. Y. (1986) J. Cell. Physiol. 128, 149-154.

9. Butley, M. S., Beer, D. G., and Malkinson, A. M. (1984) Cancer Res. 44, 2689-2697.
10. Fossberg, T. M., Doskeland, S. O., and Ueland, P.M. (1978) Arch. Biochem. Biophys. 189, 372-381.
11. Yasui, W., Sumiyoshi, H., Ochiai, A., Yamahara, M., and Tahara, E. (1985) Cancer Res. 45, 1565-1568.
12. Frattola, L., Canal, N., Ferrarese, C., Tonini, C., Tonon, G., Willani, R., and Trabucchi, M. (1983) Cancer Res. 43, 1321-1324.
13. Nakaya, K., Shimizu, T., Segawa, Y., Nakajo, S., and Nakamura, Y. (1983) Cancer Res. 43, 3778-3782.
14. Prowald, K., Fischer, H., and Issinger, O.-G. (1984) FEBS Lett. 176, 479-483.