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ALKALINE DEOXYRIBONUCLEASE ACTIVITY IN MOUSE TERATOCARCINOMA CELLS : VARIATION OF ENZYME LEVELS DURING DIFFERENTIATION

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SUMMARY

Embryonal carcinoma (EC) cells contain an alkaline DNase whose specific activity is much higher than their differentiated derivatives. After partial purification on CM-Sephadex, fractions eluted at 0.15 M NaCl contain a DNase activity which is inhibited by G-actin. The possible role of this alkaline DNase activity in maintaining the unpolymerized state of actin filaments in EC cells is discussed.

Deoxyribonuclease I is an endonuclease highly specific for DNA (1); it has been purified from bovine pancreas and well characterized (2). The enzyme with a molecular weight of 31,000 daltons (3) has been extensively studied and its aminoacid sequence has been determined (4, 5, 6). It requires Ca^{++} and Mg^{++} at pH 7,5 for optimal activity (7). Another DNAse with a similar activity has been isolated from rat liver nuclei with a molecular weight of 30,000 daltons (8, 9, 10). Its physiological role is unknown.

Eukaryotic cells contain a protein which strongly inhibits DNase I and has been recognized as a major component of the cell (11, 12). Purification to homogeneity from calf spleen was obtained by Lindberg (11). It is highly specific and does not inhibit other types of DNase from eukaryotes or prokaryotes. It has been shown that the primary structure of the inhibitor and G-actin are the same. They form a stable 1 : 1 complex with a molecular weight of about 75,000 daltons (14).

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The physiological function of the inhibition of DNAse I by actin is not known but Hitchcock and coll. (15) have shown that DNAse I causes depolymerization of F-actin and the product of this depolymerization produces a rapid inhibition of DNAse I. F-actin can be protected when complexed with heavy meromyosin. Mannherz and coll. (16) have observed a similar effect of depolymerization of F-actin by DNAse I. Paulin and coll. (18) have observed that the actin organisation is different in differentiated and embryonal carcinoma (EC) cell lines. EC cells do not show actin cables whereas the differentiated lines (fibroblasts, myocards or myoblast cells) do.

We report in the present communication that the levels and specific activity of alkaline DNAse are much higher in E.C. cells than in their differentiated derivatives. The possible role of this alkaline DNAse activity in maintaining the unpolymerized state of actin filaments in the E.C. cell lines is discussed.

MATERIALS AND METHODS

Cell cultures : All the cells used in this study are described in ref. 18 & 20. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum in a humid air - 12% CO_2 atmosphere. The E.C. cell lines were plated at high densities (1 x 10^6 cells per 100-mm diameter dish), and replated every 48 hours. Under such conditions, the cells remained in exponential phase and did not differentiate in culture (20).

All the cell cultures were routinely checked for mycoplasma contamination by two different methods: culture in specific media and fluorescent microscopy (21). Mycoplasma were absent from all the cell lines used.

Preparation of cell extracts : The method used was that of Churchill et al. (8). Cells were suspended in buffer A (50 mM Tris HCl pH 7,5, 140 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT)) and sonicated 15 sec. using a Branson apparatus and a power setting of 100 W. After centrifugation at 130,000 g for 1 hour, the supernatants were used either as crude extracts or for partial purification of alkaline DNAse activity. A carbomethyl Sephadex column (Pharmacia, Sweden) (12 x 0.8 cm) was equilibrated with buffer B (50 mM potassium phosphate pH 7,0, 1 mM EDTA, 1 mM DTT and 40% ethylene glycol). Crude extracts were dialyzed against buffer B before being put on the column which was developed with a linear 0 - 0,55 M

NaCl gradient. Fractions of 1,5 ml were collected at a flow rate of 6 ml per hour. Alkaline DNAse activity was measured in an aliquot of each fraction. Active fractions were concentrated by ultrafiltration. Protein concentration was determined either by absorption at 280 nm/260 nm or by the Lowry method using bovine serum albumin (BSA) as standard. 10^8 cells from differentiated lines yielded a 130,000 x g supernatant in which the protein concentration was 40% higher than that in the supernatant obtained from the same number of undifferentiated cells.

Preparation of labeled substrate : (^3H)-$d(A-T)_n$ was prepared with unlabeled $d(A-T)_n$ as primer template and E. coli DNA polymerase I in the presence of both (^3H)-dTTP and (^3H)-dATP (specific activities 50 Ci/mmol and 11 Ci/mmol respectively) according to a slight modification of the method described by Richardson et al (22). The polymer was extracted with phenol and purified by filtration through a G-50 Sephadex column. The final specific activity was adjusted to 7×10^4 cpm/µg with unlabeled $d(A-T)_n$.

DNAse assays : The reaction mixture contained: 10 mM Tris HCl pH 7,5, 10 mM $MgCl_2$, 2 mM $CaCl_2$, 100 ng of (^3H)-$d(A-T)_n$ and 0.1 to 100 µg of either crude extracts or purified fractions in a final volume of 100 µl. After incubation at 37°C, 900 µl of buffer C (Tris HCl pH 7,5, 1 mM EDTA) containing 120 µg of BSA was added and the reaction was stopped by the addition of 1 ml of 10% trichloracetic acid (TCA). Tubes were allowed to stand for 20 min in ice. Samples were filtered onto presoaked millipore filters which were extensively washed with 5% TCA. After drying, radioactivity was measured in a liquid scintillation spectrophotometer.

1 unit is defined as the amount of enzyme that releases 10 ng of substrate in acid-soluble forms in 15 min at 37°C. The assays were found to be reproducible and proportional to extract concentration and incubation time. Pure actin extracted from rabbit was a gift of C. Oriol.

RESULTS

Alkaline DNAse activity in different cell lines

Table I shows the specific DNAse activities in different cell lines derived from teratocarcinoma. It is about 30-fold higher in E.C. cells than in differentiated cell lines. Embryonal carcinoma cells PCC3, PCC4 and endodermal PYS-2 cells do not exhibit actin cables, whereas the three differentiated cell lines show the characteristic actin fiber arrangement. Hybrid cell lines established from PCC4 and myoblasts show cable structure, like myoblasts, and contain very low levels of DNAse activity.

Partial purification of alkaline DNAse activity from PCC3 cells

Fig. 1 shows that alkaline DNAse activity can be separated by

Table I

Alkaline DNAse activity in different teratocarcinoma cell lines

Cell lines	Specific activity units/mg protein
Embryonal carcinoma	
PCC3	450
PCC4	300
Endodermal	
PYS-2 (endodermal)	380
Differentiated	
PCD1 (myocard)	14
PCD2 (myoblast)	8
PCD3 (fibroblast)	10
Hybrid	
PCC4 + myoblast	12

5-15 µg of crude extracts are incubated at 37°C with $7x10^3$ cpm of (^3H)-d(A-T)$_n$. At different times (0, 5, 15, 30 min), aliquots are withdrawn and the remaining acid-precipitable activity is measured as indicated in Materials and Methods.

chromatography on C-M Sephadex into two peaks: the first one (A) contains DNAse activity and all the proteins which are not retained by the column and are eluted with the washing buffer; the second one (B) represents DNAse activity eluted at 0.15 M NaCl with a specific activity after concentration by ultrafiltration of 12,000 units/mg. Optimal pH for DNAse activity of this partially purified fraction is between 7.5 and 8.0, and 10 mM Mg^{++} is required for full activity. The enzyme is stimulated by30% with 1 mM Ca^{++}. Ethylene glycol bis (β aminoethylether)-N, N'-tetraacetic acid (EGTA), a selective chelating agent for Ca^{++}, reverses the stimulatory action of Ca^{++}.

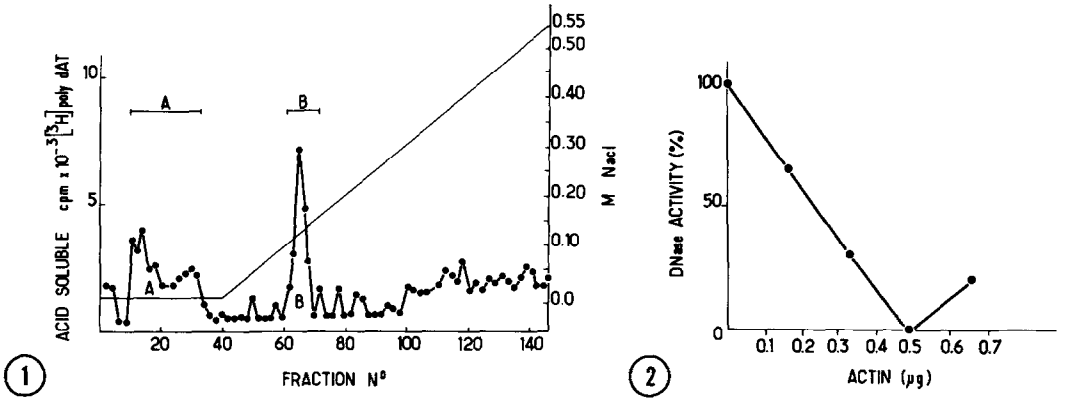

Fig. 1: C.M-Sephadex elution profile of the crude extracts from 6.10^8 E.C. cells.

0.05 ml of each fraction was tested for DNAse activity as described in Material and Methods. Active fractions A and B were concentrated by ultrafiltration and protein concentration determined.

Fig. 2: Inhibition of DNAse activity by G-actin.

6 units of fraction B (Fig. 2) were pre-incubated with increasing amounts of actin for 10 minutes at 37°C, before the addition of (^{3}H)-poly dAT. After further incubation for 15 minutes at 37°C, the acid precipitable material was determined as described in Material and Methods.

Inhibition of partially purified DNAse activity by G actin

When increasing amounts of G-actin are added to a constant amount of fraction B from the C.M-Sephadex column, a clear-cut inhibition of DNAse activity is observed. As shown in Fig. 2, maximal inhibition is obtained with 0,5 µg of actin for 6 units of partial purified enzyme.

When F-actin is added to fraction B and the mixture is pre-incubated under the same conditions as indicated above, either 18 hours at 4°C or 2 hours at 37°C are necessary to obtain the same degree of inhibition as with G-actin. Under such conditions, by electron microscopy, a depolymerisation of F-actin is observed (data not shown).

DISCUSSION

Alkaline DNAse activity has been detected in and partially purified from E.C. cells (PCC3) under mild extraction conditions (140 mM NaCl, see Material and Methods). Two forms of the enzyme have been separated by C.M-Sephadex chromatography, one (A) is eluted with the washing buffer; and the other (B) is eluted at 0.15 M NaCl. The latter represents a 30-fold purification with respect to the activity of the crude extracts. A similar result has been obtained by Churchill et al. (8) with HeLa cells. Several lines of evidence indicate that this alkaline DNAse is probably a DNAse I type activity (7, 15) (Ca^{++} and Mg^{++}-dependent, optimal pH 7.5, inhibition of DNAse activity by G-actin). Under the same conditions, it was not possible to isolate a similar activity from differentiated cell lines.

The multipotent embryonal carcinoma lines are resistant to infection by polyoma and SV 40 virus (23). As *in vitro* differentiation proceeds, the cells become progressively susceptible to viral infection. It is possible that in E.C. cell DNAse modifies the viral DNA, thus preventing the transcription of specific sequences necessary for viral development.

When F-actin and DNAse I interact *in vitro* in equimolar amounts, a striking reciprocal influence is observed, i.e., depolymerization of F-actin and inhibition of DNAse I (15, 16). It is not known whether a similar phenomenon occurs *in vivo*. It has been shown that actin polymerizability is influenced by a low molecular weight protein called profilin. The latter forms a stable complex with G-actin (profilactin) which prevents its polymerization (17). Actin could be capable of rapid mobilization to form functional microfilaments. Since the authors were unable to depolymerize F-actin by direct addition of profilin, they proposed the existence of a hypothetical depolymerizing factor. Such a role could be played by the DNAse I type activity.

Since the level of alkaline DNAse activity is much higher in E.C. cells than in differentiated ones, it is tempting to relate this high enzyme activity to actin organization previously reported in E.C. cells (18). Thus, E.C. cells do not display actin cables, whereas when cells are allowed to differentiate in vitro, actin cables appear. In this respect, alkaline DNAse in E.C. cells is a good candidate as a depolymerizing factor.

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