THE ACTIVATION OF ACTIN:DNase I COMPLEX WITH RAT LIVER PLASMA MEMBRANES

The possible role of 5′-nucleotidase

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1. Introduction

The specific interaction of bovine pancreatic DNase I and skeletal muscle or cytoplasmic actin has been well documented [1–3]. After formation of a stable 1:1 complex the biological properties of both proteins are inhibited, i.e., the DNA degrading activity of DNase I and the ability of G-actin to form high molecular weight polymers. The isolation of a secretory actin:DNase I complex from rat pancreatic juice and the reversal of the inhibitory action of actin on DNase I by a protein from rat or human bile gives evidence for the natural occurrence of this complex and for a possible physiological importance [4]. The activating protein from bile has now been identified by us as 5′-nucleotidase (in preparation). We described the interaction of snake venom 5′-nucleotidase with rabbit skeletal muscle actin:bovine pancreatic DNase I complex (synthetic actin:DNase I complex [5]). As 5′-nucleotidase is known as a marker enzyme of plasma membranes, we investigated the interaction of this complex with plasma membranes isolated from rat liver.

Abbreviations: Hepes, 2,4-(2-hydroxyethyl)-1-piperazine–ethane–sulfonic acid

Enzyme: DNase I or deoxyribonuclease I, EC 3.1.4.5; 5′-nucleotidase or 5-ribonucleotide phosphohydrolase, EC 3.1.3.5

2. Materials and methods

Rat liver plasma membranes were obtained following a modification of the method in [6]. Bovine pancreatic DNase I (EC 3.1.4.5.) was purchased from Worthington Corp. and further purified as in [7]. Rabbit skeletal muscle actin was obtained from acetone powder as in [3].

Prior to use it was extensively dialysed against 5 mM Hepes buffer (pH 7.5) plus 0.1 mM $CaCl_2$ and 0.5 mM NaN_3 (buffer A). Synthetic actin:DNase I complex was obtained by mixing rabbit skeletal muscle actin and purified bovine DNase I in stoichiometric ratio and further purification by gel filtration on an AcA 44 Ultrogel (LKB) column [8]. Protein concentrations of solutions of skeletal muscle actin, bovine pancreatic DNase I and synthetic actin:DNase I complex were determined by measuring the absorbance using the values [8]. $E^{1\%}_{290\,nm} = 6.6$, $E^{1\%}_{280\,nm} = 12.3$ and 9.6, respectively. All other protein concentrations were determined by the Lowry method.

Activation or inhibition of actin:DNase I complex with respect to DNA-splitting activity was measured as follows: 50 μl actin:DNase I complex or 50 μl DNase I were mixed with 50 μl of the cytoplasmic membrane fraction (protein concentrations see below) and incubated for periods described in a mini-incubation vessel at 37°C. This mixture, 10 μl was then tested for DNase I activity. DNase I activity was measured as in [8] with an Aminco DW 2 Spectrophotometer at 260 nm and 30°C. The assay used contained 50 μg/ml

salmon sperm DNA sodium salt, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 10 mM Tris–HCl buffer (pH 8.0).

5′-Nucleotidase activity was determined optically [9] in buffer A supplemented with 2 μg/ml adenosine desaminase (EC 3.5.4.4) obtained from Boehringer-Mannheim and 0.1 mM 5′-AMP at 30°C. Electrophoresis on SDS–polyacrylamide gels was as in [10].

Negatively stained samples of liver cytoplasmic membranes were prepared on carbon-coated grids, stained with 1.5% uranyl acetate or 1.5% phosphotungstic acid (pH 7.4) and examined in a Zeiss EM 9s 2 or a Philips 300 electron microscope. Phalloidin was a generous gift from Professor Th. Wieland. All other reagents were of analytical grade.

3. Results

The activation of the DNA-degrading activity of skeletal muscle actin:pancreatic DNase I complex by rat liver cytoplasmic membranes is illustrated in fig.1,2. The sigmoidal time dependence of DNA hydrolysis of actin:DNase complex is shown altered to a more normal progress curve, similar to that of DNase I alone, resulting in much higher activity.

This activation is strongly temperature dependant. The 5-fold activation that is reached at 37°C after 2 h is only reached after 8 h at 20°C incubation temperature. Under actin polymerising conditions, i.e., in buffer A supplemented with 0.1 M KCL, activation occurs much faster; addition of 10^{-5} M phalloidin does not result in a further increase in the reactivation although this peptide is known to increase the rate of actin polymerisation [12]. Addition of a 10 molar excess of G-actin over DNase I to the incubation mixture when full reactivation had been achieved resulted in an instantaneous and complete inhibition of the DNase I activity. The activation does not occur with rough microsomes from rat pancreas, membranes that have no or only trace activity of 5′-nucleotidase (obtained as in [17]).

Examination of the membranes after incubation with actin : DNase I complex by electron microscopy (fig.3) showed no change on intact vesicles, but more

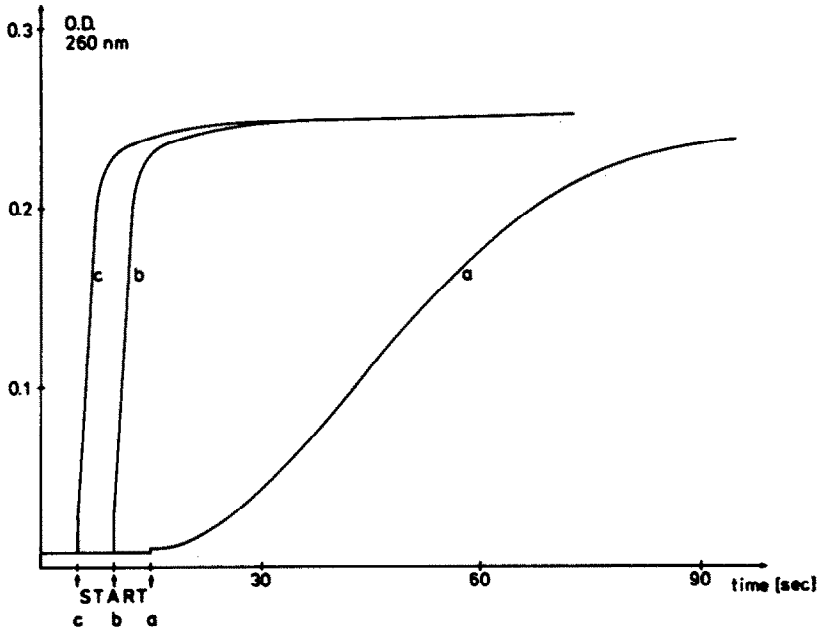

Fig.1. Change of time dependence of DNA hydrolysis with actin:DNase I complex after 3 h incubation with rat liver plasma membranes at 37°C. Incubation mixture contained 40 μl membranes (3 mg protein/ml) and 40 μl actin:DNase complex (1 mg/ml) in buffer A or 80 μl buffer A containing 400 μg/ml DNase I. Trace (a) before incubation; trace (b) after incubation; trace (c) DNase alone.

Fig.2a.

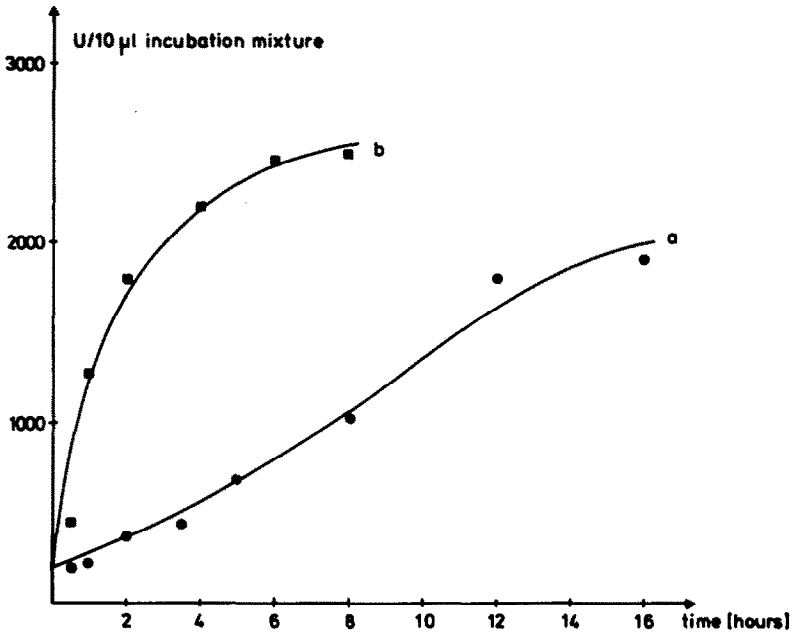

Fig.2a. Increase in DNase I activity during incubation at different temperatures. Incubation mixture see fig.1a. Trace (a) at 20°C, (b) at 37°C incubation temperature, 3 mg membrane protein/ml, 5'-nucleotidase activity = 4.33 U/mg protein (1 U = 1 µmol AMP hydrolyzed/min/ml reaction mixture).

Fig.2b.

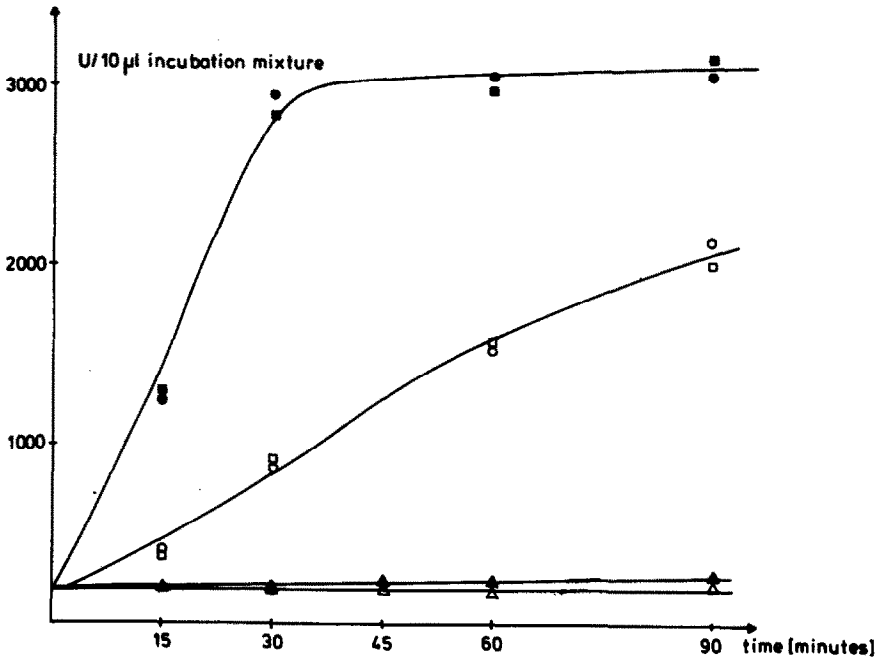

Fig.2b. Increase in DNase activity during incubation of actin:DNase I complex with rat liver cytoplasmic membranes. Incubation mixture contained 40 µl membranes (3.5 mg protein/ml) and 40 µl AD complex (1 mg protein/ml) in buffer A. (□) Membranes alone; (△) 0.1 M KCl alone; (■) membranes + 0.1 M KCl; (○) membranes + 10^{-5} M phalloidin; (●) membranes + 10^{-5} M phalloidin + 0.1 M KCl; (▲) rough microsomes from rat pancreas (2 mg protein/ml); 5'-nucleotidase activity = 0.23 U/mg protein.

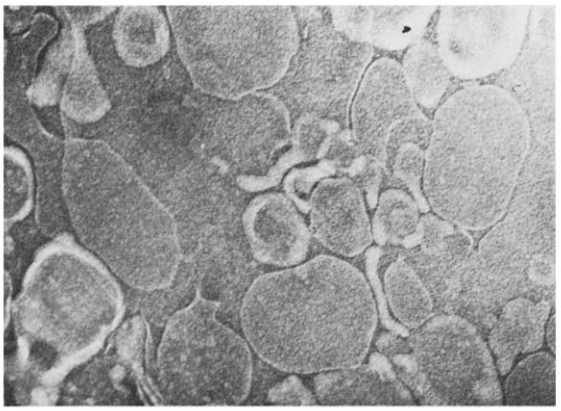

Fig.3a.

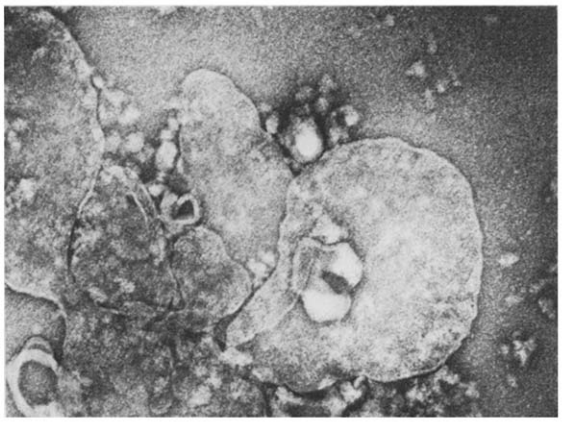

Fig.3b.

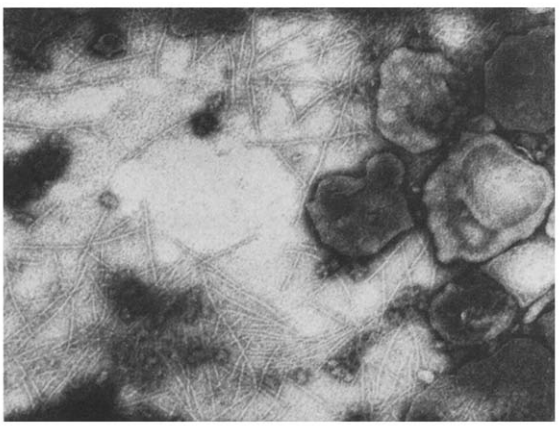

Fig.3c.

deteriorated material was found. Only when incubated with phalloidin, with or without actin:DNase I complex and/or KCl could actin filaments be visualized [12]. They also appear in the actin:DNase I complex when incubated with 5′-nucleotidase and phalloidin. We think that 5′-nucleotidase of the cytoplasmic membranes is responsible for this activation, since we found that 5′-nucleotidase from the snake *Crotalus adamanteus* venom [5] and 5′-nucleotidase isolated from rat bile (in preparation) are able to activate this complex and accelerate the rate of actin polymerisation. Neither the free 5′-nucleotidase nor the membrane-bound 5′-nucleotidase alter their enzymatic activity during activation of the actin:DNase I complex.

To answer the question whether the membrane-bound or -associated cytoplasmic actin (review [11]) could inhibit DNase I activity, we investigated the interaction of bovine pancreatic DNase I with rat liver cytoplasmic membranes. The presence of actin in the membrane fraction is demonstrated by comigration of a protein band with rabbit skeletal muscle actin on SDS–slab gels (fig.4).

This actin does not seem to occur in the filamentous state as no filaments could be visualized by electron microscopy after negative staining. Incubation of DNase I (50 μl at 0.06 mg/ml) with an equal volume of liver plasma membranes (2 mg protein/ml final conc.) at 37°C showed at the beginning of the incubation period the same activity as was found in the absence of membranes. This activity does not change during 2 h incubation at 37°C and only slightly decreases (~10%) after 10 h. Addition of equimolar concentration of either G- or F-actin leads to inhibition under these conditions.

To prove dissociation of DNase I from actin by the interaction with plasma membranes, the actin:DNase I complex was incubated with various ratios of rat liver plasma membranes for 2 h under actin polymerising conditions (0.1 M KCl) at 37°C. The DNase I activity

Fig.3. Rat liver plasma membranes negatively stained with 1.5% phosphotungstic acid (pH 7.4), 100 μl membranes (2 mg protein/ml), incubation for 1 h. (a) Without any reagents; (b) with 50 μl DNase I complex (1 mg/ml) and 0.1 M KCl; (c) with 50 μl actin:DNase I complex (1 mg/ml), 0.1 M KCl and 10^{-5} M phalloidin. Note that actin filaments appear only in (c). Magnification: (a,b,) × 65 000; (c) × 100 000.

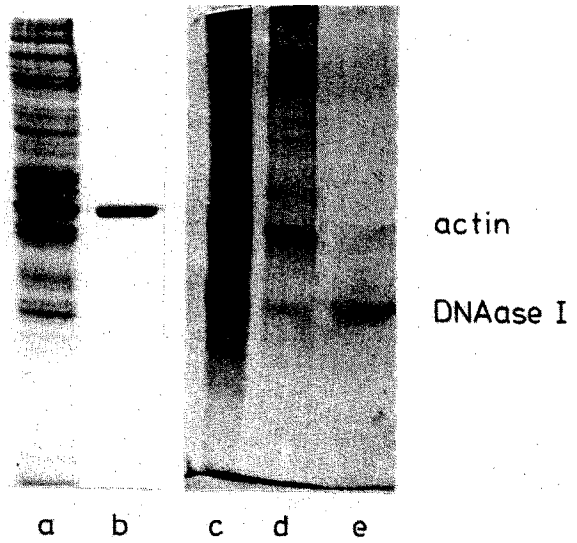

Fig.4. SDS–slab gel electrophoresis of rat liver plasma membranes. (a) Rat liver plasma membrane fraction; (b) rabbit skeletal muscle actin. Rat liver plasma membranes, 1 ml (2 mg protein/ml) incubated for 90 min with 500 μg actin:DNase I complex (1 mg/ml): (c) At the beginning of incubation; (d) pellet after 3 h centrifugation at 100 000 × *g*; (e) supernatant after 3 h centrifugation at 100 000 × *g*.

increased 20-fold. This incubation mixture was centrifuged for 3 h at 100 000 × *g*. The pellet contained membranes with unchanged 5′-nucleotidase activity whereas the DNase I activity remained mostly in the supernatant. SDS–gel electrophoresis (fig.4) showed a strong reduction of the actin concentration in the supernatant, almost 90% of the actin being pelleted with the membranes, whereas DNase I remained in the supernatant. When actin : DNase I complex was treated under the same conditions, no sedimentation was obtained.

4. Discussion

The interaction of rat liver plasma membranes with actin : DNase I complex results in a reversal of the inhibitory action of actin on DNase I, dependent on ionic conditions and temperature.

Furthermore the sedimentation experiments indicate that reactivation is due to a separation of the complex into DNase I and actin, excluding the possibility that the increase in DNA-degrading activity is caused by the actin:DNase I complex itself.

We obtained similar results with 5′-nucleotidase from snake venom of *Crotalus adamanteus* or 5′-nucleotidase from rat bile (in preparation). As 5′-nucleotidase is known to be present in plasma membranes we assumed that the membrane-bound 5′-nucleotidase is responsible for these effects. Since isolation of this enzyme from the membrane preparations would result in their destruction, this cannot be proved directly. This hypothesis however is substantiated by the observation that 5′-nucleotidase isolated from rat liver plasma membranes shows similar results (in preparation). This membrane-bound enzyme is similar if not identical with 5′-nucleotidase secreted in rat bile [13].

Assuming that 5′-nucleotidase is the reactivating factor, then this study demonstrates that this enzyme can also exert its effect on actin:DNase I complex when incorporated into membranes. It has not been demonstrated yet whether 5′-nucleotidase spans the whole membrane with parts extending into both the outer and inner face of the plasma membrane, although there is general agreement that its enzymatic activity is located on the outer face [14]. Even if this were the case it cannot be decided from the present study which part of the enzyme induces reactivation of the actin:DNase I complex, although the finding that its enzymatic activity is not altered during reactivation (see [5]) indicates a site different from its active center. The reported reactivating effect of isolated 5′-nucleotidase, however, excludes the possibility that actin still associated with membranes (see fig.1) might be solely responsible for reactivation via a mechanism postulated and termed anchorage modulation [18]. Since we have found that 5′-nucleotidase is also able to accelerate the rate of actin polymerisation (in preparation), membrane-bound 5′-nucleotidase might act as an anchoring protein for intracellular actins and/or modify their availability for cellular motile responses. The actual state in which actin is present after liberation from the complex by membrane-bound or by isolated 5′-nucleotidase need not necessarily be the classical G- or F-form. We found, that forms similar to F-actin appear only after incubation of actin and snake venom 5′-nucleotidase with phalloidin. In the absence of phalloidin an indistinct meshwork appears, whereas change in the intensity of

scattered light indicates polymerisation [5]. In rat liver plasma membrane fractions prepared in a similar manner to our procedure, no or only very few actin filaments are visible. They first appear after incubation with phalloidin and can then be decorated with heavy meromyosin [12].

This membrane bound or associated actin seems not to be in the G-form, since it does not inhibit DNase I even after 10 h incubation. This does not contradict the finding that actin can be isolated from rat liver plasma membranes with a DNase : agarose affinity column [16], since this is only possible under membrane solubilizing conditions.

There is also no contradiction to reports indicating that DNase I can extract actin from glycerinated and myosin extracted cells [17]. This takes place on the visible filaments of the cytoplasm, and there is no indication that actin is totally extracted.

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