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Degradative inactivation of cyclic AMP-dependent protein kinase by a membranal proteinase is restricted to the free catalytic subunit in its native conformation

(brush-border membranes/intestinal microvilli/enzyme regulation/limited proteolysis)

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ABSTRACT **A membranal proteinase from brush-border epithelial cells of the rat small intestine was shown to bring about a restricted and limited degradation of the free catalytic subunit (C) of cyclic AMP-dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) with concomitant inactivation of the kinase. This membranal proteinase exhibits a remarkable specificity. (*i*) It degrades C in its native conformation, but not after it has been heat-denatured. (*ii*) The degradation of C (M_r 40,000) does not proceed further, once a distinct clipped product (M_r 34,000) is formed. (*iii*) The undissociated ("stored") form of the enzyme (R_2C_2) is not attacked by the membranal proteinase, preserving both its potential catalytic activity and its molecular integrity. Only upon addition of cyclic AMP to release free C does the proteinase attack it. (*iv*) The membranal proteinase does not degrade the regulatory subunit (R), released by cyclic AMP from R_2C_2, although R is quite susceptible to degradation by other proteolytic enzymes. None of these features of the membranal proteinase could be reproduced with trypsin, chymotrypsin, clostripain, or papain. The specific, restricted, and limited action of this membranal enzyme raises the possibility that it may have a distinct physiological assignment associated with the bioregulation of cyclic AMP-dependent protein kinase.**

Cyclic AMP (cAMP)-dependent protein kinase (cAMPdPKase; ATP:protein phosphotransferase, EC 2.7.1.37) (1) can be regarded as a major intracellular sensor of changes in cAMP levels, which take place in response to extracellular hormonal stimuli. As such, it is in charge of implementing the instructions given by a hormone to its target cell. This enzyme has been found in quite a few mammalian tissues (2) and is composed of two types of subunits, one being catalytically active (C) and the other having a regulatory function (R) (3, 4). These two subunits are assembled together to yield the inactive form of the enzyme, R_2C_2, which is now believed to be activated by cAMP according to the following equation (5–7):

$$R_2C_2 + 4\text{cAMP} \rightleftharpoons R_2(\text{cAMP})_4C_2 \rightleftharpoons R_2(\text{cAMP})_4 + 2C.$$

One of the intriguing questions regarding the physiological function of cAMPdPKase arises from the fact that this enzyme can phosphorylate various proteins, at least *in vitro* and probably even in intact cells (8). Therefore, it becomes imperative to elucidate the mechanism(s) by which the kinase activity may be channeled *in vivo* to bring about a specific phosphorylation at a given time and a given locus within the cell in response to a distinct metabolic signal. Some reports have suggested that different hormones that function through cAMP may trigger different chains of metabolic events within the same cell (9). This raises the possibility that there may be additional regulatory devices for modulating the cellular response to the hormonal stimulus.

cAMPdPKase activity in brush-border membranes (from the rat small intestine) vanishes within a few minutes upon addition of cAMP (10). The inactivation was shown to be due to the existence in these membranes of an enzyme that brings about a specific, limited degradation of the catalytic subunit of cAMPdPKase. This membranal enzyme did not attack (under the same conditions) other proteins in the membrane preparation or any one of six cytosolic proteins chosen at random (10).

This paper attempts to evaluate the possible physiological significance of this degradative inactivation by comparing it with the action of other proteolytic enzymes on cAMPdPKase and by demonstrating the strict recognition between the membranal proteinase and the native conformation of the free catalytic subunit of the kinase.

MATERIALS AND METHODS

Purification and Assay of cAMPdPKase. The undissociated R_2C_2 form of the enzyme (type I) and its free catalytic subunit (C) were obtained from rabbit skeletal muscle in a homogeneous form as described (11). The assays of the enzyme were based on the phosphorylation of histone H2b with [γ-^{32}P]ATP (12) with the modifications as described (13). The potential catalytic activity of the R_2C_2 form of the enzyme was measured after preincubation with cAMP (final concentration, 5 μM). The R_2C_2 preparations had specific activities of 2.8–4 units/mg, and those of C had specific activities of >5 units/mg, one unit of enzyme activity being defined as the amount of enzyme that catalyzes the transfer (at pH 6.5 and 30°C) of 1 μmol/min of [^{32}P] from [γ-^{32}P]ATP onto histone H2b.

Purification of the C-Subunit Proteinase. Brush-border microvillus membranes were prepared from small intestines of 6- to 8-wk-old male Wistar rats (Experimental Animal Unit, Weizmann Institute of Science) by the procedure of Schmitz *et al.* (14). These membranes were further purified by the method of Hopfer *et al.* (15) and then were partially solubilized by suspension in 0.1% Triton X-100 for 15 min at 4°C. After spinning down (60 min at 4000 × *g* and 4°C), the pellet was washed once with 50 mM Hepes (pH 7.5) and then was suspended in 50 mM Hepes, pH 7.5/0.9% octyl-β-D-glucopyranoside. This suspension was allowed to stand for 15 min at 4°C and then was recentrifuged to remove undissolved components. The supernatant contained the large majority of the C-subunit proteinase activity (>80%) in a soluble form.

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Abbreviations: cAMP, cyclic AMP; cAMPdPKase, cAMP-dependent protein kinase (type I); C and R, catalytic and regulatory subunits (respectively) of cAMPdPKase.

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Other Enzymes and Proteins. Trypsin, chymotrypsin, and papain were purchased from Worthington, clostripain was from Boehringer, and bovine serum albumin and soybean trypsin inhibitor were from Sigma. Histone H2b was prepared as described (16).

Chemicals. Octyl-β-D-glucopyranoside was obtained from Calbiochem and [γ-^{32}P]ATP (2.0–2.7 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was purchased from Radiochemical Centre, Amersham, England. All other chemicals were the best available grade from commercial sources.

Analytical Polyacrylamide Gradient Gel Electrophoresis in the Presence of Sodium Dodecyl Sulfate. The electrophoresis was carried out on thin-layer slabs with a 25 mM Tris glycine buffer (pH 8.6) containing 0.1% sodium dodecyl sulfate (17). Before application, the samples (dissolved in 25 mM Tris glycine, pH 6.7/10% (vol/vol) glycerol/0.01% bromphenol blue) were treated (5 min at 100°C) with 2% (wt/vol) sodium dodecyl sulfate and a reducing agent (0.75 M 2-mercaptoethanol). Electrophoresis was allowed to proceed for ≈2 hr at 15 V/cm in 10-cm slab gels of polyacrylamide (a linear gradient of 7–20%). Gels were stained with a 0.25% Coomassie blue solution in 50% (vol/vol) methanol/7% (vol/vol) acetic acid. Destaining was carried out with 7% acetic acid/5% methanol.

Protein Concentrations. Protein concentrations were determined by the method of Lowry *et al.* (18), with bovine serum albumin as a reference standard.

RESULTS AND DISCUSSION

The Membranal Proteinase Recognizes the Native Conformation of C. The inactivation of the free catalytic subunit of cAMPdPKase by the membranal proteinase isolated from the membranal brush border of the rat small intestine (10) can be regarded as a typical example of limited proteolysis (19). When free C M_r 40,000 was exposed to the membranal proteinase, it underwent a rapid, time-dependent degradation that yielded a clipped product (M_r 34,000) which (under the conditions of our experiment) did not undergo any further degradation (Fig. 1 *Left*). This process exhibited an unusual specificity: when free C was incubated for 5 min at 56°C (pH 7.5) to lose its catalytic activity (Fig. 2) and then was exposed to the membranal proteinase, no degradation occurred (Fig. 1 *Right*), which suggests

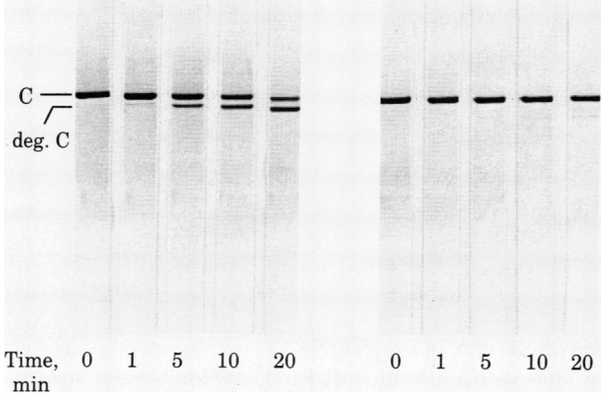

FIG. 1. Exposure of native free C and of heat-denatured C to the membranal proteinase. (*Left*) A reaction mixture composed of C (200 μg/ml) and the solubilized membranal proteinase (30 μg/ml) were incubated in 50 mM Hepes (pH 7.5) at 23°C. At the indicated times, 40-μl aliquots were removed and subjected to polyacrylamide gradient gel electrophoresis in the presence of sodium dodecyl sulfate. (*Right*) An identical experiment to that in *Left*, except that the C subunit was heat-denatured by preincubation for 5 min at 56°C in the Hepes buffer. deg. C, Degraded C.

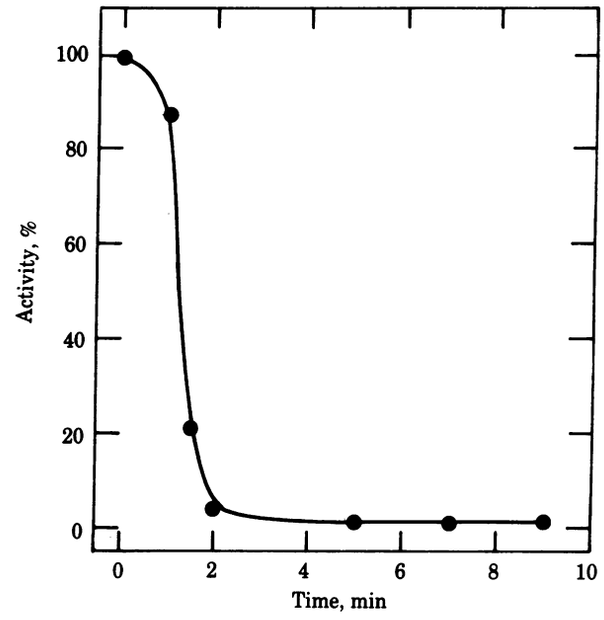

FIG. 2. Kinetics of the heat inactivation of C at 56°C. A solution of pure, free C (200 μg/ml in 50 mM Hepes, pH 7.5) was incubated at the above temperature. At the indicated times, aliquots (20 μl) were removed, diluted (1:10) into the same Hepes buffer (4°C), and assayed immediately for kinase activity. A control sample of C was allowed to stand at 23°C, and its activity (taken as 100%) was preserved (within 5%) during the experiment.

that the native conformation of C is essential for this degradation to occur.

The resistance of the heat-denatured form of C to proteolysis by the membranal enzyme is in marked contrast to the increased susceptibility to proteolysis commonly observed with denatured protein substrates. Furthermore, the heat denatured form of C readily underwent proteolysis with trypsin (Fig. 3 *Right*). In this case, a variety of degradation products were formed, as expected, because denaturation exposes a large number of trypsin-vulnerable peptide bonds (cf. Fig. 3 *Left* and *Right*).

The Degradative Inactivation Takes Place when C Is in its Free (Active) Form but not in the R_2C_2 (Inactive or "Stored") Form. The selectivity of action of the membranal proteinase is shown further by the experiments in Figs. 4*A* and 5*A*. When pure undissociated cAMPdPKase (in its R_2C_2 form) was incubated (pH 7.5, 23°C) with the solubilized membranal proteinase, it retained its potential catalytic activity (expressed upon

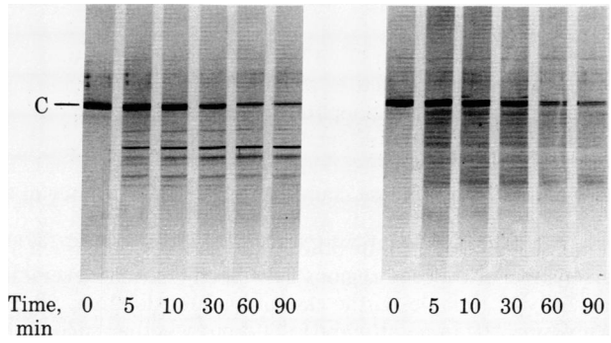

FIG. 3. Exposure of native, free C and of heat-denatured C to trypsin. This experiment was carried out as described (Fig. 1, legend) except for the fact that the membranal proteinase was replaced by trypsin (4 μg/ml).

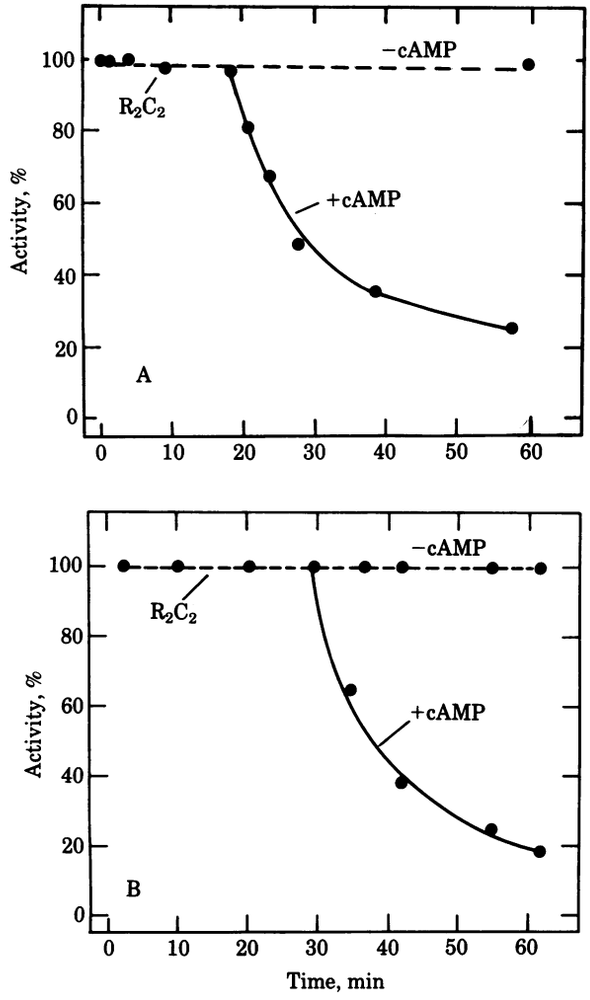

FIG. 4. Protection from loss of potential catalytic activity of the R_2C_2 form of cAMPdPKase when exposed to either the membranal proteinase (*A*) or trypsin (*B*). The reaction mixtures containing pure cAMPdPKase in its R_2C_2 form (final concentration, 100 μg/ml) and either solubilized membranal proteinase (final concentration, 15 μg/ml) or trypsin (final concentration, 3 μg/ml) were incubated at 23°C in 50 mM Hepes, pH 7.5/0.08% octyl-β-D-glucopyranoside. After 19 min (*A*) or 32 min (*B*), cAMP was added to part of each one of the reaction mixtures (final concentration, 5 μM). At the indicated times, aliquots were removed, diluted (1:10) into the same Hepes buffer (4°C), and assayed immediately for potential catalytic activity. The dashed lines represent the potential activity of the R_2C_2 form of the enzyme when exposed to the proteolytic enzymes in the absence of cAMP.

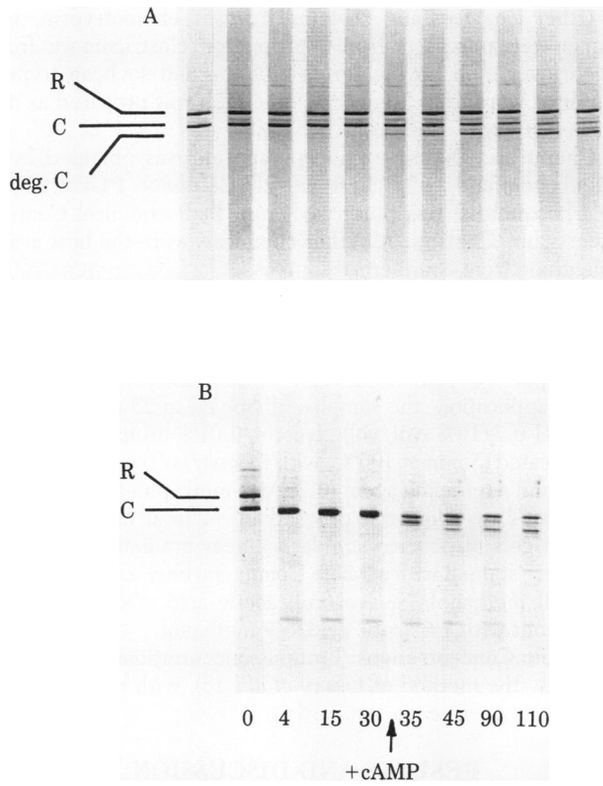

FIG. 5. A comparison of the course of degradation of R_2C_2 by the membranal proteinase (*A*) and by trypsin (*B*). The reaction mixtures and the addition of cAMP were as described (Fig. 4, legend). At the indicated times, aliquots (40 μl in all cases) were removed and subjected to electrophoresis. deg. C, Degraded C.

assay in the presence of cAMP) and as its molecular integrity. However, upon addition of cAMP to the incubation mixture, the kinase was inactivated in a time-dependent process (Fig. 4*A*) with concomitant degradation of the C subunit (Fig. 5*A*) but not (under the same conditions) of the regulatory protein R.

In principle, the restricted and limited degradation of C by the membranal proteinase could be due to the existence of an exposed region in C that is readily available to attack by this membranal enzyme and by other proteinases as well. Such hypersensitive or exposed regions have been found in several instances—for example, in the case of immunoglobulins (20, 21) or myosin (22). In an attempt to find out whether such is the case here, we tried to reproduce the restricted degradation of C with proteolytic enzymes (namely, trypsin, chymotrypsin, clostripain, and papain). The following observations were made. (*i*) None of these proteinases degraded C in the specific, restricted, and limited manner observed with the membranal proteinase (Fig. 1 *Left*). (*ii*) All of these proteinases degraded the R_2C_2 form of cAMPdPKase, whereas the membranal proteinase did not. Even in the case of trypsin, where R protected C from inactivation (Fig. 4*B*), the molecular integrity of R_2C_2 was not preserved (Fig. 5*B*). (*iii*) All of these proteinases attacked the R subunit both when bound in R_2C_2 and when in its free form (after dissociation with cAMP†), whereas the membranal proteinase did not degrade R under these conditions (Fig. 5 *A* and *B*).

Possible Physiological Implications. The physiological significance of the specific inactivation of C by the membranal proteinase is not clear yet. In principle, it might be the first step initiating its digestion (25, 26), or it might constitute a safety device to prevent phosphorylation of proteins by this kinase when and where such phosphorylation may have undesired consequences. Alternatively, the specific degradation of C might be a means of transferring a unique physiological stimulus if, for example, one or more of the degradation products will be found to possess an important function (catalysis, inhibition, activation, etc). In any case, the fact that the membranal enzyme exhibits such a specific, restricted, and limited action on C, the fact that the biodegradative inactivation takes place when C is in its free form but not in its R_2C_2 (inactive or "stored") form, the fact that it degrades C only when it is in its native conformation, and the fact that the proteinase is found in a membrane whose function (fluid and electrolyte secretion) is known to be

† The observation that free R is susceptible to degradation by a variety of proteinases is in agreement with reports from other laboratories (23, 24).

definitely affected by cAMP (27–29) make it quite probable that the remarkable recognition between the enzyme described here and the free catalytic subunit of cAMPdPKase is not fortuitous but indicates a distinct physiological (possibly regulatory) assignment. A most interesting possibility will arise, of course, if the degraded form of C will be found to translocate within the cell or to possess a new (yet unknown) catalytic activity in response to interaction with either a modulator (cf. ref. 30) or an intracellular metabolite. The specific degradation described above may then constitute the means by which the cell diverts the action of cAMPdPKase from one assignment to another.

We wish to congratulate Professor Helmut Holzer on his 60th birthday. This work constitutes part of a Ph.D. thesis (E.A.) submitted to the Weinberg Graduate School of the Weizmann Institute of Science. S.S. is the incumbent of the Hella and Derrick Kleeman Chair in Biochemistry.

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