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LIMITED PROTEOLYSIS OF THE CATALYTIC SUBUNIT OF cAMP-DEPENDENT PROTEIN KINASE - A MEMBRANAL REGULATORY DEVICE?

Eytan Alhanaty[§] and Shmuel Shaltiel*

Department of Chemical Immunology, The Weizmann Institute of Science,

Rehovot, Israel

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SUMMARY

Brush border membranes isolated from rat small intestine were found to possess a cAMP-dependent protein kinase activity. Upon addition of cAMP, a rapid, time-dependent inactivation of this enzyme occurs, which was found to be due to a proteolytic activity identified in the membranes. This activity could not be assigned to previously known brush border proteases. The inactivation and the proteolytic degradation of the kinase could be reproduced also with the pure catalytic subunit of cAMP-dependent protein kinase (C) from rabbit skeletal muscle (M.W. 40000) which was cleaved by the membranal proteolytic activity with concomitant quantitative appearance of a degradation product (M.W. 30000) devoid of kinase activity. The membranal proteolytic activity appears to be specific for C since: (1) it does not degrade the other endogenous proteins in the membrane preparation; (2) it does not degrade any of six arbitrarily chosen proteins from other sources; (3) it catalyzes a limited proteolysis of C which could not be simulated by other proteolytic enzymes such as trypsin, clostripain, chymotrypsin and papain. The attack of C by the membranal protease is blocked by the presence of the nucleotide substrate of the kinase (MgATP). In addition, the undissociated and inactive form of the enzyme (R_2C_2) does not lose its potential enzymatic activity, and neither its catalytic nor its regulatory subunits are digested by the protease. The specific, restricted and limited action of the protease, together with the prevention of its action by the substrate and the regulatory protein (R) of the kinase raise the possibility that the membranal protease may have a distinct physiological (possibly regulatory) assignment.

It is now commonly accepted that proteolytic enzymes play a key role in a large variety of physiological functions, some of which are of a regulatory nature [1,2]. For example, many enzymes and hormones are synthetized in a precursor (inactive) form, which is then prompted into action by specific and limited proteolysis in response to the appropriate biological signal. Proteolysis is thus linked with digestion, blood coagulation, fibrinolysis, the release of peptide hormones, lactation, metamorphosis, fertilization, yeast sporulation, etc. [1,2].

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* To whom correspondence should be addressed.

Abbreviations used are: cAMP, adenosine 3':5'-cyclic monophosphate; cAMPdPK, cAMP-dependent protein kinase; C, catalytic subunit of cAMPdPK; R, regulatory subunit of cAMPdPK; R_2C_2, undissociated and inactive form of cAMPdPK; PKI, cAMPdPK inhibitor protein.

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Another basic mechanism of bioregulation involves the metabolic interconversion of enzymes [3,4] in which an enzyme becomes locked in an active or inactive conformation, as a result of an attachment (or detachment) of a small, covalently bound functional group. This reversible covalent modification (e.g. phosphorylation) is catalyzed by regulatory enzymes (e.g. kinases and phosphatases) specially assigned for this task. The best known regulatory enzyme of this type is cAMP-dependent protein kinase (cAMPdPK). This kinase (EC.2.7.1.37), found essentially in all mammalian tissues [5], is composed of two types of subunits, one being catalytically active (C) and the other (R) having a regulatory function. The inhibited form of the enzyme (R_2C_2) is activated by cAMP according to the following equation [6,7]:

$$R_2C_2 + 2\ cAMP \rightleftharpoons R_2(cAMP)_2 + 2C$$

and the released free catalytic subunit (C) catalyzes the phosphorylation of enzymes (e.g. glycogen phosphorylase kinase or glycogen synthetase) and thus modulates their activity.

While attempting to purify and study a membranal cAMPdPK from rat intestinal brush border, we found that such membranal preparations exhibit a proteolytic activity which specifically clips and inactivates the free catalytic subunit of this kinase. This paper characterizes this proteolytic activity and presents evidence suggesting that free C is its physiological target. The possible implications of these findings with regard to the control of phosphorylation in brush border membranes is discussed.

MATERIALS AND METHODS

Isolation and Purification of Brush Border Membranes: Brush border microvillus membranes were prepared from small intestines of 6-8 week old male Wistar rats (The Experimental Animal Unit of the Weizmann Institute of Science, Rehovot), following the procedure described by Schmitz et al. [8]. These membranes were further purified by the method of Hopfer et al. [9] and then suspended in a buffer composed of Hepes (50 mM), pH 7.5, to yield a preparation containing 1.5 - 2 mg protein/ml.

Solubilization and Further Purification of the C Subunit Protease: Isolated brush border membranes were partially solubilized by suspension in 0.1% Triton X-100 for 15 min at 4° and then spinning down (60 min at 40000 x g, 4°). The pellet was washed once with 50 mM Hepes (pH 7.5) and then suspended in a buffer containing Hepes (50 mM), pH 7.5 and octyl-β-D-glucopyranoside (0.83%). After standing for 15 min at 4° the suspension was recentrifuged as above, to remove undissolved components. The supernatant thus obtained contained the large majority of the C subunit protease activity (>80%) in a soluble and purified form (purification factor x 10).

Purification and Assay of cAMPdPK (Type I) and of its Free C Subunit: R_2C_2 as well as C were purified from rabbit muscle following the procedure described by Beavo et al. [10]. The assay was based on the phosphorylation of histone H2b with [γ-^{32}P]ATP [11] with the modifications described previously [12]. The potential catalytic activity of the undissociated and inactive form of the enzyme (R_2C_2) was measured after preincubation in the presence of 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 which catalyzes the transfer (at pH 6.5, 30°)of 1µmol of [^{32}P] from [γ^{32}P] ATP onto histone H2b, per min.

Preparation of cAMP-dependent Kinase Inhibitor Protein: This inhibitor was prepared following the procedure described by Walsh et al. [13] up to and including the DEAE-cellulose chromatography step.

Analytical Polyacrylamide Gel Electrophoresis in the Presence of Sodium Dodecylsulfate: The electrophoresis was carried out on thin layer slabs using a 25 mM tris-glycine buffer (pH 8.6) containing 0.1% sodium dodecylsulfate [14]. Before application, the samples (dissolved in 25 mM tris-glycine, pH 6.7, 10% glycerol and 0.01% bromphenol blue) were treated (5 min, 100°) with 2% sodium dodecylsulfate 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 to 20%). Gels were stained with 0.25% Coomassie blue solution in 50% methanol and 7% acetic acid. Destaining was performed with a solvent containing acetic acid (7%) and methanol (5%). The gels were scanned at 560 nm with a Gilford 2410-S spectrophotometer and the relative areas of the various densitometric peaks were determined by cutting out each peak and weighing it.

Protein concentrations: Protein concentrations were determined by the method of Lowry et al. [15] with bovine serum albumin as a reference standard.

RESULTS AND DISCUSSION

Brush border membranes purified from rat small intestines by procedures described in the literature [8,9] possess a cAMP-dependent as well as a cAMP-independent kinase activity. As seen in Fig. 1A, upon preincubation of the membrane preparation with cAMP, the cAMP-dependent activity rapidly vanishes in a time-dependent process,which does not affect the non-dependent kinase activity. This is indicated by the fact that the time-dependent inactivation triggered by preincubation with cAMP levels off at a basal value very similar to the kinase activity remaining after inhibition by PKI - a specific cAMPdPK inhibitor characterized by Walsh et al. [13].

In an attempt to find out whether the role played by cAMP in this process is that of triggering directly an inactivating factor (e.g. an enzyme) or merely dissociating the R_2C_2 form of the kinase to release free C, which only as such is susceptible to inactivation, we tried to reproduce a similar time - dependent inactivation with pure exogenous C (from rabbit muscle). Indeed, the preparation of brush border membranes does inactivate exogenous pure C without addition of any cAMP whatsoever (Fig. 1B), strongly suggesting that the function of cAMP is to release free C into the system. At the same time, the experiments depicted in Fig. 1 show that the inactivation of the kinase found initially in the membrane preparation is not a unique property characteristic of the membrane-bound kinase, as it occurs also with the pure C subunit of cytosolic cAMPdPK.

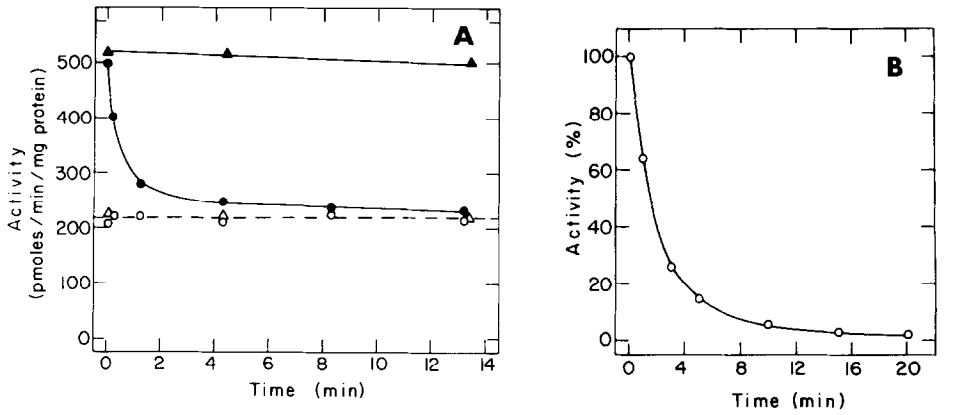

FIGURE 1: (A) Time-dependent inactivation of membrane bound cAMPdPK induced by cAMP. Brush border membranes (protein content 0.3 mg/ml) were suspended in 50 mM Hepes (pH 7.5) containing 0.1% Triton X100 and allowed to stand for 15 min at 4°. At time zero, two samples from this suspension were removed and transferred to 23°. One of these samples was preincubated as such and the other was preincubated with cAMP (final concentration 10 µM). At the indicated times, aliquots (40 µl) were removed from each sample, and assayed either in the presence or in the absence of PKI (20 µg per assay mixture (200 µl)). All assays were carried out in the presence of 5 µM cAMP. -▲-, preincubation without cAMP and assay without PKI; --Δ--, preincubation without cAMP and assay in the presence of PKI; -o-, preincubation with cAMP and assay without PKI; --●--, preincubation with cAMP and assay in the presence of PKI.

(B) Time-dependent inactivation of rabbit muscle pure C by brush border membranes from rat small intestines. A reaction mixture containing pure C subunit (final concentration 20 µg/ml) and brush border membranes (final protein concentration 0.5 mg/ml) in 50 mM Hepes, pH 7.5 was incubated at 23°. At the times indicated, aliquots (20 µl) were removed and assayed immediately for protein kinase activity (no cAMP added).

The time-dependent inactivation of pure C seems to be associated with a specific proteolytic activity in the membranal preparation. When aliquots were removed from the incubation mixture and subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate it was found (Fig. 2, ①) that the brush border membrane preparation degrades the C subunit protein (M.W. 40000) with concomitant, quantitative appearance of a clipped, inactive protein (M.W. ∿ 30000). Under the conditions of the experiment depicted in Fig. 2, no intermediary degradation products could be detected and the clipped product was not degraded any further (cf. also Fig. 4). It was also observed that while the added free C is clipped by the membranal protease, the other endogenous proteins in the preparation (marked I to XI in Fig. 2, ①) are not degraded at the same time, as indicated by the fact that these protein bands in the gel preserve the area of their densitometric peaks (Fig. 2, ② and ③).

It could be argued that the lack of proteolytic degradation in the endogenous proteins of the membrane might be due to a special resistance of these

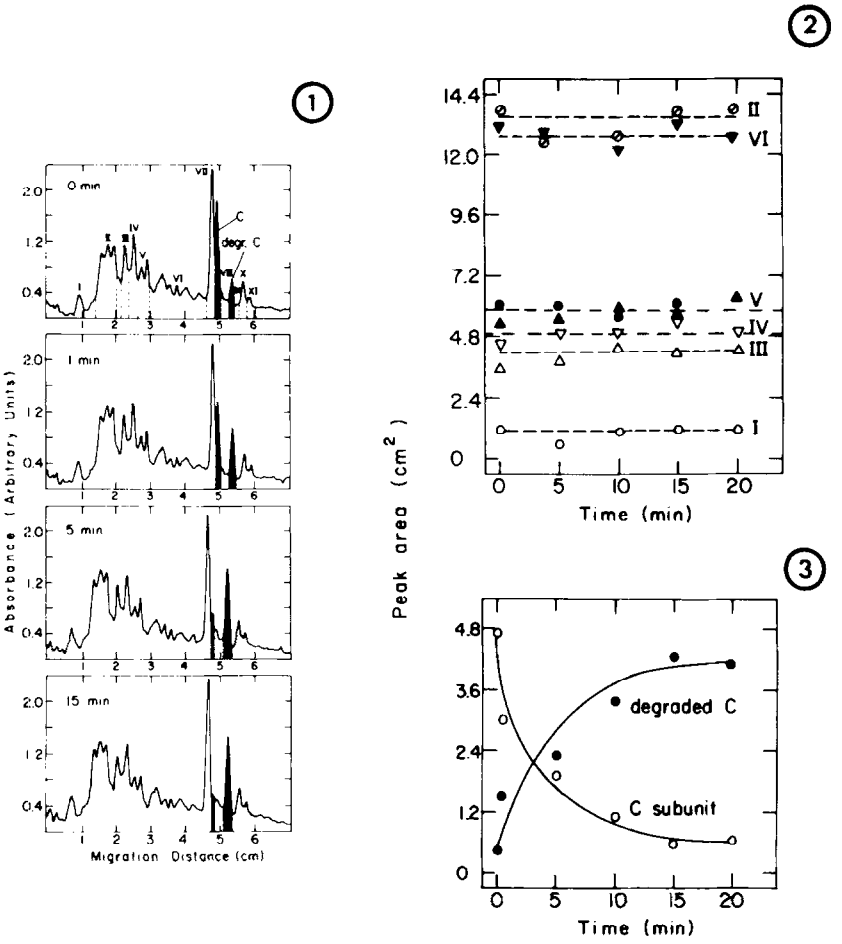

FIGURE 2: Time-dependent specific degradation of exogenous C by brush border membranes, as illustrated by gel electrophoresis in the presence of sodium dodecylsulfate, densitometric scanning of the gels, and monitoring the areas of the peaks. A reaction mixture containing pure rabbit muscle C subunit (final concentration 100 µg/ml) and brush border membranes (final protein concentration 0.5 mg/ml) in 50 mM Hepes, pH 7.5, was incubated at 23°. At the times indicated, aliquots (30 µl) were removed and subjected to electrophoresis as described under Materials and Methods.

Panel (1) : Densitometric scans of the samples removed at the indicated times. The peaks of the C subunit (M.W. 40000) and of its degradation product (M.W. ∿ 30000) are shaded for emphasis. All scans were carried out under identical settings of the instrument.

Panel (2) : The area of the various peaks marked I to XI in panel (1) as a function of time. These areas are obtained from densitometric scans of the type shown in panel (1) and are associated with endogenous protein components in the membrane preparation.

Panel (3) : Time-dependent disappearance of C (M.W. 40000) and concomitant formation of degraded C (M.W. 30000). Peak areas obtained from the scans described in panel (1) and two additional scans taken at 10 min and 20 min.

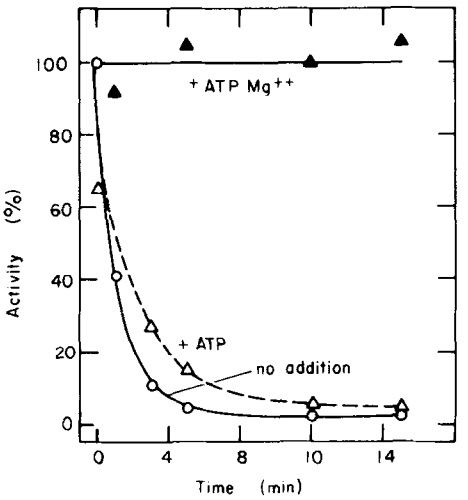

FIGURE 3: Effect of MgATP on the time-dependent inactivation of rabbit muscle pure C by brush border membranes. Three samples of C (40 µg/ml, in 50 mM Hepes, pH 7.5) were incubated (5 min, 23°) as such (o), in the presence of 1 mM ATP (Δ), or in the presence of 1 mM ATP and 10 mM $Mg(CH_3COO)_2$ (▲). At time zero, each of these samples was mixed (1:1) with a sample of brush border membranes (protein content 1 mg/ml) prepared as described under Materials and Methods. Aliquots (20 µl) were removed from each reaction mixture at the indicated times and assayed for protein kinase activity.

proteins, essential for their co-existence with the protease in the membrane, or that these proteins represent resistant "cores" of proteins already degraded in the course of purification. However,this membranal protease also failed to digest (under the same conditions) any of six arbitrarily chosen proteins from different sources. These exogenous proteins included: glycogen phosphorylase b (from rabbit muscle), bovine serum albumin, human hemoglobin, myoglobin (from whale skeletal muscle), ovalbumin (from chicken egg) and histone H2b (from calf thymus). Furthermore, the restricted and limited action of the membranal protease could not be simulated by other proteolytic enzymes such as clostripain, trypsin, chymotrypsin or papain (Alhanaty, Patinkin and Shaltiel, to be published).

The inactivation of the C subunit by the brush border membranal protease is blocked in the presence of MgATP, the nucleotide substrate of the kinase (Fig. 3). This complete protection from inactivation and from degradation could not be achieved by either the nucleotide or the metal ion alone,but was very effective when both were present.

The substrate-induced protection of the kinase from degradative inactivation could be due either to the fact that the protease attacks a site on the kinase which is shielded by MgATP, or to the fact that upon binding, this

substrate brings about a conformational change of the kinase which tightens its structure and buries an otherwise exposed site of attack of the protease [16,17]. Several cases of substrate- or cofactor-induced resistance to proteolytic degradation are known in the literature [18].

The C-specific proteolytic activity could not be attributed to other well known proteases which are found in brush border preparations. This specific protease could be separated, for example, from aminopeptidase [19,20] and from enterokinase [21] since these two activities were solubilized (> 80%) with 0.1% Triton, while the C-specific protease (as well as the membranal marker alkaline phosphatase [22]) was not (> 80% of these two activities were found in the pellet). In this connection it should be noted that a similar C-specific proteolytic activity was also found in renal brush border membranes (from rat) prepared by the same procedure [8,9]. This finding is not surprising in view of the similarity between the brush border membranes from these two sources with respect to their histological appearance, adsorption function and enzyme activities [19,23].

Solubilization of the C-specific membranal protease could be achieved with octyl-β-D-glucopyranoside (0.83%). With this purified preparation the selectivity of the protease was further illustrated in the experiment depicted in Fig. 4. Pure undissociated cAMPdPK in its R_2C_2 form was incubated with the solubilized protease preparation. The kinase retained its potential catalytic activity† as well as its molecular integrity. However, upon addition of cAMP to the incubation mixture the enzyme becomes readily inactivated (-●-, Fig. 4A) with concomitant limited proteolysis of the C subunit but not of the regulatory protein (Fig. 4A and B). This preferential degradation of C over R and R_2C_2 emphasizes the selectivity of action of the membranal protease, since in general the R subunits of cAMPdPK are known to be unusually susceptible to proteolytic attack by other proteases [24].

The protease described here may provide a very selective tool for the preparation of a well-defined and homogeneous fragment of the catalytic subunit of a key enzyme. Such fragments are very useful in structure-function studies similar to the classical experiments carried out with ribonuclease [25]. Moreover, in view of the fact that the protease does not seem to inactivate the non-dependent protein kinase(s) in the system (cf. Fig. 1) it might also be used as an additional criterion (like the Walsh-Krebs inhibitor [13]) for discriminating between cAMP-independent kinases and free C subunits of cAMPdPK.

The physiological significance of the specific inactivation of C through proteolysis is not clear yet. In principle it might be the first step initiating

† expressed when the assay is carried out in the presence of cAMP

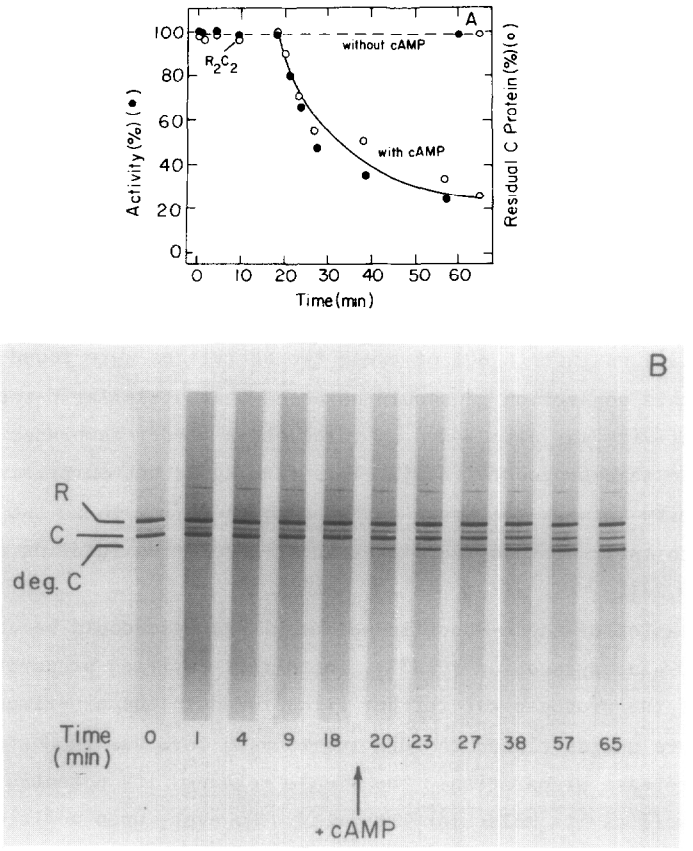

FIGURE 4: Illustration of (A) the protection from proteolysis afforded by the regulatory protein (R_2) to the C subunits of cAMPdPK and (B) The remarkable susceptibility to degradation exhibited by C compared with the resistance to proteolysis exhibited (under the same conditions) by R. A reaction mixture containing pure rabbit muscle cAMPdPK in its R_2C_2 form (final concentration 100 µg/ml) and purified soluble C subunit protease (final protein conc. 15 µg/ml) was incubated at 23° in a buffer containing 50 mM Hepes, pH 7.5, and 0.083% octyl-β-D glucopyranoside. After 19 min, two samples were removed. One of these was further incubated as such (----) and cAMP (final concentration 5 µM) was added to the other (——). Aliquots were removed at the indicated times and assayed (• in panel A) or subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (panel B). The percentage of residual C subunit protein (o in panel A) was determined from densitometric scanning of the gels illustrated in panel B.

its biodegradation [26,27] or constitute a safety device to prevent phosphorylation of proteins where and when 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 formed will be found to possess an important function (inhibition, activation, amplification, etc.). In any case, the fact that the

membranal protease exhibits such a specific, restricted and limited action on C, the fact that the biodegradative inactivation takes place when C is in its free (active) form but not in its R_2C_2 (inactive or "stored") form, the fact that the substrate MgATP protects C from inactivation, and finally the fact that the protease is found in a membrane whose function (fluid and electrolyte secretion) is known to be definitely affected by cAMP [28-30], make it quite probable that the biorecognition between the protease described here and the free catalytic subunit of cAMPdPK is not fortuitous but indicates a distinct physiological (possibly regulatory) assignment of this protease.

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