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Evidence of a yeast proteinase specific for elongation factor 2

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Two proteinases active on elongation factor 2 have been found in yeast. The former hydrolyzes the factor producing a single ADP-ribosylatable fragment, whereas it does not produce any fragment when incubated with different proteins. The latter, less specific, is active in cleaving both EF-2 and other proteins giving rise to a noticeable number of fragments. Moreover, when native EF-2 is incubated with the most specific of the two proteinases, the amount of the ADP-ribosylatable fragment increases with time, while no fragments are evident when ADP-ribosylation of EF-2 comes before its incubation with the proteolytic enzyme. A possible regulatory role of this proteinase on EF-2 turnover is hypothesized.

Elongation factor; Proteinase

1. INTRODUCTION

The elongation factor 2 (EF-2) promotes the shift of the ribosome of one codon along the messenger RNA in the course of protein biosynthesis in eukaryotic cells. Although it has the same function as the prokaryotic EF-G, it displays some fundamental differences. In fact, the EF-G activity is regulated only by the GTP/GDP ratio in the cell, whereas the EF-2 activity can also be affected by an endogenous enzyme which catalyzes, like the diphtheria toxin fragment A, the ADP-ribosylation of the factor [1–3].

In a preceding paper [4] we showed that EF-2 is subjected to an endogenous proteolysis in all the organisms examined. As far as the yeast is concerned, we also found that the EF-2 proteolysis is dependent on the growing phase of the cells. In this paper we report experimental evidence that this proteolysis is specific for EF-2. A possible regulatory role for EF-2 cleavage is discussed.

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2. MATERIALS AND METHODS

Partially purified yeast proteinase was obtained as follows: yeast cells (*Saccharomyces carlsbergensis*) were grown in a Sabouraud-glucose medium (Serva) at early exponential phase (A_{660} = 0.3–0.5). The cells from 2 l culture were harvested, washed twice with cold buffer (20 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM EDTA, 5 mM mercaptoethanol) and then disrupted by grinding them with twice their weight of acid-washed glass beads (Sigma) in the same buffer. After centrifugation at 30000 × *g* for 30 min, the supernatant was loaded on a Sephadex G-25 column equilibrated with the same buffer at 4°C and the eluted bulk of proteins stored at −80°C in 500 μl portions until used.

500 μl of yeast extract prepared as above were injected in a Mono Q column of an FPLC system (Pharmacia). The column was equilibrated with 20 mM Tris-HCl, pH 7.5; the elution was made in the same buffer with a linear gradient from 0 to 350 mM KCl in 10 min at a constant flow rate of 1 ml/min. 200 μl fractions were collected.

The active fractions were pooled, concentrated at 500 μl using a Centricon tube (Amicon) with a molecular mass cutoff of 30000 Da and loaded on a Superose 12 HR 16/50 (Pharmacia) gel-filtration column equilibrated in 20 mM Tris-HCl, pH 7.5, 100 mM KCl. The column was previously calibrated for molecular mass determination by using alcohol dehydrogenase, bovine serum albumin, ovalbumin, carbonic anhydrase and myoglobin as standards. 500 μl fractions were collected.

EF-2 was purified from yeast as previously described [4].

The mixture for testing proteinase activity (50 μl) contained: 20 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM EDTA, 5 mM mercaptoethanol, 10 μM phenylmethylsulfonyl fluoride,

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25 pmol EF-2 and 25 μl proteinase solution. The incubation was at 37°C for 30 min unless otherwise specified. After incubation 1 μg of diphtheria toxin fragment A and 30000 dpm of nicotinamide [U-^{14}C]adenine dinucleotide (NEN) were added to the mixture and the incubation was protracted for another 10 min at the same temperature. The reaction was stopped by adding SDS sample buffer and the slab-gel electrophoresis in denaturing conditions (SDS-PAGE) was performed according to Laemmli [5] on a 12.5% gel. The gel was treated for fluorography according to Bonner and Laskey [6].

3. RESULTS

Recently [4] we have reported the finding of a proteinase acting on EF-2 in various eukaryotic sources. In particular, in the case of the yeast cells, we also showed that this proteolysis is related to the growing phase: i.e., the amount of the native factor is more than 95% in the early-exponential phase, but its abundance decreases in the mid-exponential phase and becomes negligible in the resting cells. As a consequence of the EF-2 decrease, the production of ADP-ribosylatable fragments was observed. This evidence led us to suppose the existence of a specific proteinase acting on EF-2.

With the aim to investigate the existence of such a specific enzyme, the yeast cells were grown in early-exponential phase, then the soluble fraction obtained after cell disruption was subjected to the chromatographic steps described in section 2. In fig.1, the elution pattern of an exponentially grown cell extract, obtained on a Mono Q column in an FPLC chromatographic system, is reported. The fractions were assayed for their ability to promote EF-2 proteolysis using purified EF-2 as a substrate. In fig.2, an SDS-PAGE fluorography of the fractions constituting the dashed peaks (see fig.1) incubated with purified EF-2 and then ADP-ribosylated is shown. The fluorography exhibited at least two proteinase activities. In fact fractions 1–5 showed the same fragmentation pattern, differing only in the relative abundance of a single fragment, due to the increasing concentration of the proteinase. Starting from the sixth fraction, the EF-2 fragmentation pattern changed, showing a complex fragmentation due to a new proteinase activity which partially overlapped with the first one.

The two fractions (4 and 7) showing different behaviour in the EF-2 fragmentation were tested for their ability to cleave proteins different from EF-2. The following proteins were tested:

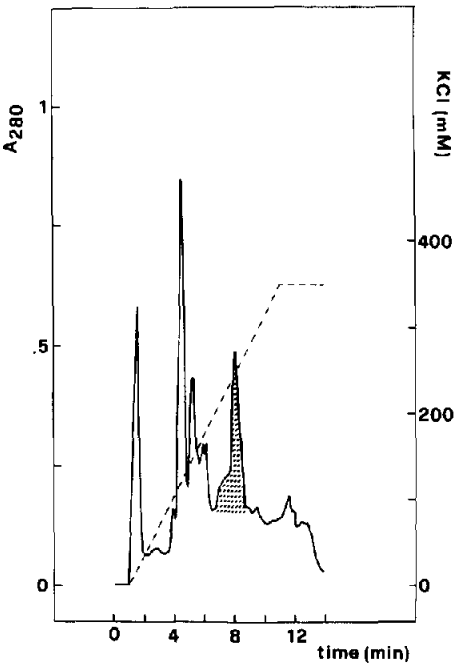

Fig.1. FPLC chromatography of yeast extract on a Mono Q column. The elution was performed with a linear salt gradient in 10 min. Solvent A: 20 mM Tris-HCl, pH 7.5. Solvent B: A + 350 mM KCl. Flow rate: 1 ml/min. The fractions containing proteinase activity were those constituting the dashed peak.

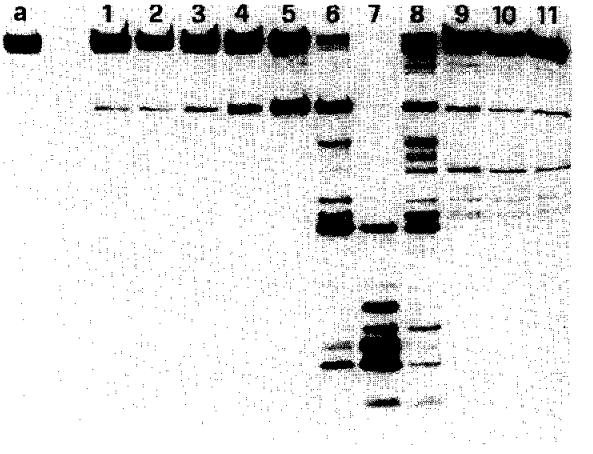

Fig.2. SDS-PAGE fluorography of the mixtures obtained by incubating the fractions constituting the dashed peak of fig.1 with 25 pmol of purified EF-2. The EF-2 fragments were labeled by ADP-ribosylation using diphtheria toxin and [^{14}C]NAD as described in section 2. Lane a: Purified EF-2 incubated 30 min at 37°C and then ADP-ribosylated. Lanes 1–11: Purified EF-2 incubated with 25 μl of each fraction constituting the dashed peak of fig.1 for 30 min at 37°C and then ADP-ribosylated.

phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, myoglobin and cytochrome *c*. In all the experiments performed, fragmentation occurred when the proteins were incubated with fraction 7, whereas no fragments were evident when fraction 4 was used. However, the experiment was performed using the Coomassie staining technique which does not possess a high sensitivity. To improve the assay sensitivity we used [^{14}C]methylated bovine serum albumin (Amersham) with a specific activity of 42 μCi/mg. In fig.3 the SDS-PAGE fluorography of the labeled BSA incubated with fraction 4 (lane a) and with fraction 7 (lane b) is shown. In lane a no proteolytic effect is detected, whereas in lane b a noticeable BSA fragmentation appears.

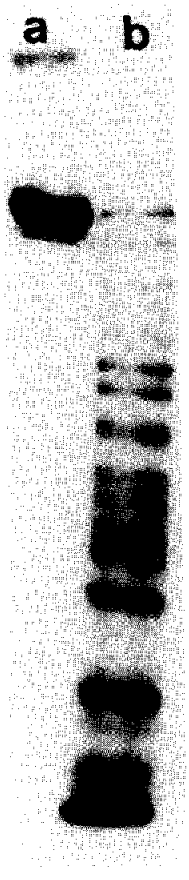

Fig.3. SDS-PAGE fluorography of the mixtures obtained by incubating [^{14}C]methylated BSA with the fractions 4 and 7 obtained by Mono Q chromatography. Radioactive BSA was incubated with 25 μl of the fraction 4 (lane a) and fraction 7 (lane b) for 30 min at 37°C.

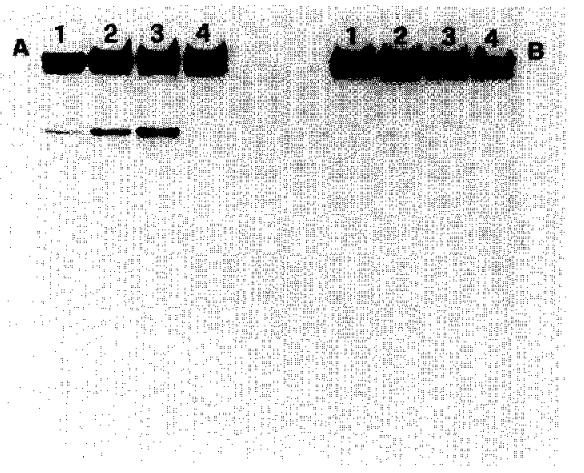

Fig.4. SDS-PAGE fluorography of the mixture obtained by incubating the proteinase purified by gel-filtration with EF-2. (A) The same amount (25 μl) of proteinase solution was added to EF-2 and the mixture was incubated for 10 min (lane 1), 20 min (lane 2) and 30 min (lane 3) at 37°C, followed by 10 min of ADP-ribosylation reaction with [^{14}C]NAD. The same amount of EF-2 was incubated for 30 min without proteinase and then ADP-ribosylated (lane 4). (B) EF-2 was ADP-ribosylated for 10 min with [^{14}C]NAD and then incubated with 25 μl of proteinase solution for 10 min (lane 1), 20 min (lane 2) and 30 min (lane 3) at 37°C. The same amount of EF-2 was ADP-ribosylated and then incubated for 30 min in the absence of proteinase (lane 4).

Fractions 1–4 were pooled, concentrated at 500 μl and loaded on a Superose 12 column. From the calibration of this column we estimated a molecular mass of about 60 kDa. Since this proteinase activity is rather specific toward EF-2, we investigated (fig.4) its capacity to discriminate between native (panel A) and ADP-ribosylated EF-2 (panel B). Using native EF-2 as a substrate proteolysis, increasing with time, is shown to occur. No fragmentation is evident when an ADP-ribosylated factor is used in the mixture incubated for the same time.

4. DISCUSSION

In prokaryotic cells the production of newly synthesized proteins is regulated at the initiation level and by the amount of mRNAs to be translated. No evidence of other particular regulation mechanisms in protein biosynthesis has been provided so far. Conversely, in eukaryotic systems, many

reports lead one to believe that some additional regulatory action may be exerted on the elongation cycle at translocation level. In fact, EF-2 seems subjected to at least two systems of regulation, both of which depend on different post-translational modifications. One of these modifications is related to a histidine residue of EF-2 that is transformed in diphthamide by a pathway involving five enzymes [7], the other is represented by the phosphorylation [8–10]. Considering that no difference in the EF-2 activity was found when the histidine is transformed to diphthamide [11,12], the post-translational modification, represented by diphthamide residue, could be considered as a regulation site acting as a target for the endogenous ADP-ribosylation which leads to the factor inactivation.

In this paper we report evidence that in yeast cells a proteinase which is specific for EF-2 is present. The specificity is demonstrated by the finding that the EF-2 proteolysis produces a single ADP-ribosylatable fragment from EF-2 and, when it is assayed on proteins different from EF-2, none of them were found to be cleaved.

In the last few years, a growing number of studies on the yeast have reported on the existence of proteinases not located in vacuoles. A regulatory role on the cellular activity of these proteinases [13–15] has been postulated. In our case, the existence of specific proteolysis of EF-2 could be considered another way to control the protein biosynthesis, or even a mechanism acting synergistically with the ADP-ribosylation and/or the phosphorylation. This hypothesis, at least as far as the ADP-ribosylation is concerned, is supported by the observation that the ADP-ribosylated EF-2 seems to be more resistant to the proteinase activity than the native factor. In fact, when native EF-2 is incubated with the proteinase, its fragmentation increases with time, while no fragments are evident when in the incubation mixture the native factor is substituted by the ADP-ribosylated one. A similar observation was also reported by Bodley [16], who found that ADP-ribosylated EF-2 is more resistant, with respect to the native factor, to trypsinolysis. Therefore, considering that the amount of EF-2 depends on the growing phase of the cell [4], the endogenous ADP-ribosylation could be a way of saving the factor from the action of this specific proteinase when the cell decreases its biosynthetic activity. Such a mechanism, indeed, requires, for the occurrence, the regeneration of native factor by an ADP-deribosylating enzyme whose activity has not yet been demonstrated.

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