Processing of yeast exoglucanase (β-glucosidase) in a KEX2-dependent manner

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Received 19 March 1990; revised version received 15 May 1990

We have detected proteolytic processing of a form of exoglucanase representative of the endoplasmic reticulum (form A). This processing did not take place when form A was obtained from protoplasts lysed in the presence of either EDTA or leupeptin, two wel-characterized inhibitors of KEX2 endoprotease from *Saccharomyces cerevisiae*. Sequencing of the amino terminus of an A-like form of enzyme secreted by a kex2 mutant indicated the presence of 4 amino acids, with a pair of basic residues (Lys-Arg) at their carboxyl side, preceding the amino terminus of the wild-type external exoglucanase.

Exoglucanase (β-glucosidase); Proteolytic processing; Secretory pathway; Posttranslational modification

1. INTRODUCTION

Maturation of many eukaryotic glycoproteins involves processing of both carbohydrate and protein moieties. Final carbohydrate composition confers important physical as well as biological properties to the molecules [1]. On the other hand, proteolytic processing has been related to physiological regulation of many important processes [2].

Proteolytic processing of precursors is well documented in yeast. Thus, many zymogenic precursors of vacuolar proteases are converted to the final active form by PEP4 protease, or protease A [3,4]. However, the best-studied examples of proteolytic processing in yeast concern the maturation of the glycoprotein precursors of α-factor and killer toxin [5]. Central to the maturation of both precursors is the product of the KEX2 gene, an endoprotease located somewhere in the Golgi body [6,7]. The KEX2 protease cleaves on the carboxyl end of dibasic residues with the sequence X-Lys-Arg [8]. In addition to these naturally synthesized yeast products, the KEX2 protein also processed an amyloglucosidase from *Aspergillus* and pre-α-factor-somatostatin hybrids cloned in yeast [9,10].

Saccharomyces cerevisiae secretes two exoglucanases, which share a common protein portion but differ in their carbohydrate moieties [11,12]. The major isoenzyme (exoglucanase II) contains two short N-linked oligosaccharides [13] whereas the minor one (exoglucanase I) adds an outer chain to at least one residue [12]. In a previous report [14] we have described the presence of two ionic representatives of exoglucanase II during its transport to the cell surface. At that time it was suggested that the transformation of one into the other could be due to the addition of phosphate to the carbohydrate moiety. In the present report we present evidence indicating that central to the transformation is a proteolytic cleavage, in a kex2-dependent manner, of at least 4 residues at the NH_2-terminus of the less acidic form.

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Abbreviations: endo H, endo-β-N-acetylglucosaminidase H; PAGE, polyacrylamide gel electrophoresis; HPLC, high-pressure liquid chromatography; SDS, sodium dodecyl sulphate; pNPG, paranitrophenol-β-D-glucoside

2. MATERIALS AND METHODS

2.1. *Microorganisms and culture conditions*

S. cerevisiae haploid strain X2180-1A and its derivative sec7, which is blocked in transport of secretory and plasma membrane proteins from Golgi body (and, accordingly, accumulates those proteins at the restrictive temperature of 37°C) were supplied by Dr Schekman (University of California, Berkeley). A kex2 mutant strain α ural kex2-1 Arg1 His7 was kindly supplied by Dr del Rey (University of Salamanca, Spain). All these strains were grown in a synthetic medium [15] with the appropriate supplement of growth factors.

2.2. *High-pressure liquid chromatography*

For ion-exchange chromatography, dialyzed samples (20–100 μl) were applied to a Bio-Gel TSK DEAE-5-PW column (75 × 7.5 mm). The column was first washed with two column volumes of 0.2 M NaCl in 20 mM sodium acetate buffer, pH 5.2. Then, the enzymes were eluted with a linear gradient of NaCl (0.2–0.5 M) in the same buffer. Elution was performed at a flow rate of 0.5 ml/min and a pressure of 12 bars. For gel filtration, 20–100 μl samples were applied to an Ultro-Pack TSK-G3000 SW (60 cm) column. They were eluted with 0.1 M of acetate buffer, pH 5.2, at a flow rate of 0.5 ml/min and at a pressure of 18 bars.

2.3. *Purification of enzymes*

Purification of the major external exoglucanase was performed as described before [11]. An A-like form of enzyme [14] (see also section

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00145793/90/$3.50

3) was purified from the culture medium of exponentially growing kex2 cells. One peak of activity eluted from the ion-exchange column (HPLC) at 0.23 M salt (the major external exoglucanase eluted at 0.27 M). The corresponding material was further purified by gel filtration (HPLC).

2.4. *Sequence determination*

The purified A-like form of exoglucanase (500 pmol) was sequenced on polybrene-coated filters using an Applied Biosystems 477A microsequencer. The sequence of the purified major external exoglucanase will be reported in extent elsewhere [12].

2.5. *Other methods*

Transformation of cells into protoplasts and enzymatic analyses using pNPG as a substrate were performed as described [15]. Protoplasts were lysed in 25 mM sodium acetate buffer, pH 5.2, containing 10 mM sodium azide (lysis buffer). When indicated EDTA (10 mM) or leupeptin (10 mM) were included in the lysis buffer. High-speed supernatants (soluble fractions) of protoplast lysates were obtained by centrifugation at 46000 rpm in a 70Ti Beckman rotor (120000×*g*). Standard endo H treatments (5 mU of endo H and about 15 U of exoglucanase in a final volume of 280 μl) were performed for 5 h in the presence of PMSF (1 mM) and pepstatin A (20 μM). Immunoblots were performed as described [14].

3. RESULTS

In a previous work we have described the presence of two ionic forms of the secretory yeast exoglucanase inside the protoplast [14]. The less acidic form (form A) was typical from the endoplasmic reticulum (i.e. it was the major enzyme form accumulated by sec18 cells) whereas the most acidic one was present in both secretory vesicles (i.e. it was the predominant form accumulated by sec1) and culture medium. The transformation of A into B appears to occur at the level of the Golgi complex, since sec7 cells accumulated similar amounts of both enzyme forms. Forms A and B were indistinguishable by SDS-PAGE; moreover, their deglycosylated (endo H-treated) products also comigrated in the same system.

Similar results were obtained when the analysis of the deglycosylation reaction was carried out by ion-exchange chromatography at high pressure. As shown in Fig. 1a, high-speed supernatants of sec7 cells that have accumulated secretory products at the non-permissive temperature gave two peaks of exoglucanase which represented 60% and 40% of the activity recovered from the column (90%). As expected, the immunoblot analysis revealed the presence of a single band that roughly co-migrated with the mature exoglucanase II [11]. Treatment of the whole supernatant with endo H under standard conditions (see section 2) converted both peaks into a single one; the corresponding material not only eluted from the HPLC column at the same position as the in vitro deglycosylated external exoglucanase (Fig. 1c) but also comigrated with it in SDS-PAGE [14]. Surprisingly, when sec7 protoplasts were lysed in the presence of EDTA, the analysis of the deglycosylated products (originated by endo H treatment of the high speed supernatant) in the ion-exchange

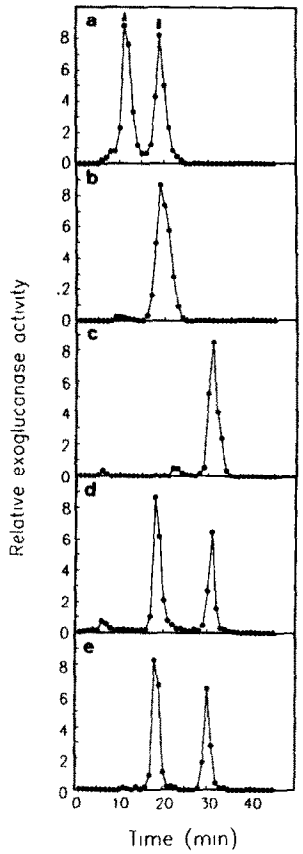

Fig. 1. Analysis of ionic forms of exoglucanase accumulated by sec7 at the restrictive temperature. Sec7 cells were incubated at 37°C for 2 h. Then they were transformed into protoplasts which were lysed in the lysis buffer (50 mM acetate buffer, pH 5.2) alone (a,b,c) or supplemented with EDTA (d) or leupeptin (e). High-speed supernatants were analyzed without further treatment (a) or after incubation at 37°C for 5 h in the absence (b) or in the presence of endo H (c,d,e) as indicated in section 2. The resulting samples were applied in the ion exchange column (HPLC) and eluted as indicated in section 2.

column indicated the presence of two (instead of a single one) peaks of activity (Fig. 1d). Endo H treatment of isolated individual peaks (obtained in the presence of EDTA) indicated that peak B gave rise to the most acidic deglycosylated compound whereas peak A was the source of the less acidic counterpart (not shown).

In order to gain information on the nature of forms A and B, their deglycosylated products were subjected to immunoblot analysis. As shown in Fig. 2, deglycosylated B appeared as a well-defined band (lane 2) that comigrated with the protein portion of the mature enzyme whereas deglycosylated A (lane 1) was heterogeneous: a well-defined front (running just behind deglycosylated B) was followed by a rather diffuse tail. The most likely explanation of all the above-mentioned results was that the protein portion of form A was converted into its counterparts of form B by an endoprotease(s) which is inactive when protoplasts are lysed in EDTA. Such a conversion also occurred by in-

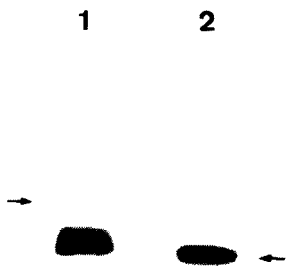

Fig. 2. Immunoblot analysis of the isolated deglycosylated peaks A (lane 1) and B (lane 2) from Fig. 1d. Upper arrow = secreted major exoglucanase and native forms A and B; lower arrow = deglycosylated exoglucanase.

cubation of the isolated form A (obtained in the absence of EDTA) at 37°C in the absence of endo H, indicating that deglycosylation was not necessary for the action of the protease (Fig. 1b).

A good candidate for playing a role in the above-mentioned transformation was the KEX2 protein, which has been reported to be inhibited by EDTA [16]. That this could be the case was reinforced by the fact that leupeptin, another well-known inhibitor of the KEX2 endoprotease [16], prevented to a great extent, transformation of A into B (or their deglycosylated products) during the deglycosylation reaction (Fig. 1e).

If that is true, a mutant lacking the protease should secrete into the culture medium an exoglucanase with the ionic properties of form A. An A-like form was indeed found in the growth medium of a kex2 mutant. This enzyme was purified by conventional methods used by us in the purification of exoglucanases ([11], see section 2). Sequencing of the new protein indicated the presence of a group of 4 amino acids (Asn-Lys-Lys-Arg) preceding the amino-terminus of the wild-type mature protein (Fig. 3). It is noticeable that the extrasequence contains 3 (out of 4) positively charged amino acids whose cleavage should account for the drastic change in the ionic behaviour of the molecule. In addition, in support of our expectations a dipeptide with the sequence Lys-Arg was found at the carboxyl end of the extra-peptide. As mentioned before this pair of basic residues are cleaved at its carboxyl side by the past KEX2 protein.

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1.-    Asn-Lys-Lys-Arg-Tyr-Tyr-Asp-Tyr-Asp-His
2.-                    Tyr-Tyr-Asp-Tyr-Asp-His
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Fig. 3. NH_2-terminal sequence of purified A-like form of exoglucanase secreted by the kex2 strain (1) as compared with that of the purified external exoglucanase (2).

4. DISCUSSION

In the present report we have presented results indicating that yeast exoglucanase is proteolytically processed during its transit to the cell surface. Two lines of evidence support a role for the KEX2 protein in such a processing. First, a kex2 mutant secretes an enzyme with an extrasequence of 4 amino acide bearing at its carboxyl end a pair of dibasic residues potentially cleavable by the protease. Second, a form of exoglucanase representative of the endoplasmic reticulum (form A) is proteolytically cleaved in vitro to give a product indistinguishable from the mature enzyme; this reaction did not proceed when form A was obtained in the presence of two inhibitors of KEX2 protease. As shown before, a similar, if not identical, processing also occurs in vivo and takes place before the execution point of sec7 mutation [14]. The fact that KEX2 protease is also located in some compartment of the Golgi complex can thus be taken as additional circumstantial evidence of the action of this protease in the processing of the form A of exoglucanase in vivo. It should be noted that, in contrast to KEX2 protease, our in vitro transforming enzyme is soluble. This does not exclude the KEX2 protein from direct participation in the in vitro transformation since the only potential transmembrane segment of KEX2 is located in the last 120 amino acids and KEX2 protease appears to present an autoproteolytic removal of about 100 amino acids [16]. We should not forget, however, that previous studies have indicated the existence of yeast proteases other than KEX2, capable of cleaving not only artificial pheromone substrates [17,18] but also the MFα propheromone itself at a cleavage site identical to that of the KEX2 protease [19].

In a previous work we have shown that both form A (isolated from a protoplast lysate) and the external major exoglucanase migrate very close in SDS-PAGE [14]. We have also reported elsewhere [13] that the major yeast exoglucanase secreted into the culture medium contains about 27 mannoses and one phosphate (distributed in two short N-linked oligosaccharides) per molecule of enzyme. On the other hand, as a representative of the endoplasmic reticulum, form A should contain 8 mannoses in each oligosaccharide [20]. The difference (11 mannoses plus one phosphate) accounts for a molecular mass of near 2000. This value is far above the molecular mass of the extrasequence found in the A-like form secreted by kex2 mutant (about 500) and it suggests the existence of an additional extrasequence at the NH_2-terminus in the form A present in the endoplasmic reticulum. Proteolytic cleavage (either in vivo or in vitro) at several sites of the extrasequence would be the source of the heterogeneity shown by the

deglycosylated form A (Fig. 2, lane 1). Work is in progress to isolate enough amounts of form A accumulated by sec18 in order to analyze its amino-terminus.

In summary, the role of KEX2 protease in the processing of yeast precursors in vivo has now been extended to the major exoglucanase from *S. cerevisiae*. We do not know yet whether the in vitro cleavage of exoglucanase is caused by KEX2 or a different protease. However, it is noticeable that the availability of a quantitative and simple assay for this exoglucanase as well as the facility to separate the substrate (form A) from the product (form B) will allow a definitive characterization of the in vitro cleaving enzyme.

Acknowledgements: This work was supported by grant PB88/435 from CICYT to G.L. We wish to thank to Ms. Michele Harrell for correction of the English version of the manuscript.

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