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Processing and Secretion of the *Yarrowia lipolytica* RNase

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Secretion of the extracellular RNase from the yeast *Yarrowia lipolytica* was studied in pulse-chase and immunoprecipitation experiments. A polypeptide of 45,000 daltons was immunoprecipitated from [^{35}S]methionine-labeled cell extracts and supernatant medium by rabbit anti-RNase antiserum. The RNase was secreted rapidly; the time between synthesis and appearance in the extracellular medium was about 5 min. In pulse-chase experiments, about 50% of the RNase was still cell associated 30 min after labeling. A polypeptide of 73,000 daltons whose immunoprecipitation was blocked by an excess of purified RNase was also detected. It broke down to a polypeptide with the same mobility and same peptide map as the mature RNase. Peptide maps of the undegraded 73-kilodalton polypeptide and the intracellular mature RNase contained several peptides of identical mobility. Immunoprecipitates from cells labeled in the presence of tunicamycin contained 66- and 45-kilodalton polypeptides. Endoglycosidase H treatment of the 73-kilodalton polypeptide converted it to a 66-kilodalton form, but did not change the apparent molecular weight of the mature form of the RNase. Labeling kinetics from pulse-chase experiments did not clearly support a precursor-product relationship between the 73-kilodalton polypeptide and the intracellular 45-kilodalton form of the RNase, and other relationships between the two polypeptides are possible.

Yarrowia lipolytica provides a model system for the study of protein secretion in eucaryotes which is amenable to genetic (12, 20) and biochemical analysis. *Y. lipolytica* is quite different from *Saccharomyces cerevisiae*, which is the most thoroughly studied lower eucaryote with respect to protein localization and secretion (7, 9, 25, 26, 28). *Y. lipolytica* has been used industrially (14), and the recent development of transformation systems (6, 11) has increased its potential as a host for the secretion of foreign proteins.

Y. lipolytica secretes an RNase (4), an alkaline extracellular protease (AEP) (23), and at least three acid proteases (35) into the extracellular medium. We have isolated pleiotropic mutations which affect secretion of all these enzymes (21, 22). This study of RNase processing and secretion was undertaken to complement our studies on the processing and secretion of the AEP. Also, little is known about the intracellular events involved in the processing and secretion of RNases from lower eucaryotes. In *Neurospora crassa*, an intracellular 53,000-molecular-weight (53K) endoexonuclease distributed in vacuoles and in mitochondria was found to be derived from an inactive 94K precursor by proteolytic action (10).

In this study we show that an intracellular 73K polypeptide related to the mature RNase exists, and we investigate the possibility of a precursor-product relationship between the two polypeptides. We also demonstrate that the transit time for secretion is longer and the rate of secretion is lower for the RNase than for the AEP.

MATERIALS AND METHODS

Strains. *Y. lipolytica* (formerly *Saccharomycopsis lipolytica* or *Candida lipolytica* [34]), suitable for genetic studies (20), was obtained from J. Bassel and R. Mortimer, University of California, Berkeley. *Y. lipolytica* PS6069 (ATCC 46028), *ade1 xpr2-34 A*, which contains a mutation in the AEP structural gene, was isolated by UV mutagenesis of strain CX161-1B (27).

Media. Cultures were maintained on YM medium (4) and grown in GPPP2 medium (4). GC medium contained (per liter): 6.7 g of glycerol, 1.7 g of yeast nitrogen base (Difco Laboratories) without amino acids and ammonium sulfate, 0.4 g of casein (Hammersten, ICN), 20 mg of adenine, and 1 ml of antifoam in 40 mM phosphate buffer, pH 6.8. Two grams of casein was boiled for 15 min in 1 liter of 0.2 M phosphate buffer, pH 6.8; portions were frozen at −20°C and thawed before use in GC medium.

Antiserum. Preparation of the ammonium sulfate-precipitated rabbit anti-RNase antiserum has been described previously (4). The level of antiserum used for immunoprecipitation of cell extracts was empirically determined to ensure a severalfold antibody excess for wild-type cell extracts.

Preparation of labeled cell extracts and supernatant media. Cells grown at 23°C in GPPP2 medium were inoculated into 50 ml of GPPP2 medium in a 500-ml baffled flask. Cell density was measured with a Klett-Summerson photoelectric colorimeter with a green filter. A Klett reading of 250 corresponded to 1 mg of cells (dry weight) per ml. The culture was grown at 23°C at 300 rpm in a New Brunswick Psycrotherm incubator shaker. Cells were collected 15 to 16 h later by centrifugation at a cell density near 500 Klett units. The cells were washed once in GC medium and suspended in GC medium at a cell density of 1,000 Klett units. From 6 to 50 ml (depending on the experiment) of this cell suspension was incubated in a 125-ml baffled flask at 23°C at 300 rpm. After 30 min of incubation, radioactive label was added. The isotopes (Amersham Corp.) used to label the cultures were L-[4,5-^{3}H]leucine (40 to 60 Ci/mmol), L-[^{35}S]cysteine (>600 Ci/mmol), and [^{35}S]methionine (>800 Ci/mmol). For cultures in which intracellular or extracellular samples were to be immunoprecipitated, 100 μCi of [^{35}S]methionine (or [^{3}H]leucine in preliminary experiments) was added for every 6 ml of suspended cells. For cultures in which the extracel-

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lular proteins were precipitated with trichloroacetic acid and no immunoprecipitation was done, 50 μCi of isotope was used per 6 ml of suspended cells. Chase with unlabeled methionine was done, when appropriate, by addition of L-methionine at a final concentration of 5 mM. Protein synthesis was stopped by pouring 5 ml of the cell suspension into 2 g of crushed ice and 0.77 ml of 100 mM sodium azide. If labeling was done without a chase, L-methionine was added to the ice and sodium azide mixture to a final concentration of 5 mM.

For determination of total protein synthesis, two 50-μl samples of the remaining 1.0 ml of cell suspension were mixed with 2.0 ml each of ice-cold 5% trichloroacetic acid and boiled for 30 min, and the pecipitates were collected on HAWP filters (0.45-μm pore size; Millipore Corp.). The filters were washed with cold 5% trichloroacetic acid, air-dried overnight, and counted in 10 ml of PCS liquid scintillation cocktail (Amersham).

After the ice had melted, the cell suspension was centrifuged at 8,000 × *g* for 10 min at 4°C. The supernatant liquid was transferred to a Corex centrifuge tube, adjusted to contain 2 mM phenylmethanesulfonyl fluoride (PMSF), and incubated for 10 min at room temperature. Supernatant samples to be immunoprecipitated were often frozen at −20°C at this step. Samples to be precipitated with trichloroacetic acid were cooled, adjusted to 5% trichloroacetic acid with a 50% solution, and incubated at 4°C for 1 h. The precipitate was collected by centrifugation (12,000 × *g*) for 10 min, washed twice with cold 5% trichloroacetic acid, redissolved in 200 μl of Laemmli gel-loading buffer (15), neutralized with 1 M sodium hydroxide until the bromphenol blue indicator turned blue, adjusted to 300 μl final volume with Laemmli gel-loading buffer, and frozen at −20°C.

The cell pellet was washed once with cold homogenization buffer (50 mM Tris hydrochloride, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM L-methionine, pH 6.8) and transferred in three 175-μl portions of homogenization buffer to a 2-ml cryotube (Vangard International, Neptune, N.J.). The cell suspension was adjusted to contain 2 mM PMSF, and 1.0 g of acid-washed glass beads (0.45 to 0.50 mm) was added. Cells were disrupted for 2 min in a Braun homogenizer (Bronwill Scientific, Rochester, N.Y.), fitted with a custom fabricated cryotube adaptor. Short bursts (2 s) of expanding CO_2 from a pressure tank were used about every 20 s for cooling. Based on microscopic examination, over 95% of the cells were damaged or broken. The cell extract plus the material from two 125-μl washes (in homogenization buffer) of the beads were adjusted to 2 mM PMSF and 1% sodium dodecyl sulfate (SDS) (BDH, electrophoresis grade), incubated for 8 min at room temperature, and centrifuged at 23,500 × *g* for 1 h at 4°C. The clarified cell extract (ca. 800 μl) was transferred to a 1.5-ml Microfuge tube and adjusted to 2% Triton X-100 with a 25% stock solution. The immunoprecipitation procedures were adapted from those described by Anderson and Blobel (1). Thirty microliters of RNase antiserum was added to the clarified cell extract for every 5 ml of suspended cells. The mixture of cell extract and antiserum was incubated for 1.5 h at room temperature on a rotating drum and then for 16 h at 4°C. Antigen-antibody complexes were precipitated by incubation with protein A-Sepharose CL-4B (Sigma Chemical Co.) (22.5 mg [dry weight], preswollen and washed in 50 mM Tris hydrochloride, pH 6.8) for 3 h at room temperature on a rotating drum. The Sepharose beads were washed five times with 1 ml of an SDS solution (0.1% SDS, 50 mM Tris hydrochloride, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.05% Triton X-100). The beads were then washed once with 1 ml of a similar buffer which did not contain SDS or Triton X-100. Then, 100 μl of Laemmli gel-loading buffer was added, and the beads were boiled for 3.5 min. The mixture was centrifuged, and the supernatant liquid was removed and frozen at −20°C. The protein A-Sepharose beads were washed three times with 250 μl of 50 mM Tris hydrochloride, pH 6.8. The washes were pooled, dialyzed overnight against 5 mM Tris hydrochloride, pH 6.8, and dried. The dried pellet was dissolved in the supernatant liquid obtained earlier; the sample volume was brought up to 100 μl with Laemmli gel-loading buffer and frozen at −20°C.

For immunoprecipitation of extracellular RNase, 2 ml of PMSF-treated supernatant medium was adjusted to contain 1% SDS and incubated for 8 min at room temperature. The additional steps were identical to those for immunoprecipitation of cell extracts, except that 12 μl of RNase antiserum and 12 mg of protein A-Sepharose were used for each sample. The amount of antiserum to be used was determined by mixing various ratios of supernatant medium and antiserum, precipitating the antibody-antigen complexes with protein A-Sepharose, and examining the supernatant medium for remaining RNase activity by the RNase-RNA agar diffusion plate assay (4).

Immunocompetition. Competition experiments with unlabeled, purified RNase were done to examine the specificity of the immunoprecipitation reactions. These were similar to the immunoprecipitation experiments described above except that 30 μl of the antiserum was first incubated with 50 or 100 μg of RNase previously denatured with either 0.1 or 1% SDS. The RNase samples were made 2 mM in PMSF and then adjusted to contain 0.1 or 1% SDS and incubated at room temperature for 8 min. Triton X-100 (25% solution) was added to adjust the concentration to twice the SDS concentration. Then 30 μl of antiserum was added, and the mixture was made 0.02% in sodium azide. The antigen-antibody mixture was incubated for 1.5 h at room temperature and 16 to 20 h at 4°C before it was added to the cell extract.

PAGE and fluorography. Proteins were analyzed by polyacrylamide gel electrophoresis (PAGE) in 12% polyacrylamide slab gels (1.5 mm; 14 by 16 cm) in the presence of SDS with the system described by Laemmli (15). A 3% stacking gel was used, and the gels were run at 10 mA in the stacking gel and at 20 to 30 mA in the running gel. The samples were thawed, and all samples were boiled for 2 to 3 min before being loaded on the gel. For proteins precipitated by trichloroacetic acid, 100 μl of the redissolved sample, equivalent to 1.7 ml of suspended cells, was loaded per lane; for immunoprecipitates, 50 μl of redissolved sample, equivalent to 2.5 ml of suspended cells, was applied per lane.

After PAGE, the gels were fixed overnight in the prestaining solution (25% methanol, 10% acetic acid), stained for 10 min with Coomassie brilliant blue R250 (0.25% [wt/vol] Coomassie brilliant blue R250, 45.4% methanol, 9.2% acetic acid), and destained for 3 to 5 h in 45.4% methanol–9.2% acetic acid. The gels were dried after treatment with dimethyl sulfoxide and the scintillator 2,5-diphenyloxazole and exposed to preflashed X-Omat AR X-ray film at −80°C (3). For quantitation, labeled bands were detected by fluorography, cut out, and rehydrated in 100 μl of distilled water, and 10 ml of Econofluor/Protosol (New England Nuclear Corp.) (95:5 vol/vol) was added. The glass vials were incubated at room temperature for 4 to 6 h and shaken overnight at 37°C to solubilize the gel pieces before scintillation counting.

Peptide mapping. Peptide mapping was done by the method of Cleveland et al. (5). Labeling was done for 2 min as described above except that 100 μCi of [^{35}S]cysteine and 100 μCi of [^{35}S]methionine were used for 6 ml of cell suspension, and L-cysteine at 5 mM final concentration was added to the ice-sodium azide mixtures containing L-methionine. In the first experiment 160 ml of suspended cells was divided into four flasks before labeling, and in the second experiment 60 ml was divided into two flasks. The cell extracts were immunoprecipitated, and the samples were subjected to electrophoresis on a 12% SDS–polyacrylamide gel. Gels were loaded with twice the concentration of cell extract normally used. The RNase-related polypeptides were located in the gel by two methods. First, two vertical strips were cut from the gel, cut into 3-mm slices, solubilized, and counted. The second method was only used in the second experiment. A 7-mm-wide vertical strip was cut into 3-mm slices, and slices 5 to 21 from the top were equilibrated with 0.5 ml of Laemmli gel-loading buffer supplemented with 1 mM EDTA for 1 h at 10°C. The gel slices were inserted into the wells of another SDS–12% polyacrylamide gel, SDS-PAGE was performed, and the gel was fluorographed. Labeled immunoprecipitated antigens were run as markers. Once the positions of the desired polypeptides were determined, the appropriate sections were cut from the remainder of the lane, and the gel slices were electroeluted and concentrated with an ISCO electrophoretic concentrator. Digestions with *Staphylococcus aureus* V8 protease (Miles Scientific Co.) were carried out as described by Cleveland et al. (5). Concentrations and incubation conditions are listed in the legends to Fig. 3 and 4. SDS-PAGE (15% gel) and fluorography were done as described above.

Tunicamycin inhibition. Cells were prepared for suspension in GC medium as for a normal immunoprecipitation experiment. Tunicamycin (Sigma), dissolved in dimethyl sulfoxide at 1 mg/ml, was added at a final concentration of 10 μg/ml to 5 ml of cell suspension in GC medium, and the cells were incubated at 23°C for 30 min. Cells were then labeled with 83 μCi of [^{35}S]methionine for 90 s without a chase, and immunoprecipitation was performed as described above. Incorporation of [^{3}H]leucine into cells was largely unaffected by 10 μg of tunicamycin per ml, but [^{3}H]mannose incorporation decreased at least 60% (data not shown).

Endo H. Endoglycosidase H (endo H; Miles Scientific) was used to remove N-linked oligosaccharides from immunoprecipitated samples. Modifications of procedures recommended by Miles Scientific and another reported by Julius et al. (13) were used. Immunoprecipitates labeled with [^{35}S]methionine were prepared and reacted with protein A-Sepharose. Proteins to be treated with endo H were eluted by boiling with 120 μl of 1% SDS (32) or with the normal elution buffer lacking the glycerol and dye. Endo H digestion of samples was carried out at 37°C in sodium citrate buffer (either 50 or 300 mM), pH 5.5, containing 0.1% SDS and 5 mM sodium azide. The enzyme concentrations and digestion times are given in the legend to Fig. 6. Samples were boiled for 3.5 min and cooled, and PMSF was added to 2 mM final concentration immediately before addition of endo H. Samples eluted with 1% SDS were diluted 10-fold before endo H treatment. After treatment they were dialyzed against 5 mM Tris hydrochloride, pH 6.8, dried, and dissolved in 50 μl of Laemmli gel-loading buffer. Other samples after endo H treatment were adjusted to 50 mM Tris hydrochloride (pH 6.8)–10% glycerol–1% SDS–1% mercaptoethanol–0.001% bromphenol blue. Invertase (Sigma, grade VII) from *S. cerevisiae* served as a positive control for endo H treatment.

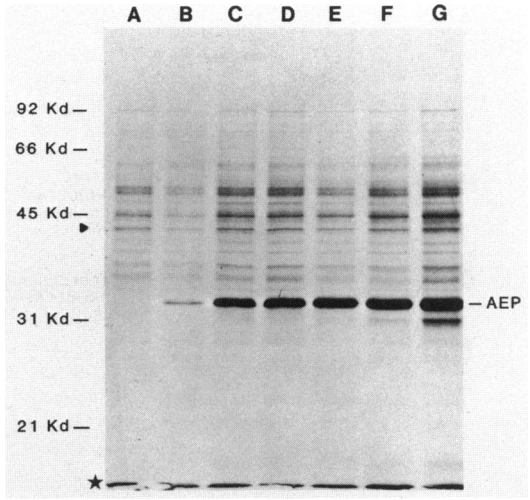

FIG. 1. Time course of secretion of [^{35}S]methionine-labeled polypeptides. Cells suspended in 22 ml of GC medium were labeled for 1.5 min with 183 μCi of [^{35}S]methionine, and the label was chased with excess unlabeled L-methionine for 2 (A), 4 (B), 6 (C), 8 (D), 12 (E), 20 (F), or 30 (G) min. Extracellular proteins were precipitated with trichloroacetic acid, resolved by SDS-PAGE, and visualized by fluorography. The film was exposed for 3 days. The position of the dye front is indicated by the star, and the position of the 43K purified RNase is indicated by the arrowhead. Sizes are indicated in kilodaltons.

The invertase was purified as described by Trimble and Maley (31) and was a gift from J. Fukayama.

RESULTS

Labeling and immunoprecipitation conditions. The initial labeling medium used was a glycerol-casein (GC) medium which had been developed for immunoprecipitation studies of the AEP (J. Fukayama, personal communication). Attempts were made to find other conditions in which RNase production was increased. The use of different nitrogen sources substituted for casein, RNA in minimal medium, and the *xpr2-34* strain carrying the AEP structural gene mutation were examined, but each of these substitutions resulted in a substantial decrease in the production of RNase.

The band corresponding to AEP was easily distinguished on fluorograms of SDS-polyacrylamide gels of trichloroacetic acid-precipitated extracellular proteins in the culture medium (Fig. 1). However, the RNase was secreted at much lower levels, and it ran into the same area on the gel as several other polypeptides (Fig. 1). Also, the purified RNase used as a marker was 43K (4) and so did not indicate the exact location of the 45K RNase. Similar results were obtained whether [^{3}H]leucine or [^{35}S]methionine was used to label the polypeptides and in an experiment in which the serine protease inhibitor PMSF was added before labeling to inactivate the AEP.

Immunoprecipitation studies. Several polypeptides were immunoprecipitated from the *Y. lipolytica* [^{35}S]methionine-labeled cell extracts with the rabbit anti-RNase antibodies (Fig. 2, lane A). The major polypeptide had an apparent molecular weight of 45,000. Another polypeptide of 73K was also detected. The less distinct bands running below the 45K polypeptide were assumed to be degradation products. Immunoprecipitation of RNase-related polypeptides from [^{35}S]methionine-labeled supernatant medium gave similar

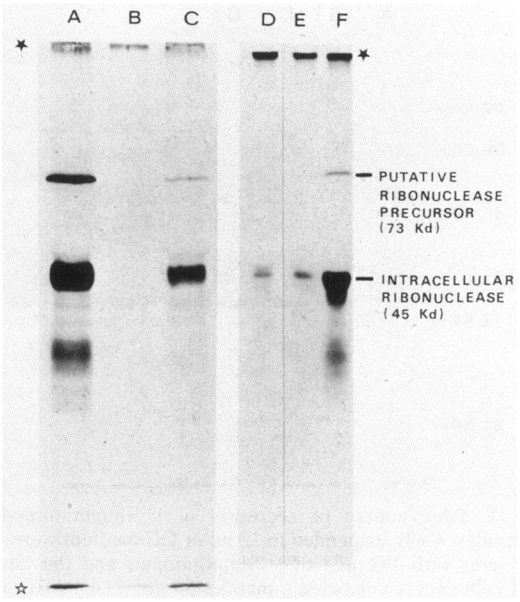

FIG. 2. Immunoprecipitation of cell extracts with anti-RNase antibodies and characterization of the antibodies. Cells were labeled for 1.5 min with 83 μCi of [^{35}S]methionine, and cell extracts were prepared. The immunoprecipitates from 2.5 ml (lanes A to C) or 1.88 ml (lanes D to F) of cells were resolved on 10- or 15-lane SDS gels, respectively, and visualized by fluorography. Lanes A and F, immunoreactive products from wild-type cells; lane B, cells immunoprecipitated with preimmune serum; lanes C, D, and E, similar to lanes A and F except that antiserum was preincubated with 50 μg (denatured with 0.1% SDS), 100 μg (denatured with 1% SDS), or 100 μg (denatured with 0.1% SDS) of purified RNase, respectively. Total trichloroacetic acid-precipitable counts (in cells and supernatant medium) incorporated by 1 ml of cell culture per μCi of label added were 1,627,000 cpm for lanes A to C and 723,000 cpm for lanes D to F. The films were exposed for 3 days. The top of the separating gel is indicated by the closed star and the dye front by the open star. Kd, Kilodalton.

results except that the 73K polypeptide was not detected (data not shown). The specificity of the immunoprecipitation was indicated by the ability of excess purified 43K RNase to block immunoprecipitation (Fig. 2, lanes C to E) and the inability of the preimmune serum to immunoprecipitate any polypeptides (Fig. 2, lane B).

73K polypeptide related to RNase. The facts that (i) the 73K polypeptide was larger than the mature RNase, (ii) it was immunoprecipitated by antiserum raised against the 43K RNase, (iii) its immunoprecipitation was blocked by excess 43K RNase (Fig. 2, lanes C to E), and (iv) it was not immunoprecipitated by preimmune serum all suggest that the 73K polypeptide is related to and possibly a precursor of the mature RNase.

Two peptide maps were done to confirm the relationship of the intracellular 73 and 45K polypeptides. Cells were labeled with [^{35}S]cysteine and [^{35}S]methionine for 2 min without a chase, and immunoprecipitates of the cell extract were resolved by SDS-PAGE. For the first peptide map the 73K polypeptide and 45K mature form of the RNase were electroeluted at room temperature, dialyzed, and digested for 1 h at 37°C with various amounts of *S. aureus* V8 protease. The controls without the protease were also incubated for 1 h at 37°C. The polypeptides and their digested peptide fragments were resolved by SDS-PAGE (15% gel) and visualized by fluorography (Fig. 3). Lane D supposedly contained the undigested 73K polypeptide. However, the 73K band was not present, and the major band present had the same mobility as the 45K RNase. The very similar peptide maps of the digested 73K polypeptide sample (lanes H and J) and the digested 45K RNase (lanes E to G, I, and K to M) strongly suggested that the 45K band observed in the undigested 73K polypeptide sample (lane D) was the same as the mature RNase.

The 73 and 45K polypeptides were separated by 2 cm on the preparative SDS-polyacrylamide gel, so it was unlikely that this result was due to incomplete separation of the two polypeptides. It seemed more likely that the 45K major band in lane D resulted from natural breakdown during handling of the sample.

Several modifications of the sample-handling procedures were included in the second peptide-mapping experiment in an attempt to prevent degradation of the 73K polypeptide. First, samples from the preparative gel were run directly without electroelution by inserting gel slices containing the 45 or 73K polypeptide into the wells of the 15% gel. Second, samples were electroeluted at 10°C instead of at room temperature. Third, the electroeluted control sample was not incubated at 37°C for 1 h. Otherwise, the second peptide-mapping experiment was done under conditions similar to the first.

The changes in sample-handling procedures greatly reduced the degradation of the 73K polypeptide. The samples applied in gel slices without electroelution contained predominately the 73K polypeptide and very little of the 45K polypeptide (Fig. 4, lanes B and C). The electroeluted sample contained a somewhat higher proportion of the 45K

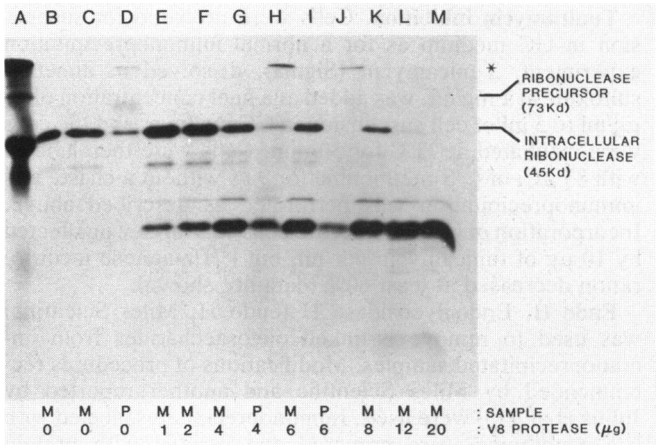

FIG. 3. Peptide maps of intracellular 45K and 73K polypeptides immunoprecipitated by anti-RNase antiserum. Cells suspended in 160 ml of GC medium were labeled with 2.71 mCi each of [^{35}S]cysteine and [^{35}S]methionine for 2 min without a chase. The 73K polypeptide (P) and 45K mature (M) forms of the RNase were electroeluted at room temperature from gel slices obtained from a preparative SDS–12% polyacrylamide gel and digested for 1 h at 37°C with various amounts of *S. aureus* V8 protease, and the samples (50 μl final volume) were resolved by SDS-PAGE (15% gel) and visualized by fluorography. Lane A contained RNase-related polypeptides immunoprecipitated from labeled cell extracts. The type of sample and amount of V8 protease used are indicated at the bottom of each lane. The amounts of radioactivity applied were 18,000 cpm for lane A, 600 cpm for lane B, 800 cpm for lanes C and D, and 1,800 cpm for lanes E to M. Total trichloroacetic acid-precipitable counts incorporated by 1 ml of cell culture per μCi of label added ranged from 446,000 to 600,000 cpm. The film was exposed for 14 days. The top of the separating gel is indicated by the star. Kd, Kilodalton.

polypeptide, but the 73K polypeptide was by far the major band. These results strongly support the hypothesis that in the first experiment the 73K polypeptide had been broken down to the 45K polypeptide. Comparison of the peptide maps of the electroeluted 73K and 45K polypeptides revealed that of the 8 and 10 bands generated from the two polypeptides (the 45K band is excluded since it does not seem to be a product of V8 protease digestion), at least 4 bands lined up. Therefore we conclude that the 73K polypeptide has peptide homology with the mature RNase (see Discussion) and that the 73K polypeptide naturally breaks down to the mature RNase.

73K polypeptide contains N-linked carbohydrate but 45K mature RNase does not. The drug tunicamycin blocks the synthesis of N-linked glycosyl chains (17) and the enzyme endo H removes N-linked oligosaccharide chains from the peptide backbone (29). Pulse-labeling of tunicamycin-treated cells (Fig. 5) and digestion of immunoprecipitated RNase-related polypeptides with endo H (Fig. 6) showed that the 73K polypeptide contained N-linked carbohydrate, while the 45K mature RNase did not. The difference in apparent molecular size for the treated and untreated 73K polypeptides was about 6,000 daltons, which corresponds to two to three core oligosaccharide chains (16, 33).

Kinetic studies. The time needed for the RNase to be secreted was determined by pulse-chase experiments. Cells were grown in GPPP2 medium, suspended at a higher cell density in GC medium, and incubated for at least 30 min before labeling. Cells were labeled with [^{35}S]methionine for 30 s, and excess unlabeled methionine was added; one sample was taken immediately, and other samples were taken at various times.

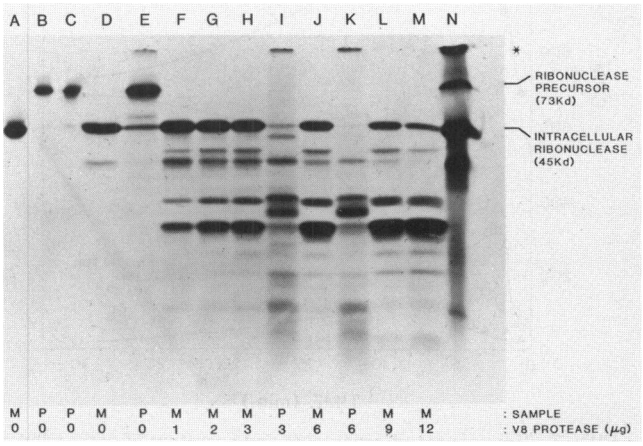

FIG. 4. Peptide maps of intracellular 45K and 73K polypeptides immunoprecipitated by anti-RNase antiserum. Cells suspended in 60 ml of GC medium were labeled with 1 mCi each of [^{35}S]cysteine and [^{35}S]methionine for 2 min without a chase. For samples in lanes A, B, and C the 73K polypeptide (P) and 45K mature (M) forms of the RNase were cut out of the preparative SDS-polyacrylamide gel, and the gel slices were inserted into the wells of the peptide-mapping gel. The other samples were electroeluted and treated as described in the legend to Fig. 3 except that electroelution was done at 10°C and the control samples were not incubated at 37°C for 1 h. Lane N contained RNase-related polypeptides immunoprecipitated from labeled cell extracts. The type of sample and amount of V8 protease used are indicated at the bottom of each lane. The amount of radioactivity applied was 3,000 cpm for lane A, 380 cpm for lane B, 370 cpm for lane C, 780 cpm for lanes D and E, 1,700 cpm for lanes F to M, and 9,000 cpm for lane N. Total trichloroacetic acid-precipitable counts incorporated by 1 ml of cell culture per μCi of label added were 515,000 cpm. The film was exposed for 105 days. The top of the separating gel is indicated by the star.

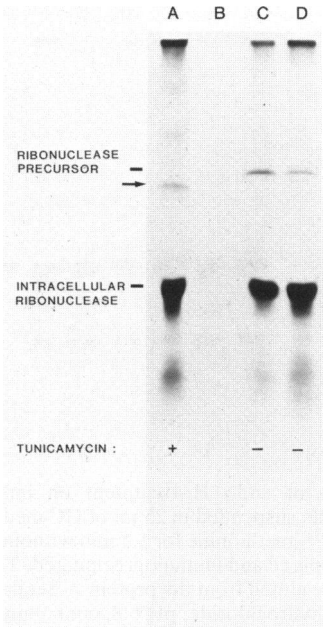

FIG. 5. Effect of tunicamycin on immunoprecipitated polypeptides. Cells were suspended in 5 ml of GC medium containing tunicamycin (10 μg/ml), incubated for 30 min, and labeled with 83 μCi of [^{35}S]methionine for 1.5 min without a chase, and cell extracts were immunoprecipitated. Lanes: A, tunicamycin-treated; B, blank; C, control immunoprecipitation; D, control immunoprecipitation in which the same volume of dimethyl sulfoxide (solvent for tunicamycin) used for the sample in lane A was added. Total trichloroacetic acid-precipitable counts incorporated by 1 ml of cell culture per μCi of label added were 651,000 cpm for lane A, 723,000 cpm for lane C, and 578,000 cpm for lane D. The film was exposed for 3.5 days.

The labeled 73K polypeptide reached its peak level between 2 and 5 min after the initiation of labeling, and it could no longer be detected at 30 min (Fig. 7). The intracellular 45K polypeptide reached its peak level at 8 min. Little or no extracellular 45K polypeptide was present for the initial several minutes, but between 8 and 12 min there was a sharp increase in its level. Thus the timing of the rise and fall of the level of the intracellular and extracellular 45K polypeptides was consistent with the 45K polypeptide being secreted to the supernatant medium. When the curve for the appearance of the extracellular RNase was extrapolated to 0 cpm, rapid secretion of the RNase was shown to begin at about 5 min. At 30 min about half of the RNase was still associated with the cells.

A second pulse-chase experiment was done in which the appearance of AEP in the supernatant medium was also measured. This experiment confirmed several observations from the first experiment. First, the ratio of the intracellular 73K and 45K polypeptides observed in the first experiment still held true, i.e., the intracellular 45K polypeptide was present at higher levels even at the earliest time points. Second, the start of rapid secretion of the RNase was comparable in the two experiments. Rapid secretion of AEP began earlier, at between 3 and 4 min. Third, less than 50% of the RNase was secreted to the extracellular medium by 30 min.

A third pulse-chase experiment was done with modified procedures for handling the intracellular samples. Half the

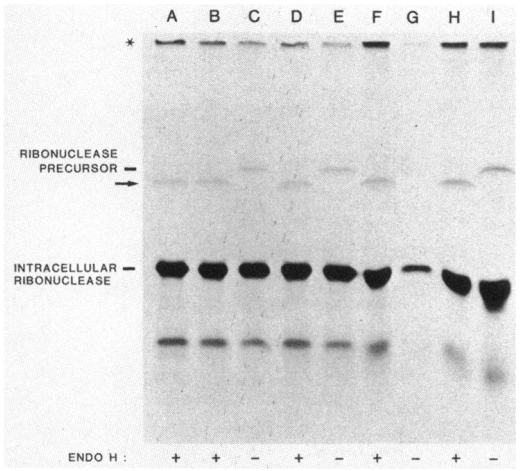

FIG. 6. Effect of endo H treatment on immunoprecipitated polypeptides. Cells suspended in 25 ml of GC medium were labeled with 415 μCi of [^{35}S]methionine for 1.5 min without a chase, and cell extracts were prepared and immunoprecipitated. The RNase-related polypeptides were eluted from the protein A-Sepharose beads either with 50 mM Tris hydrochloride, pH 6.8, containing 1% SDS and 1% mercaptoethanol (lane A) or with 1% SDS (lanes B to I). Control samples in lanes C, E, G, and I were not digested with endo H. Samples were digested with 1.3 mU of endo H for 24 h (lanes A, D, and H) or with 2.3 mU for 30 h (lanes B and F). The polypeptides were resolved by SDS-PAGE and visualized by fluorography. Total trichloroacetic acid-precipitable counts incorporated by 1 ml of cell culture per μCi of label added were 723,000 cpm. The film was exposed for 3.5 days. The top of the separating gel is indicated by a star, and the position of the endo H-treated 73K polypeptide by an arrowhead.

cell extract was immunoprecipitated with antibody raised against the RNase and half with antibody raised against AEP. At the earliest time points there were higher levels of the AEP precursors than mature AEP, but the intracellular 45K RNase was still present at higher levels than the 73K RNase-related polypeptide.

DISCUSSION

The finding that the major intracellular and extracellular immunoprecipitated polypeptides had apparent molecular weights of 45,000 confirms that the 45K polypeptide and not the 43K polypeptide (the major form of the RNase in the extracellular medium under certain conditions) is the form of the RNase which is initially secreted (4). Probably little or no 43K polypeptide was detected in the extracellular medium in these experiments because of the short time between labeling and collection of samples (30 min or less).

Various media were used in an attempt to increase RNase production. The medium supporting the highest level of RNase production supported much higher levels of AEP production (Fig. 1). Based on a comparison of [^{35}S]methionine incorporated into cell protein and radioactivity recovered in gel slices from immunoprecipitated intracellular and extracellular RNase-related polypeptides, RNase synthesis represented about 0.01% of total protein synthesis. For cells labeled with [^{35}S]methionine, AEP synthesis represented about 0.2% of total protein synthesis, and a severalfold-higher value was obtained when the cells were labeled with [^{3}H]leucine (unpublished data). These values are probably underestimates since they assume 100% recovery.

In pulse-chase experiments, extrapolation of levels of extracellular proteins to 0 cpm suggested that the RNase first appeared at significant levels in the supernatant medium at about 5 min and AEP at about 3 to 4 min. The half-time for secretion of RNase was also at least twice that for AEP. The transit times (the time between synthesis and translocation of a protein to its final destination) for the *Y. lipolytica* RNase and AEP were comparable to the transit times (3 min to 1 h or more) reported for secretory and cell surface proteins of *S. cerevisiae* (13, 18, 19). The longer transit time for the RNase from synthesis to appearance in the supernatant medium does not necessarily mean that transit time between synthesis and secretion across the cytoplasmic membrane is longer than for AEP. In the pulse-chase experiments about 50% of the labeled RNase was still retained by the cells 30 min after labeling. Some of this material might be outside the cytoplasmic membrane but retained by the cell wall.

The two peptide maps provide complementary proof that the 73K and 45K polypeptides are related. The natural breakdown of the 73K polypeptide to the 45K mature RNase provided very strong evidence for this relatedness. Most probably this natural breakdown is due to a protease(s)

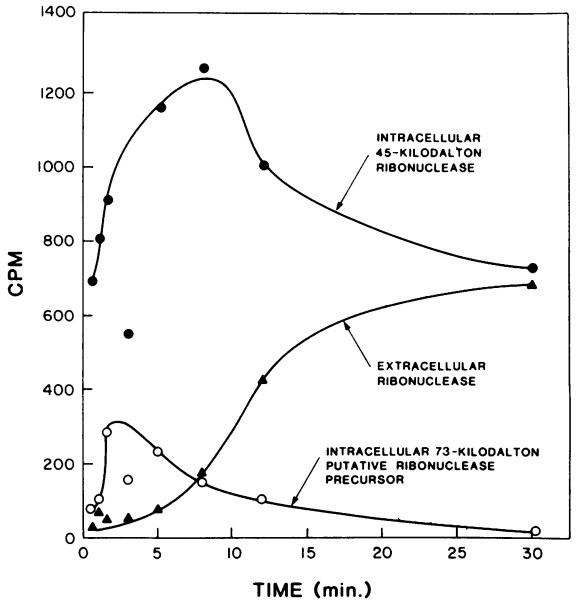

FIG. 7. Time course of processing and secretion of extracellular RNase by wild-type *Y. lipolytica*. Cells suspended in 38 ml of GC medium were labeled with 633 μCi of [^{35}S]methionine for 30 s and then chased with 5 mM L-methionine (final concentration) for 30 min. Four milliliters of culture was harvested at 40 s, 70 s, 100 s, 3 min, 5 min, 8 min, 12 min, and 30 min after the initiation of labeling. The cell extracts were immunoprecipitated with 24 μl of RNase antiserum, and samples equivalent to 2.5 ml of suspended cells were applied to each lane. The extracellular RNase in half the volume of every supernatant sample was immunoprecipitated with 12 μl of RNase antiserum. The immunoprecipitates were resolved by SDS-PAGE and visualized by fluorography. The films were exposed for 5 days. Bands containing the RNase-related polypeptides were identified, and gel slices were cut out and solubilized. The radioactivity contained in the solubilized gel slices was measured, normalized to that in 2.5 ml of suspended cells, and plotted against the time of harvest of the samples. Total trichloroacetic acid-precipitable counts incorporated by 1 ml of cell culture per μCi of label added were 515,000, 484,000, 503,000, 496,000, 467,000, and 517,000 cpm at 2.3, 3.7, 5.7, 8.7, 12.7, and 30.7 min, respectively.

present at such low levels that it is not detectable on the SDS-polyacrylamide gels (24).

The second peptide map also suggests a relatedness of the 73K and 45K polypeptides. Several peptides from the digested precursor on the first map were not present on the second map, and this indicates that the natural cleavage site between the precursor portion and mature portion of the RNase is not an *S. aureus* V8 protease cleavage site. Because of the large size difference between the precursor and mature form, several bands on the peptide maps would not be expected to line up. However, when numerous peptides are formed, some of them would line up by chance. Based on an estimate that the smallest difference in mobility which we could detect was 1 mm, we took the distance (57 mm) between the largest peptide (smaller than 45K) and the smallest peptide which we could detect and assumed it was divided into 57 windows. Then, given the number of detectable bands (8 and 10), we calculated the probability that 4 or more bands would line up in these windows by chance. The value obtained from this simple model was 0.024, and given that one could reasonably argue that a fifth and possibly a sixth band lined up (Fig. 4), these results strongly support the relatedness of the two polypeptides.

The level of 73K polypeptide in the pulse-chase experiments peaked before that of the 45K polypeptide, and this fits a precursor-product relationship. However, the facts that at the earliest time point there was much more 45K than 75K polypeptide and that the label in the precursor never reached more than 20% of the total label in RNase-related polypeptides do not fit a precursor-product relationship. It was expected that at early time points most of the label would be in the precursor. The pulse-chase data fit other models of RNase processing and secretion. For instance, the majority of the RNase might be synthesized in its mature form and secreted as such. The 73K polypeptide could be the product of a different gene or an alternative transcript of the RNase gene. It might be degraded intracellularly, or more interestingly, considering that half of the RNase remained cell associated during a 30-min period, the 73K polypeptide might be a form of RNase that is not secreted but instead is targeted to the vacuole.

The possibility that the 73K polypeptide is a direct precursor of the RNase should not be completely discounted. Perhaps processing is not completely stopped after sampling, or the amount of precursor was underestimated. The first peptide-mapping experiment demonstrated that the 73K polypeptide could break down to the RNase. Much more radioactivity was found at the top of the SDS gel in the precursor lanes than in the mature RNase lanes (Fig. 3 and 4), and possibly the percentage recovery of the mature form during immunoprecipitation is greater than for the precursor. If the 73K polypeptide is a precursor of secreted RNase, then its processing resembles that of *S. cerevisiae* α-factor and killer toxin much more than that of invertase. The secreted form of invertase contains large amounts of N-linked carbohydrate (8). The precursor forms of the α-factor and killer toxin molecules contain N-linked oligosaccharide, but their mature forms do not (13, 30). Very little is known about the carbohydrate portions of *Y. lipolytica* glycoproteins. If the glycosyl side chains are assumed to be similar to those of *S. cerevisiae*, then the N-linked oligosaccharide presumably consists of core oligosaccharide chains added in the endoplasmic reticulum (8).

In conclusion, there is strong evidence that the 73K polypeptide and the 45K RNase are related, but the nature of that relationship is still unclear. We are presently attempting to clone the RNase gene(s) by using the antibody as the probe. The number of genes obtained and the sequences of these genes should help define the relationship.

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