Signal Processing, Glycosylation, and Secretion of Mutant Hemagglutinins of a Human Influenza Virus by *Saccharomyces cerevisiae*

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We investigated the nature of signal recognition, transport, and secretion of mutant hemagglutinins (HAs) of a human influenza virus by the yeast *Saccharomyces cerevisiae*. The cDNA sequences encoding variant forms of influenza HA were expressed in *S. cerevisiae*. The HA polypeptides (HA500 and HA325) that were synthesized with their N-terminal signal peptides were correctly targeted to the membrane compartment where they were glycosylated. In contrast, the HA polypeptides (HA484 and HA308) lacking the signal peptide were expressed in the cytoplasm and did not undergo any glycosidic modification, demonstrating the importance of the heterologous signal sequence in the early steps of translocation in *S. cerevisiae*. The analysis of the N-terminal amino acid sequence of HA500 and HA325 polypeptides demonstrated the correct cleavage of the signal peptide, indicating the structural compatibility of a heterologous signal peptide for efficient recognition and processing by the yeast translocation machinery. The membrane-sequestered and glycosylated HA polypeptides were relatively stable in *S. cerevisiae* compared with the signal-minus, nonglycosylated HA molecules. Although both the anchor-minus HA (HA500) and HA1 (HA325) polypeptides were targeted efficiently to the membrane, their glycosylation and transport patterns were shown to be different. During pulse-chase, the HA500 remained cell-associated with no detectable secretion into the extracellular medium, whereas the HA325 secreted into the medium. Furthermore, only the cell-associated and secreted forms of HA325 and not HA500 appeared to have undergone hyperglycosylation with the extensive addition of high-molecular-weight outer-chain mannans. Possible reasons for the observed phenotypic behavior of these two mutant HAs are discussed.

Hemagglutinin (HA) of influenza virus is probably the best characterized viral membrane protein. The three-dimensional structure of the protein, including the antigenic epitopes and the receptor binding site, has been elucidated (58). It has served as a model membrane protein in probing the membrane biogenesis of eucaryotic cells (38, 46) as well as in understanding the function of various domains of the protein in the vectorial transport process (12, 13, 32). cDNA copies of the gene encoding the HA have been expressed in both procaryotic and eucaryotic cells (32). Full-length HA, when expressed, is properly glycosylated and transported to the cell surface in eucaryotic cells (14, 20). Deletion of the C-terminal transmembrane portions of the protein results in the secretion of the anchor-minus HA into the medium (3, 15, 50), suggesting the role of the transmembrane domain as a stop-transfer sequence in anchoring the protein on the membrane (2). On the other hand, deletion of the N-terminal signal sequence results in the intracellular cytoplasmic expression of the signal-minus HA (15, 44; D. P. Nayak, unpublished data). The signal peptide of HA is also shown to target a heterologous simian virus 40 T antigen to the rough endoplasmic reticulum (RER) of mammalian cells, although further transport of the T antigen beyond the RER is blocked (45). Recent studies have also demonstrated that the ectodomain, but not the transmembrane or the cytoplasmic domain, may contain the signal(s) for the polarized apical transport of HA (31a). These studies have delineated some of the important functional domains of the HA polypeptide

in the intracellular transport process of higher eucaryotic cells.

Studies on the secretory mutants of the yeast *Saccharomyces cerevisiae* have identified a number of genes that may be involved in the transport pathway (35, 43). The ability of the yeast secretory pathway to process the secretory and membrane proteins of heterologous origin has recently been tested (8, 22, 24, 25, 33, 56, 60). Even though some foreign secretory proteins can be processed and secreted by yeasts, they are often processed improperly (22). Only in a few instances have signal sequences of foreign secretory proteins been shown to be recognized by the yeast secretory system with the eventual delivery of the mature protein into the medium (24, 60). Hence, the majority of the heterologous proteins are expressed and made to secrete from the yeast cells by providing a yeast mating type α -factor signal peptide (4, 16, 47, 61). In addition to the secretory proteins, a few heterologous membrane proteins have also been found to be translocated and glycosylated in *S. cerevisiae* (25, 56).

We have reported earlier the expression of the influenza viral HA gene in *S. cerevisiae*, but the level of expression was rather low (25), which could be due to a number of factors, including the lack of proper recognition and processing of the HA signal by the yeast translocation system. To determine if the heterologous signal sequence is properly recognized and correctly processed by yeasts, as well as to elucidate the role of topogenic domains of a heterologous protein in the yeast transport pathway, we have expressed variant forms of HA deleted in specific domains of the protein. The data presented here demonstrate that the signal sequence of HA is efficiently recognized and correctly

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FIG. 1. Schematic representation of the HA mutants. Symbols: $\square$, hydrophobic regions; 1, signal peptide (17 aa); 2, fusion peptide (25 aa) at the amino terminus of HA₂; 3, carboxyl-terminus transmembrane anchor region (24 aa); $\uparrow$, potential glycosylation sites (21) that are shown on the full-length WSN HA565. In the signal-minus HAs (HA484 and HA308), aa 2 to 17 were deleted. Asp is the first aa of the mature protein, and the signal cleavage occurs between Ala (17 aa) and Asp (18 aa) as shown by arrowhead. The last aa Leu* in HA500 and HA484 was coded by a linker sequence. The aa Thr in HA500 and HA484, as well as the last aa Ser in HA325 and HA308, are the aa 482 and 308 of the mature HA protein (21), respectively. For details, see Materials and Methods.

processed by the yeast signal peptidase and is critically important for targeting the ectodomain of HA to the membrane compartment. However, the transport and glycosylation patterns of the two HA deletion mutants appear to be qualitatively different. For example, HA500 lacking the C-terminal transmembrane anchor and cytoplasmic domains, although efficiently translocated, remains cell associated with little or no extracellular secretion. On the other hand, the other HA325, containing only the HA1 region but lacking the entire HA2 region including the transmembrane and cytoplasmic domains, is not only efficiently translocated across the RER membrane but also appears to be hyperglycosylated with an apparent M_r of >250,000 and secreted into the medium.

MATERIALS AND METHODS

Cells. The yeast strain 20B-12 (α *trp1 pep4-3*) was used to introduce recombinant plasmids selecting for Trp⁺ transformants. The bacterial strain *Escherichia coli* 294 (*thi endA hsdR*) was used to propagate all plasmid DNA used in this study. Growth conditions and media used for the growth of yeast transformants were described previously (25).

Construction of recombinant plasmids. The plasmid pSH482 (3) was cut with *Bam*HI to release the HA insert. The insert was then treated with *Eco*RI methylase to protect an internal *Eco*RI site present in the HA gene (21) and was filled in by the Klenow fragment, and the *Eco*RI linker (dGGAATTCC) was added to generate *Eco*RI cohesive ends on the inserts. The *Eco*RI HA inserts were cloned into a unique *Eco*RI site of pWY4, a yeast shuttle vector described before (25), and the resulting plasmid is called pWYHA500. To clone HA cDNA encoding 308 amino acids (aa) of HA1 into the yeast shuttle vector, the plasmid pHA1308 (7) was cut with *Eco*RI and *Bam*HI, and the *Eco*RI-*Bam*HI fragment was cloned into pBR322. The resulting plasmid, pBRHA308, was linearized with *Bam*HI and filled in by Klenow DNA polymerase. *Eco*RI linker was added, and the HA308 insert was isolated and cloned into pWY4. The resulting plasmid is called pWYHA308. Subsequently, by using unique restriction sites *Ssr*I and *Sal*I on the HA insert and the vector, respectively, the plasmids pWYHA325 and pWYHA484 were constructed. The cDNA HA insert HA500 encodes a polypeptide of the first 500 aa of the complete HA (565 aa) but missing the last 66 aa of HA2 that includes 12 aa of cytoplasmic domain, the entire transmembrane domain (24

aa), and 30 aa of the ectodomain (Fig. 1). One additional Leu residue was coded because of the linker before the termination codon TAG. The protein coded by HA500 DNA is also referred to as anchor-minus HA. HA484 DNA encodes a protein that lacks the signal sequence (aa 2 to 17) but possesses the first 482 aa of the mature HA (21) plus the first Met residue and the last Leu residue from the linker. The protein HA484 is referred to as signal-minus anchor-minus HA. HA308 DNA codes for a protein containing the first 308 aa of the mature HA1 without the signal peptide, whereas HA325 DNA codes for the same sequence plus the HA signal peptide (Fig. 1). All four recombinant plasmids were introduced into the yeast strain selecting for tryptophan-independent recombinants (25), and the transformants harboring the plasmids were named 20B12(pWYHA325), 20B12(pWYHA308), 20B12(pWYHA500), and 20B12(pWYHA484), and the corresponding polypeptides are referred to as HA325, HA308, HA500, and HA484 (Fig. 1).

Labeling and immunoprecipitation of HA-specific protein. The yeast transformants harboring various recombinant plasmids were grown in SD Trp⁻ selective medium as described before (25). Briefly, the yeast transformants were grown at 26°C to an optical density at 600 nm of 1.0 and concentrated to an optical density at 600 nm of 2.0. For the steady-state labeling, the yeast cells were incubated with either [³⁵S]methionine or [³⁵S]cysteine (50 μ Ci/ml) for 2 h at 26°C. The labeled cells were washed and lysed in 50 to 100 μ l of immunoprecipitation buffer (50 mM NaCl, 10 mM Tris hydrochloride [pH 7.4], 0.5% deoxycholate, 0.5% Triton X-100, 10 mM sodium azide [NaN₃], 1 mM phenylmethylsulfonyl fluoride) by vortexing vigorously for 5 min with glass beads. Additional immunoprecipitation buffer (450 μ l) was added, and the lysates were clarified in a Microfuge (Beckman Instruments, Inc., Fullerton, Calif.) for 5 min. Sodium dodecyl sulfate (SDS) was added to 0.1%, and the lysates were taken through the treatment steps before the addition of anti-WSN antibodies, as described before (25).

To analyze HA-specific proteins in the medium, the cells were labeled with [³⁵S]methionine (50 μ Ci/ml) in SD Trp⁻ medium containing 100 μ g of bovine serum albumin per ml and 50 mM leupeptin. The medium was recovered from the cells and concentrated in a Centricon 30 concentrator (Amicon Corp., Lexington, Mass.). The concentrated medium was adjusted with immunoprecipitation buffer containing 0.1% SDS and immunoprecipitated.

For pulse-chase experiments, the yeast transformants

were pulse-labeled for either 15 or 30 min with [35 S]methionine (100 μ Ci/ml; 1,400 Ci/mmol). At the end of the pulse, unlabeled methionine was added to 20 mM, and samples of cells were withdrawn at different times of incubation. The pulse-labeled cells were quickly chilled and lysed.

For immunoprecipitation, the lysates were incubated with antibodies for 2 h on ice, protein A-Sepharose was added, and the mixture was shaken for another 2 to 3 h. Immune complexes were collected and washed three times with the immunoprecipitation buffer. The immunoprecipitated protein samples were dissolved in 50 μ l of sample buffer (2 M urea, 6.5 mM Tris hydrochloride [pH 6.8], 10% glycerol, 2% SDS, 5% mercaptoethanol) and electrophoresed on SDS-10% polyacrylamide gel (29) containing 2 M urea. Gels were fixed in 300 ml of 30% methanol-10% acetic acid-10% trichloroacetic acid and washed thoroughly with water. The washed gel was soaked in 1 M sodium salicylate for 20 min and dried for fluorography. The gels were exposed to XAR-5 film (X-Omat; Eastman Kodak Co., Rochester, N.Y.) with an intensifying screen at -70°C .

Fractionation of yeast transformants. Spheroplasts of yeast cells were made, and fractionations were done as described earlier (25).

Endo H treatment of HA polypeptides and labeling of yeast cells in the presence of tunicamycin. The immune complex with the protein A-Sepharose beads was suspended in 20 μ l of 1% SDS and heated at 100°C for 3 min. H_2O (180 μ l) was added, and the sample was heated again for 1 min. The beads were removed by spinning in a Microfuge for 5 min, and the supernatant containing the proteins was adjusted to 50 mM sodium acetate, pH 5.5. The samples were divided into two aliquots; one was treated with 5 to 10 mU of endo- β -N-acetylglucosaminidase H (endo H; Miles Laboratories, Inc., Elkhart, Ind.) for 17 h at 37°C , and the other was mock treated. Both samples were dried, dissolved in sample buffer, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). For the tunicamycin treatment, the yeast transformants were grown to an optical density at 600 nm of 1.00 and concentrated twofold, as described above. The yeast cells were pretreated with tunicamycin (10 μ g/ml) for 30 min at 26°C . The treated cells were quickly spun down, incubated in fresh medium containing tunicamycin (10 μ g/ml), and labeled with [35 S]methionine (50 μ Ci/ml) for 60 to 90 min. Lysis and immunoprecipitation of the tunicamycin-treated cell lysates were done as described above.

NH_2 -terminal aa analysis of HA polypeptides made in *S. cerevisiae*. HA-specific polypeptides were immunoprecipitated from the ^{35}S -labeled yeast lysates and treated with endo H as described above. The treated samples were suspended in 50 μ l of sample buffer (10 mM Tris hydrochloride [pH 6.8], 10% glycerol, 2% SDS, 2% β -mercaptoethanol) and analyzed by SDS-PAGE (10% polyacrylamide). The Tris-glycine-SDS buffer that was run had 0.1 mM sodium thioglycolate to destroy free radicals in acrylamide, and recrystallized SDS was used as described previously (23). The gel was fixed in 300 ml of 30% isopropanol-10% acetic acid for 15 min and dried for autoradiography. The gel region corresponding to the HA polypeptide position was excised and allowed to swell in 10 ml of 25 mM Tris hydrochloride (pH 8.3)-0.1% SDS-0.1 mM sodium thioglycolate. The gel was washed with 10 to 20 ml of the same buffer and sliced into small pieces with a razor blade. The gel pieces were loaded onto a 5-ml pipette tip, and the proteins were electroeluted into a dialysis tube (Spectrum Medical Industries, Inc., Los Angeles, Calif.) at 4°C for 15 to 20 h. To minimize any adsorption of the labeled protein to the dialysis

FIG. 2. Expression of HA-specific polypeptides in *S. cerevisiae*. Yeast transformants were labeled with [35 S]methionine (50 μ Ci/ml) for 2 h, and cell lysates were immunoprecipitated: 20B12(pWYHA500) (lanes 1, 4), 20B12(pWYHA484) (lanes 2, 3), pWYHA308 (lane 5), and pWYHA325 (lane 6). Arrowheads indicate the HA-specific polypeptides immunoprecipitated either with polyclonal (IgG fraction of anti-WSN antibody) (lanes 1 and 3 to 6) or with monoclonal anti-HA antibody (lane 2). Molecular sizes (in kilodaltons) as estimated from marker proteins are shown.

membrane, 50 μ g of bovine serum albumin was added to the gels during electroelution. The eluent was recovered from the dialysis bag and dried in a Speed Vac concentrator (Savant Industries, Inc., Hicksville, N.Y.). The dried samples were suspended in 200 μ l of 10 mM NH_4HCO_3 -0.02% SDS by heating at 65°C for 10 to 15 min. Six volumes of acetone mix (acetone-acetic acid-sodium triethanolamine-water [88:5:5:2]) were added to precipitate the protein overnight at -20°C . The precipitate was collected by centrifugation (20 min) in a Microfuge in the cold room. The protein pellet was washed three times with the acetone mix and once with acetone alone. The final pellet was dried and suspended in 40 to 50 μ l of 10 mM NH_4HCO_3 -0.02% SDS. The protein samples were applied to a microsequencer (Applied Biosystem, Foster City, Calif.) and subjected to Edman degradation. Anilino thioglycoline derivatives of the aa residues were collected and counted in a liquid scintillation counter.

RESULTS

Biosynthesis of HA-specific proteins in *S. cerevisiae*. To determine the nature of the HA-specific polypeptides expressed in *S. cerevisiae*, transformants harboring different deletion mutants of HA cDNA (Fig. 1) were labeled with [35 S]methionine. Labeled HA-specific proteins were immunoprecipitated either with the immunoglobulin G (IgG) fraction of anti-WSN antibodies or with anti-HA monoclonal antibodies and analyzed by SDS-PAGE (Fig. 2). The yeast transformant [20B12(pWYHA500)] harboring the plasmid pWYHA500 synthesized a protein of M_r 69,000 (HA500; anchor-minus HA) that was precipitable by both polyclonal (Fig. 2, lane 1) and monoclonal (Fig. 2, lane 4) antibodies. In contrast, the plasmid pWYHA484 directed the synthesis of a protein of M_r 58,000 (HA484; signal-minus anchor-minus HA) that was poorly recognized by monoclonal antibodies (Fig. 2, lane 2) but efficiently recognized by the polyclonal antibodies (Fig. 2, lane 3). The yeast cells carrying the plasmid pWYHA308 made a polypeptide of M_r 40,000 (HA308; Fig. 2, lane 5), but the plasmid pWYHA325 did not appear to direct the synthesis of a polypeptide of expected

FIG. 3. Stability and processing of HA484 and HA500 polypeptides expressed in *S. cerevisiae*. Transformants were labeled with [35 S]methionine (100 μ Ci/ml) for 15 min and chased in the presence of unlabeled methionine (20 mM) for different time periods as noted on the top of the gel (Min). Cell lysates were immunoprecipitated with anti-WSN IgG: 20B12(pWYHA484) (lanes 1 to 5) or 20B12(pWYHA500) (lanes 6 to 10). Arrowheads indicate various forms of HA polypeptides synthesized in pulse-labeled lysates (lanes 1 and 6) and in the subsequent chase (lanes 2 to 5 and 7 to 10). Upward arrowhead (lane 6) points to a protein that probably represents nonglycosylated full-length anchor-minus HA (HA500). Molecular size marker proteins (lane M) are shown (in kilodaltons).

size (Fig. 2, lane 6). However, a protein of higher M_r (>250,000) is found at the top of the gel (HA325; Fig. 2, lane 6). Analysis of HA-specific polypeptides made in the yeast cells demonstrated that viral genes, when placed under the proper yeast regulatory signal, would direct the synthesis of corresponding viral proteins and that the yeast translation system recognized the proper translation-initiation and -termination signals encoded by the viral mRNA. Furthermore, a difference in the M_r (11,000) between HA500 (Fig. 2, lanes 1 and 4) and HA484 (Fig. 2, lane 3) was observed and was unlikely to be due to the M_r of the signal peptide (17 aa) coded by the HA500 DNA (Fig. 1). Similarly, the difference in the M_r of HA325 (Fig. 2, lane 6) and HA308 (Fig. 2, lane 5) was even much greater. In fact, the apparent molecular weight of HA325 (Fig. 2, lane 6) was even greater than that of HA500 (Fig. 2, lanes 1 and 4) and was quite unexpected. We present evidence below that the differences in molecular weights between these forms of HA polypeptides made in the yeast cells reflected the additional processing during their transport in the secretory pathway of yeast.

Stability and processing of HA500 and HA484 polypeptides made in the yeast cells. The yeast transformants were pulse-labeled with [35 S]methionine for 15 min and chased for different time periods in the presence of excess (20 mM) unlabeled methionine (Fig. 3). The cell lysates were immunoprecipitated with the IgG fraction of anti-WSN polyclonal antibodies. Figure 3 (lanes 1 to 5) shows that the HA polypeptide (signal-minus HA484; M_r , 58,000) synthesized in 20B12(pWYHA484) cells during a 15-min pulse did not undergo any further increase in molecular weight during the 3-h chase but was progressively degraded with a half-life of 30 min, reflecting the instability of the signal-minus HA484 in *S. cerevisiae*. HA484 was predominantly localized in the cytoplasm and not associated with the membrane (data not shown). Therefore, the plasmid pWYHA484 directed the synthesis of a signal-minus HA polypeptide that was made in the cytoplasm, remained unglycosylated, and was unstable.

Essentially similar properties of the signal-minus HA have been demonstrated in mammalian cells (15, 44). However, the HA500 polypeptide (M_r , 69,000) made in the cell 20B12(pWYHA500) (Fig. 3, lanes 6 to 10) was quite stable compared with the signal-minus HA484 (M_r , 58,000) with a half-life of 2.5 h, which is the same as the generation time of yeast cells harboring pWYHA500. Moreover, a 15-min pulse-labeling of 20B12(pWYHA500) showed a major polypeptide of 69 kilodaltons (kDa) and a minor polypeptide of 60 kDa (Fig. 3, lane 6). During the subsequent chase, the minor polypeptide (M_r , 60,000) disappeared with an approximate half-life of less than 15 min (Fig. 3, lanes 6 and 7), which was faster than the degradation kinetics of HA484 (Fig. 3, lanes 4 and 5). The 60-kDa protein that was slightly larger (1 to 2 kDa) than the signal-minus HA484 (58 kDa) might correspond to the full-length unglycosylated HA500, whereas the predominant 69-kDa polypeptide might represent the glycosylated form of HA500.

Analysis of mutant HA polypeptides (HA500, HA484, HA325, and HA308) for glycosylation, transport, and secretion. Since the HA has potential glycosylation sites (Asn-X-Thr/Ser), these polypeptides are expected to be modified by the transfer of dolichol phosphate to the polypeptide backbone when targeted to the RER membrane of yeasts. In yeasts, the addition of core high mannose and outer-chain mannose (mannans) residues are known to occur in separate compartments of the secretory pathway (1, 5, 10, 34). Glycosylation patterns of the HA500 and HA484 polypeptides were analyzed by their sensitivity to endo H, which is known to remove the mannose-rich oligosaccharides from proteins (52). Figure 4A shows that the HA484 protein made in 20B12(pWYHA484) was unaffected by the endo H treatment (Fig. 4, lanes 1 and 3). In contrast, the HA500 polypeptide made in 20B12(pWYHA500) was sensitive to the endo H treatment, demonstrating that the HA500 polypeptide was glycosylated. Since, unlike higher eucaryotes (28), *S. cerevisiae* does not add endo H-resistant complex sugars (1, 5), the HA polypeptide (HA500) made in *S. cerevisiae* maintained its endo H sensitivity even after a 90-min chase. However, the HA500 polypeptide treated with endo H (Fig. 4, lanes 5, 7, and 9) moved slightly slower than did the signal-minus HA484 (Fig. 4, lanes 1 to 4).

Fractionation studies of the yeast transformant synthesizing HA500 (anchor-minus HA) showed that the protein was predominantly associated with the membrane, but absent either in the periplasmic space or in the cytoplasm (Fig. 4B). The majority of the HA500 in the membrane fraction (Fig. 4B, lane 4) appeared to be cleaved into two polypeptide components. Since the cleavage of HA500 was not observed in the pulse-chase experiments (Fig. 3 and 4A), it must have occurred during the Zymolyase treatment, suggesting a possible protease contamination of the Zymolyase mixture. Therefore, we cannot completely rule out the presence of a small amount of HA500 in the periplasmic fraction. A relatively homogeneous glycosylation pattern as observed from the sharpness of the band, as well as only a slight increase in molecular weight during the chase (Fig. 3 and 4A), are suggestive of the RER residence of HA500 (9), although its transport to the Golgi compartment without the extensive addition of outer-chain mannans cannot be ruled out. No HA-specific protein could be immunoprecipitated from the cell-free medium of 20B12(pWYHA500) that was labeled at the steady state for 2 h (data not shown).

Unlike the plasmid pWYHA500, the plasmid pWYHA325 appears to direct a protein of extremely high molecular weight that is even retarded from entering into SDS-PAGE

FIG. 4. (A) Analysis of HA484 and HA500 polypeptides for glycosylation. Yeast transformants were labeled with [35 S]methionine (100 μ Ci/ml) for 15 min and chased for the time periods (in minutes) shown at the top of the gel. Other conditions are the same as those for Fig. 3. 20B12(pWYHA484) (lanes 1 to 4) and 20B12(pWYHA500) (lanes 5 to 10) were analyzed. The end of a 15-min pulse-label is indicated (0'). Endo H-treated polypeptides (lanes 1, 3, 5, 7, and 9) and mock-treated polypeptides (lanes 2, 4, 6, 8, and 10) are shown. Molecular weight markers (lane M) are shown (in kilodaltons). (B) Membrane association of HA500. The yeast transformants harboring the plasmid pWYHA500 [20B12(pWYHA500)] were labeled with [35 S]methionine (50 μ Ci/ml) for 2 h, spheroplasts were made, and subcellular fractionations were carried out as described previously (25). Immunoprecipitation was done with monoclonal anti-HA antibodies. Total cell lysate (T; lane 1), soluble cytoplasmic fraction (C; lane 2), periplasmic supernatant (S; lane 3), and insoluble membrane fraction (M; lane 4) are shown. Arrowheads indicate the HA-specific polypeptides.

(10% polyacrylamide) (Fig. 2, lane 6). Since outer-chain mannose residues are added in the Golgi complex of the yeast secretory pathway (10), it is likely that HA325 has traversed the same pathway as does a yeast secretory protein. Therefore, we analyzed both the intracellular and extracellular forms of HA308 and HA325 polypeptides made in the yeast transformants 20B12(pWYHA308) and 20B12(pWYHA325) (Fig. 5). As expected, the HA308 had a molecular size of 40 kDa and was not affected by the endo H treatment (Fig. 5, lanes 1 and 2). HA308 was also not found in the medium, suggesting that in the absence of the N-terminal signal peptide, HA308 failed to be targeted to the membrane compartment. Therefore, both HA308 and HA484 possessed similar properties; i.e., they were cytoplasmic and unglycosylated. In contrast, the HA325 protein made in 20B12(pWYHA325) was found to be secreted into the medium (Fig. 5, lane 8). Furthermore, two intracellular forms of HA325 were present. One form (47 kDa) possibly represents the core-glycosylated form in the RER, and the other (>250 kDa), possessing outer-chain mannans, might have traversed the Golgi complex (Fig. 5, lane 6). When treated with endo H, both forms migrated as 42 to 44 kDa species (Fig. 5, lane 5). However, the medium contained only the large-molecular-size form (>250 kDa) with the added outer-chain mannose (Fig. 5, lane 8) and was also sensitive to endo H (Fig. 5, lane 7). After endo H treatment of HA325, the majority of the large-molecular-size proteins (Fig. 5, lane 6) were converted to smaller species (42 kDa; Fig. 5, lane 5), whereas the same endo H treatment of 20B12(pWYHA500) lysate did not have any effect on the proteins present at the top of the gel (Fig. 5, lanes 9 and 10). These results show that the HA325 polypeptide traversed the yeast secretory pathway and secreted into the medium, whereas the HA500 protein, although translocated across the RER, remained cell associated with no detectable extracellular secretion.

Processing and kinetics of secretion of mutant HA polypeptides. To further investigate the nature and rate of secretion of the HA325 polypeptide in the yeast transform-

ant, pulse-chase experiments were carried out (Fig. 6A). Accordingly, the yeast transformant 20B12(pWYHA325) was pulse-labeled for 15 min (Fig. 6A, lane 3) and chased for different time periods in the presence of unlabeled methionine in excess. Both the intracellular lysates (Fig. 6A, lanes 3, 5, 7, and 9) and the media (Fig. 6A, lanes 4, 6, 8, and 10) were analyzed. After 15 min of pulse-labeling (Fig. 6A, lane 3), two intracellular forms were present. As observed for

FIG. 5. Analysis of HA308 and HA325 polypeptides for glycosylation and secretion. The yeast transformants were labeled for 2 h with [35 S]methionine (50 μ Ci/ml). Media and cell lysates were used for immunoprecipitation with anti-WSN IgG and for endo H treatment. Cell lysates used were 20B12(pWYHA308) (lanes 1 and 2), 20B12(pWYHA325) (lanes 5 and 6), and 20B12(pWYHA500) (lanes 9 and 10). Growth media used were 20B12(pWYHA308) (lanes 3 and 4) and 20B12(pWYHA325) (lanes 7 and 8). Arrowheads indicate various forms of HA polypeptide before and after endo H treatment. Endo H-treated polypeptides (lanes 1, 3, 5, 7, and 9) and mock-treated polypeptides (lanes 2, 4, 6, 8, and 10) are shown. Marker proteins (lane M) are shown (in kilodaltons).

FIG. 6. (A) Secretion of HA325 into the medium. Yeast transformant 20B12(pWYHA325) was pulse-labeled with [35 S]methionine (100 μ Ci/ml) for 15 min and chased for the time indicated at the top (min). HA-specific polypeptides immunoprecipitated from cell lysates (lanes 3, 5, 7, and 9) and media (lanes 4, 6, 8, and 10) and from the cell lysate of 20B12(pWYHA308) (lanes 1 and 2) are shown. Arrowheads indicate different forms of HA made in *S. cerevisiae*: HA308 signal-minus HA (lanes 1 and 2) and HA325 nonglycosylated full-length HA and the RER form (lane 3). Arrowhead at the top indicates added high-molecular-weight outer-chain HA325 polypeptide present in both cell lysate and extracellular medium. (B) Analysis of HA-specific polypeptides in extracellular medium. The yeast transformant harboring pWYHA500 was pulse-labeled for 15 min and chased for 3 h in the presence of excess unlabeled methionine. The cells were collected by centrifugation, and the medium was concentrated by using a Centricon concentrator. The cell lysis and immunoprecipitation of the samples were done as described in Materials and Methods. Immunoprecipitation of cell lysates (lanes 1, 3, and 6) and medium (lanes 2, 4, and 7) are shown. Immunoprecipitation of pulse-labeled proteins (15 min) from cell lysate (lane 1) and medium (lane 2) are shown. After a 3-h chase, immunoprecipitation of pulse-labeled proteins from cell lysate (lane 3) and medium (lane 4) are shown. Longer exposure of lanes 3 to 5 is shown (lanes 6 to 8). Markers (lanes 5 and 8) are shown (in kilodaltons).

HA500, one form was apparently nonglycosylated full-length HA325 (M_r , 42,000; Fig. 6A, lane 3, arrow) that disappeared at a faster rate. The other major form (M_r , 47,000), presumably the core-glycosylated RER form of HA325, matured into higher-molecular-weight species, and the maturation process appeared to be completed in about 60 min (Fig. 6A, lane 7). When the medium was examined, only the higher-molecular-weight polypeptide was found (M_r , >250,000). Some higher-molecular-weight form was already present in the medium at the end of a 15-min pulse (Fig. 6A, lane 4), demonstrating the relatively high efficiency of the processing and secretion. However, 30 to 40% of the higher-molecular-weight HA325 polypeptide remained cell associated even after 2 h of chase (Fig. 6A, lane 9) and is likely to be present in the Golgi or post-Golgi compartment. It was not detected in the periplasmic space (data not shown). However, as mentioned earlier, because of the possibility of protease contamination in the Zymolyase, we cannot rule out the presence of a small amount of HA325 in the periplasm.

Similar pulse-chase kinetics were done for the yeast transformants that synthesized HA500 protein. The HA500 made at 15 min of pulse (Fig. 6B, lane 1) remained cell associated even after 3 h of chase (Fig. 6B, lane 3), and the medium fraction was found to be negative for any HA-specific polypeptide (Fig. 6B, lane 4). The longer exposure of the gel is included to show that there is little or no secretion of HA500 polypeptide (Fig. 6B, lanes 6 and 7). The medium was also found to be negative for HA-specific polypeptides during 1 or 2 h of chase (data not shown). Furthermore, as noted earlier, the fact that the intracellular form of HA500 is a stable protein and did not show any appreciable decrease during a 3-h chase (Fig. 3) also indicates that the HA500 polypeptide was not secreted into the extracellular medium. These data demonstrate that the HA325 was efficiently packaged into secretory vesicles and transported to the cell

surface for its eventual delivery into the medium, whereas the HA500 appears to be predominantly localized intracellularly with no apparent extracellular secretion.

Analysis of HA polypeptides made in the yeast transformants in the presence of tunicamycin. Although the endo H treatment of HA325 and HA500 increased the mobility of the proteins, they migrated slightly slower on the gel than did the corresponding signal-minus HA polypeptides (Fig. 4A and 5). This could be due to the retention of one *N*-acetylglucosamine residue on the polypeptide backbone or to the lack of signal cleavage or both. To distinguish between these possibilities, HA polypeptides were synthesized in the presence of tunicamycin, which inhibits the addition of dolichol phosphate and thus prevents the glycosylation of translocated polypeptides (30). Figure 7 shows that, as in the case with endo H treatment, both signal-minus HAs (HA308 and HA484) were unaffected by the tunicamycin treatment (lanes 1, 2, 5, and 6). However, the tunicamycin treatment caused a considerable reduction in the synthesis of HA325 and HA500 polypeptides, and high-molecular-weight glycosylated forms of HA were completely absent (Fig. 7, lanes 3 and 7). Furthermore, tunicamycin-treated HA325 (Fig. 7, lane 3) and HA500 (Fig. 7, lane 7) exhibited a faster mobility, but they still migrated slightly slower than did the corresponding signal-minus HA polypeptides (compare Fig. 7, lanes 2 and 3, and 6 and 7) and comigrated with the small fraction of presumably nonglycosylated HA polypeptides present in untreated control samples (compare Fig. 7, lanes 3 and 4, and 7 and 8 [arrowheads]). The difference in the apparent molecular size of the signal-minus HA and the HA made in the presence of tunicamycin is approximately 2.0 kDa, which is expected for the mass of the 17-aa signal peptide. These results suggest that either the HA polypeptides were undergoing some tunicamycin resistance processing (e.g., *O*-glycosylation) or, although recognized by the yeast

translocation machinery, the HA signal was not cleaved by yeast signal peptidase.

N-terminal aa analysis of HA polypeptides made in *S. cerevisiae*. To unequivocally determine whether the signal peptide of HA is cleaved in *S. cerevisiae*, sequence analyses of the N termini of HA500 and HA325 were undertaken. However, because of the degradation of the secreted HA325 polypeptide in the medium, we were unable to obtain any meaningful sequence analysis of the N terminus of the secreted form of HA325 in several attempts. Therefore, we have determined the N-terminal aa sequence of the major intracellular form of HA325 and HA500 polypeptides made in *S. cerevisiae*. The yeast transformants harboring the plasmids pWYHA325 and pWYHA500 were pulse-labeled (30 min) separately with either [35 S]methionine or [35 S]cysteine and chased for 90 min in excess of the corresponding unlabeled aa. Immunoprecipitated HA polypeptides from the yeast cell lysates were subjected to endo H treatment to remove oligosaccharides and analyzed by SDS-PAGE (10% polyacrylamide). The proteins were electroeluted from the gels corresponding to the HA protein bands as described in Materials and Methods. The [35 S]methionine- and [35 S]cysteine-labeled proteins were mixed together and subjected to Edman degradation by using a microsequencer. Figure 8 shows the amount of radioactivity recovered from each Edman degradation cycle. The peak of radioactivity was present at position 4 of the sequencing cycle with both HA325 and HA500 polypeptides. The Cys residue is aa residue 4 in the mature HA protein after signal cleavage (Fig. 8). Since each polypeptide contained the same amount of radioactivity (1,500 cpm per residue) for either methionine or cysteine, the amount of label in position 4 is significant and suggests the correct processing of HA signal peptide by yeast signal peptidase. This view is further confirmed by the absence of a significant amount of radioactivity in position 1 (methionine), although we could not rule out the presence of a small fraction of uncleaved cytoplasmic HA polypeptides. Moreover, the sequence analysis of HA polypeptide labeled with both [35 S]methionine and [3 H]leucine failed to recover radioactivity at the expected positions (position 1 for methionine and positions 5, 6, 8, and 9 for leucine; Fig. 8) in the

FIG. 7. Analysis of the HA polypeptide made in yeast transformants treated with tunicamycin. Yeast transformants were pulse-labeled for 15 min with [35 S]methionine (100 μ Ci/ml) in the presence (lanes 1, 3, 5, and 7) or absence (lanes 2, 4, 6, and 8) of tunicamycin (Tm; 10 μ g/ml). Arrowheads indicate signal-minus HA308 (lanes 1 and 2) and presumably nonglycosylated full-length HA325 (lane 4) and HA500 (lane 8). Molecular size markers (lane M) are shown (in kilodaltons).

FIG. 8. N-terminal aa analysis. Intracellular HA500 or HA325 polypeptides were labeled separately with [35 S]methionine or [35 S]cysteine, and the labeled HA polypeptides were purified, mixed together, and subjected to Edman degradation as described in the text. Radioactivity from each cycle of the reactions was counted as described in the text. HA500 and HA325 indicate the anchor-minus HA and HA1, respectively. Analysis was done up to seven cycles for HA325 and five cycles for HA500. The N-terminal sequence of the HA signal peptide and cleavage site (arrow) are shown below the graph. The cysteine (Cys) residue at position 4 of the mature HA is underlined.

signal peptide (data not shown) and thus demonstrated the absence of the signal peptide in the processed HA polypeptides. Furthermore, the analysis of [35 S]cysteine-labeled signal-minus HA484 released radioactivity predominantly at cycle 5 (data not shown), corresponding to the position of cysteine in the signal-minus HA (Fig. 1). Because of the decreased synthesis and increased instability of the unglycosylated HA325 and HA500 polypeptides made in the presence of tunicamycin, their N-terminal aa sequence could not be determined. These data show that the yeast signal peptidase is capable of recognizing the HA signal and processing it at the correct cleavage site, as in the case of

mammalian signal peptidase. However, we are left with the paradox of slower mobility of HA500 and HA325 in the presence of tunicamycin when compared with the corresponding signal-minus HA polypeptides, and this could be due to other modifications (e.g., *O*-glycosylation) or aberrant mobility of the polypeptides.

DISCUSSION

Recently, a great deal of interest has been focused on studying the yeast secretory process because of the functional and structural similarities between the transport pathways of higher and lower eucaryotes. Because *S. cerevisiae* is genetically easily manipulable, its secretory pathway has been dissected in greater detail by the analysis of conditional lethal mutants defective in various steps of protein transport (35, 43). Additionally, *S. cerevisiae* is being used in the biotechnology industry for the biosynthesis of medically important proteins which require posttranslational processing and modification (e.g., glycosylation) for preserving either the functionally active sites or correct antigenic epitopes. Translocation across the RER membrane is an early step in directing secretory, lysosomal, and membrane proteins into the transport pathway (37, 53, 57), and a major impediment in using *S. cerevisiae* in the biosynthesis of foreign glycoproteins appears to be poor recognition of the heterologous signal sequence by the yeast translocation system. Although a number of heterologous proteins have been expressed and shown to be secreted by the yeast cells (47), the majority of them utilize the signal sequence of yeast mating type α factor (4, 47). In a few cases in which the foreign signal sequence is recognized, it is often improperly cleaved, resulting in poor extracellular secretion of the polypeptides (22). Even though signal sequences have been shown to be interchangeably recognized among procaryotes or eucaryotes (51, 55). *S. cerevisiae* appears to be more stringent in signal recognition of foreign secretory proteins (39, 43). Data presented in this report show that the HA signal is not only efficiently recognized but also correctly processed, demonstrating a possible structural compatibility of the HA signal for the yeast translocation machinery, and therefore may be of potential use in expressing other foreign glycoproteins in *S. cerevisiae*.

The signal sequence, although critically required for targeting the proteins to the translocation-competent membranes (RER), is not sufficient for subsequent transport and secretion of a polypeptide. Two variant forms of HA lacking anchor domains, when expressed in *S. cerevisiae*, behave differently in the intracellular protein traffic. The HA500 remains cell associated and cannot be detected in the extracellular medium, whereas HA325 is efficiently processed and extracellularly secreted. Clearly, the different transport behavior of HA500 cannot be explained because of its instability. In fact, the intracellular form of HA500 is stable and does not appreciably decrease in amounts after a 3-h chase (Fig. 3). These observations would also corroborate the observation that there is little or no secretion of HA500 into the extracellular medium (Fig. 6B). The transport behavior of the HA500 in *S. cerevisiae* appears to be similar to its properties observed in mammalian cells. In CV1 cells, HA500, when expressed under the control of the late simian virus 40 promoter, is secreted very slowly, and the majority of the intracellular HA500 was endo H sensitive and localized by indirect immunofluorescence to be in the RER or *cis* compartment of the Golgi apparatus (3).

HA325, on the other hand, is not only cotranslationally glycosylated in the RER, but becomes hyperglycosylated

and extracellularly secreted. Since only the extensively glycosylated form is secreted, the secreted HA325 is most likely routed via the Golgi complex of the yeast transport pathway. However, the secretion of HA325 appears to be slower when compared with the constitutive secretion of native yeast proteins, such as invertase and α -factor pheromone (27). In addition to the signal sequence, other structural features are likely to play an important role in efficient transport and secretion of proteins in the membrane traffic of both higher and lower eucaryotes. However, the nature of these structural features (traffic or transport signals) remains elusive. Experiments to date have mostly documented the negative aspects, i.e., factors causing a block in the transport or secretion. For example, changes in the primary structure of proteins are known to affect the protein transport (3, 9, 31, 59). In some cases, glycosidic modifications might confer a positive signal for vesicular protein transport (17). In the present report, we have clearly demonstrated that a deletion of 175 aa from the C terminus of HA500 had allowed a predominantly cell-associated protein to be secreted extracellularly. Although the nature of the structural changes caused by the deletion remains unclear, the removal of the hydrophobic N terminus of HA2 (fusion peptide) may have increased the solubility of HA1 and thereby allowed the protein to be transported to the cell surface for eventual secretion into the medium. In the case of rotavirus VP7 protein, which remains localized in the RER, the deletion of a hydrophobic sequence makes the protein move from the RER to the cell surface and be secreted in the medium (36). Whether HA325 will exhibit a similar transport behavior (i.e., extracellular secretion) in mammalian cells remains to be determined.

Clearly, the intracellular retention of HA500 is not due to the lack of cleavage of the signal sequence, although a defect in signal cleavage can retard the intercompartmental transport of proteins (6, 18, 42). Analysis of the N-terminal aa sequence of both HA500 and HA325 polypeptides shows correct signal processing in the yeast cells. Other structural features such as protein topology and protein conformation may have affected the intracellular transport of HA500. Again, the protein conformation, in addition to affecting transport, may determine the extent of glycosidic modification of a protein. For example, carboxypeptidase Y, a lysosomal enzyme, is not as extensively glycosylated as the yeast invertase is, even though both enzymes are transported through the same Golgi compartment during their vesicular transport (48, 49).

The secreted HA325 is so extensively glycosylated that it fails to enter into SDS-PAGE gel (10% polyacrylamide) and has an apparent molecular size of >250 kDa. Proteins with such a large mass are not usually secreted in the medium. Among the large-molecular-mass secretory proteins, unprocessed glycosylated mating α -factor which is secreted in the Kex2 mutant has a mass of 150 kDa (26). On the other hand, another glycoprotein, invertase (M_r , 120,000), although secreted into periplasm, does not pass through the cell wall, probably because of the oligomer formation in the periplasmic space (R. Schekman, personal communication). In fact, hyperglycosylation may have aided in the efficient secretion of HA325 in the medium, possibly by increasing its solubility and stability. After tunicamycin treatment, no unglycosylated HA325 is found in the medium, which may reflect reduced synthesis as well as increased degradation.

Stability of the HA polypeptides made in *S. cerevisiae* suggests that they are not recognized as "junk" proteins which would be routed to the vacuoles for degradation.

However, the signal-minus, unglycosylated HA is relatively unstable compared with its glycosylated counterpart, as is also seen in mammalian cells (15, 43). It is possible that the signal-minus HA is targeted to the vacuoles for degradation, as the mature λ chain was found to be localized in the vacuolar compartment of yeasts (60).

Our studies of influenza viral HA expression in a heterologous system have reinforced the importance of an efficient signal recognition mechanism in targeting the proteins to the cellular membrane compartment *in vivo*. Recent studies of the *in vitro* reconstitution of translocation assay of yeasts (19, 40, 41, 54) have uncovered some novel features in the initial steps of the translocation process. Use of the *in vitro* system in the translocation of a heterologous protein like HA would be useful in elucidating the subtle differences between the homologous and heterologous translocation processes.

Isolation and characterization of temperature-sensitive mutants in the secretory pathway of the yeast *S. cerevisiae* have identified a large number of genes (gene products) involved in various steps of the transport process (35, 43). However, mutations affecting the initial steps of translocation, e.g., signal recognition and targeting to the membrane, have not yet been identified. Availability of the HA mutants and yeast mutants that are defective in the protein transport process would allow us to analyze the details of the signal recognition step in *S. cerevisiae*.

The intracellular retention of HA500 does not appear to cause any deleterious effect on the normal physiology of the yeast cells. This could be due to a low-level expression of the polypeptide (around 0.3% of the total protein) and may not be sufficient to inhibit the global protein traffic saturating the transport components. In the present study, we used the alcohol dehydrogenase 1 promoter, which because of its constitutive nature yields a low level of expression. However, with a regulated promoter it should be possible to increase the yield of the protein and thereby "poison" the traffic system. If so, such a system would provide us with a valuable tool to isolate mutants by selecting for revertants which would be useful in identifying additional components in the transport pathway.

In conclusion, we have shown that the HA signal sequence is obligatory for the translocation of the ectodomain of HA in *S. cerevisiae*. The HA signal peptide is not only recognized efficiently by the yeast translocation system but also cleaved correctly by the yeast signal peptidase. We have also demonstrated that one of the HA mutants (HA325) traverses the entire yeast secretory pathway, becomes hyperglycosylated, and is efficiently secreted in the medium, whereas the other (HA500) appears to remain cell associated with little or no secretion into the extracellular medium. Heterologous signal sequences like HA may be potentially useful in the biosynthesis of functionally active and medically important foreign secretory or glycoproteins in yeasts as well as in further understanding of the yeast secretory pathway.

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