The heat shock protein (Hsp) 70/Hsp40 chaperone system plays an essential role in cell physiology, but few of its in vivo functions are known. We report that biogenesis of Axl1p, an insulinase-like endoprotease from yeast, is dependent upon the cytosolic Hsp40 protein Ydj1p. Axl1 is responsible for cleavage of the P2 processing intermediate of pro-a-factor, a mating pheromone, to its mature form. Mutant ydj1 strains exhibited a severe mating defect, which correlated with a 90% reduction in a-factor secretion. Reduced levels of a-factor export were caused by defects in the endoproteolytic processing of P2, which led to its intracellular accumulation. Defective P2 processing correlated with the reduction in the steady state level of active Axl1p. Two mechanisms were uncovered to explain why Axl1p activity was diminished in ydj1 strains. First, AXL1 mRNA levels were reduced ydj1 strains. Second, the half-life of newly synthesized Axl1p was greatly diminished in ydj1 strains. Collectively, these data indicate Ydj1p functions to promote Axl1p mRNA accumulation and in addition appears to facilitate the proper folding of nascent Axl1p. This study is the first to suggest a role for Ydj1p in RNA metabolism and identifies Axl1p as an in vivo substrate of the Hsp70/Ydj1p chaperone system.

Molecular chaperones in the Hsp701 class play an essential role in cell physiology (1–3). However, the in vivo substrates and cellular functions of Hsp70 are not well defined (4–6). Many functions of Hsp70 are specified through its interactions with Hsp40 co-chaperone proteins (7–9). Therefore, analyzing the functions of its Hsp40 co-chaperone proteins can identify the reactions that are catalyzed by Hsp70. Ydj1p is an Hsp40 protein that is localized in the yeast cytosol and acts to regulate Hsp70 Ssa protein function (10–12). Genetic studies indicate that Ydj1p acts with Ssa proteins to promote protein translocation across membranes (13, 14), ubiquitin-dependent protein degradation (15, 16), and signal transduction to the nucleus (17, 18). To facilitate these reactions, Ydj1p functions as a molecular chaperone to bind and deliver non-native polypeptides to Hsp70 (19). In addition, Ydj1p is responsible for regulation of the Hsp70 ATP hydrolytic cycle (10, 20). Regions within the J-domain of Ydj1p act to regulate Hsp70 ATPase activity (19, 21). On the other hand, independent regions within the zinc finger-like domain and carboxyl terminus of Ydj1p carry out its chaperone functions (19, 22). The combined activities of both the J-domain and polypeptide-binding domain are required in order for Ydj1p to assist Hsp70 in protein folding (19).

To further define the cellular functions of the Hsp70 Ssa1/ Ydj1p chaperone system, we examined how mutations in Ydj1p influence the mating efficiency of MATa yeast strains. The yeast mating reaction was chosen as a model test system because Ydj1p and Hsp70 Ssa1 were previously shown to function in biogenesis of a-factor (13, 23, 24), the pheromone secreted by MATa cells. However, whether Ydj1p could also function to promote a-factor biogenesis was not known.

To mate efficiently, MATa and MATa yeast must secrete their respective mating pheromones, which triggers signaling events that promote the fusion of haploid cells (25). In MATa and MATa cells, these pheromones are synthesized in a proform and are processed to mature forms by peptidases that are homologous to the convertases that process prohormones and growth factors in higher eukaryotes (26, 27). Prepro-a-factor is processed by endoproteases that are localized within the lumen of the endoplasmic reticulum and Golgi apparatus and then is secreted via the classical secretory pathway (28, 29). Ydj1p functions to promote the post-translocational translocation of prepro-a-factor across the endoplasmic reticulum membrane (13).

MATa cells produce the propheromone pro-a-factor. a-Factor is a member of a growing family of secreted proteins, which include interleukin (IL) 1α and IL1β and the fibroblast growth factor 1 and 2, that are endoproteolytically processed in the cytosol and then secreted via a mechanism that does not involve the classical secretory pathway (30–32). Whether cytosolic chaperone proteins function to facilitate the processing, folding and/or secretion of any of these of these propeptides is an open question.

Pro-a-factor is encoded by two genes: MFA-1 and MFA-2. Biogenesis of the MFA-1 gene product is most frequently studied and has been shown to occur in multiple steps. MFA-1-derived pro-a-factor is synthesized as a 36-amino acid residue propeptide, which contains a single copy of mature a-factor (33). Pro-a-factor contains an amino-terminal extension and a CAAX box (C is cysteine, A is typically aliphatic, and X can be one of many amino acids) on its carboxyl terminus. The CAAX box serves as a site for the covalent modification of pro-a-factor with the isoprenoid farnesyl. Like other farnesylated proteins, pro-a-factor undergoes endoproteolytic cleavage of its carboxyl-terminal three amino residues (34, 35). The terminal cysteine residue that is exposed by endoproteolysis is then carboxymethylated, and this form of pro-a-factor represents a process-
ing intermediate termed P1 (34, 35). The amino-terminal extension of P1 is cleaved in two steps. First, Ste24p converts P1 to P2 by cleaving pro-a-factor between residues 7 and 8 (35). Then mature a-factor is generated by cleavage of a 14-amino acid residue peptide from the amino terminus of P2 in a reaction that is catalyzed by Ax11p (26). Finally, the ATP binding cassette protein Ste6p (30, 36) secretes the 12-residue a-factor lipo-peptide across the plasma membrane. We report that MATa ydl1 strains exhibited severe mating defects that can be accounted for by a dramatic reduction in their ability to secrete a-factor. Defects in a-factor secretion result from diminished processing of P2 to mature a-factor. Inhibition of P2 processing was accomplished by reductions in the level of Ax11. Reduced Ax11 levels were resultant from a drop in the quantity of Ax11 mRNA and instability of newly synthesized forms of this endopeptidase. In the absence of functional Ydl1p, loss of Ax11 activity appears to hinder flux through the a-factor biogenic pathway and reduce mating efficiency of MATa cells. These data identify Ax11p biogenesis as a process that is dependent upon Hsp70 SsaIp/Ydj1p chaperone system.

**Materials and Methods**

**Mating Assay**—Standard techniques were utilized to monitor the mating of the haploid MATa strains GMY210, GMY200, and GMY214 with the MATa partner GPY60 (37). MATa strains were each grown in appropriate selective minimal media to mid-log phase. The GPY60 culture was grown to mid-log phase, and then 1 OD600 (units/ml) of these cells was plated on YPD plates. The various MATa strains were harvested and resuspended to an OD600 of 1.0. Then, 4 µl of each strain was spotted onto the lawn of GPY60 and incubated for 5 h at 30 °C to allow for mating to occur. Mating mixtures were then replica plated onto selective semi-synthetic dextrose plates to select for diploids. The plates were incubated at 30 °C to allow for outgrowth of the diploid colonies, and mating was visualized after 48 h.

**Halo Assay**—A halo assay (37) was utilized to monitor a-factor secretion in the strains GMY210, GMY200, and GMY214. Briefly, yeast strains were grown overnight in selective minimal medium at 30 °C. The cells were then washed once in medium, and 5 ODs of each strain were spotted onto a YPD plate containing a lawn of 0.2 ODs of the a-factor tester strain XBI9–2C. Plates were then incubated at 30 °C, and halos were visualized after 48 h.

**Metabolic Labeling and Immunoprecipitation of a-Factor**—Processing and maturation of pro-a-factor were monitored by pulse-chase and immunoprecipitation analysis as described previously (38). Yeast strains harboring the 2µ MFA1 expression plasmid, pYK17 (a gift from Dr. Jeffery Becker of the University of Tennessee), were cultured in selective semi-synthetic minimal medium at 30 °C overnight. Cells were then diluted into 250 ml of the same selective medium and incubated until an OD600 between 0.2 and 1.0 was reached. Cells were then harvested and resuspended at 7.0 OD600/ml. Cells were then harvested and resuspended in a total volume of 3 ml at an OD600 of 7.0. Cells were then washed once in medium, and 5 ODs of each strain were spotted onto a YPD plate containing a lawn of 0.2 ODs of the a-factor tester strain XBI9–2C. Plates were then incubated at 30 °C, and halos were visualized after 48 h.

Cells were then washed once in medium, and 5 ODs of each strain were spotted onto a YPD plate containing a lawn of 0.2 ODs of the a-factor tester strain XBI9–2C. Plates were then incubated at 30 °C, and halos were visualized after 48 h.

**Metabolic Labeling and Immunoprecipitation of a-Factor**—Processing and maturation of pro-a-factor were monitored by pulse-chase and immunoprecipitation analysis as described previously (38). Yeast strains harboring the 2µ MFA1 expression plasmid, pYK17 (a gift from Dr. Jeffery Becker of the University of Tennessee), were cultured in selective semi-synthetic minimal medium at 30 °C overnight. Cells were then diluted into 250 ml of the same selective medium and incubated until an OD600 between 0.2 and 1.0 was reached. Cells were then harvested and resuspended in a total volume of 3 ml at an OD600 of 7.0. Cells were then washed once in medium, and 5 ODs of each strain were spotted onto a YPD plate containing a lawn of 0.2 ODs of the a-factor tester strain XBI9–2C. Plates were then incubated at 30 °C, and halos were visualized after 48 h.

**Western Blot Analysis**—For Western blot analysis of the steady state levels of indicated proteins, strains were cultured overnight in the appropriate selective semi-synthetic minimal medium. Cultures were then diluted and allowed to grow into log phase. A total of 7.5 OD units were harvested from cultures of each respective strain, fixed in 5% trichloroacetic acid, rinsed with acetone, and dried. Pellets were resuspended in 100 µl of a buffer that contained 5% SDS, 0.5% or Tris, 40 mM dithiothreitol, 15% glycerol, 1 mM phenylmethylsulfonyl fluoride, and a 2-fold concentration of a protease inhibitor mixture named Complete Tab that was purchased from Roche Molecular Biochemicals. Glass beads were then added and the cells were lysed by vortexing the tubes three times for 1 min at 100 °C. The extracts were then transferred to new 1.5-ml centrifuge tubes and incubated at 55 °C for 15 min prior to loading on SDS-PAGE gels. Proteins were transferred to nitrocellulose and probed with an affinity-purified antibody against Ste6p (38) or with anti-HA antibody.

**Northern Analysis**—RNA extraction and Northern analysis were carried out as described previously (42). Strains were grown in 2% nitrogen in 2× AA’s, 2% glucose medium buffered with 50 mM potassium phosphate (pH 7) to ~1 OD600/ml at 30°C. A 500-kilobase probe for the AXL1 mRNA was generated by polymerase chain reaction using primers GGAAG-TACTACGACCACGGC and GTCGGATCTCCCCTCGTT. The ACT1 probe was generated using the primers DB-154 and DB-155 (43). The probes were labeled with [γ-32P]dATP using the random hexamer method. The specific band for the AXL1 messenger RNA was deter-
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**RESULTS**

*Ydj1p Function Is Required for Efficient Mating of MATα Yeast*—To initiate our studies on the functions of Ydj1p in a-factor biogenesis, we examined the ability of ydj1 mutant strains to mate with the MATα tester strain GMY210 (Fig. 1; Table I). Mating assays were carried out under conditions where MATα strains were incubated with an excess of GPY60. Under these conditions, the efficiency of diploid formation is dependent upon the mating competence of the MATα strain under study. We observed ydj1 strains to exhibit a severe mating defect. Since ydj1 strains exhibit a slow growth phenotype (44), we needed to exclude the possibility that decreased mating was due to some general defect in protein metabolism. Therefore, the mating efficiency of a MATα strain that harbors the ydj1-g315d allele of YDJ1 was examined. This ydj1-g315d allele was chosen for study because haploid yeast strains that harbor it exhibit temperature-sensitive growth, but under permissive conditions these strains grow at near normal rates and do not exhibit signs that are characteristic of the stress response (18). The mating efficiency of the ydj1-g315d strain was higher than that of the ydj1 strain, but remained about 50% lower than that of the wild type strain.

**Loss of Ydj1p Function Causes a Defect in α-Factor Secretion**—To test whether the mating defect observed in ydj1 strains was related to aberrations in α-factor biogenesis, the ability of ydj1 strains to secrete this pheromone was monitored in halo assays (Fig. 1B). A lawn of the MATa tester strain YBH8–2C (which undergoes growth arrest when exposed to a-factor) was spread on a YPD plate. Then, aliquots of MATa strains that harbored YDJ1 or mutant copies of it were spotted onto the lawns. Plates were incubated at 30 °C for 2 days, and the size of the zones of growth inhibition formed around the different MATα strains, which are proportional to the quantity of α-factor secreted (37), was determined. The colonies formed by ydj1 and ydj1-g315d strains were of similar size, but the halos, which surrounded the mutants, were markedly smaller than the one around the YDJ1 strain. Comparison of the size of halos formed around the different ydj1 mutants indicates that α-factor secretion was reduced to a greater extent in ydj1 strains than in ydj1-g315d. In mating assays, the ydj1 strain was markedly less efficient at forming diploids than the ydj1-g315d strain (Fig. 1B). Thus, there appears to be a correlation between the differences in the mating efficiency of ydj1 and ydj1-g315d and the ability of these mutants to secrete α-factor. These data demonstrate that Ydj1p action is required for yeast to secrete α-factor with maximum efficiency.

*Ste6p Levels Appear Normal in ydj1 Strains*—Next, we investigated whether the defects in α-factor secretion observed in ydj1 strains were due to a reduction in the steady state level of Ste6p. To address this question, we examined the levels of Ste6p in YDJ1, ydj1Δ, and ydj1-g315d strains by Western blot and found them to be similar (Fig. 2A). In addition, we found that the half-life of Ste6p was not changed when Ydj1p function was compromised (Fig. 2B). Finally, the overexpression of Ste6p could not ameliorate the α-factor export defect in observed in ydj1 strains (data not shown). These results suggest that a loss of Ste6p function is not likely to be the major cause of the α-factor secretion defect observed ydj1 strains.

*Pro-α-factor Processing Is Defective in ydj1 Mutants*—Next, we examined whether loss of Ydj1p function causes a defect in pro-α-factor synthesis and/or processing. To monitor α-factor biogenesis, Ydj1 and ydj1Δ strains were transformed with a 2-μm plasmid that contains the MFA1 gene to promote expression of pro-α-factor to levels that are high enough to allow its processing to be monitored by biochemical techniques (30). Cells were metabolically labeled for 5 min with [35S]cysteine to generate 35S-pro-α-factor, and then its maturation and export were monitored during the course of a 30-min chase reaction (Fig. 3, A and B). Immediately after the labeling period, the total pool of pro-α-factor was similar in YDJ1 and ydj1Δ cells. However, the YDJ1 and ydj1Δ strains exhibited striking differences in their ability to process pro-α-factor. In the YDJ1 strain, pro-α-factor was initially found in intracellular pools in the P1, P2 and mature forms; the P2 form was predominant. At the end of the chase period, the P2 pool was largely depleted and a significant quantity of mature α-factor was found outside of the cell (Fig. 3B). In the case of ydj1Δ, just after labeling, the P2 form of pro-α-factor was predominant within cells and little mature α-factor was detected. During the chase period, the pool of P2 appeared stable, little matured, and the export of α-factor was reduced by greater than 90% (Fig. 3B). These data demonstrate that loss of Ydj1p function causes a kinetic defect in pro-α-factor processing. This processing defect reduces the efficiency at which the P2 intermediate is processed to the mature form and appears severe enough to account for the reductions in α-factor secretion observed in halo assays (Fig. 1B).

**A Functional J-domain and Polypeptide Binding Domain Are Required for Efficient Pro-α-factor Processing**—What regions of Ydj1p are required for pro-α-factor to be processed efficiently? Does Ydj1p act alone or in combination with Hsp70 to promote pro-α-factor maturation? To address these questions, we analyzed pro-α-factor processing in strains that harbor the ydj1-h34q and ydj1-g315d alleles instead of YDJ1 (Fig. 4). Ydj1-h34q has a mutation in the J-domain (21), and Ydj1-g315dp has a mutation that causes a defect in polypeptide binding (19). In experiments where the processing of pro-α-factor by YDJ1, ydj1Δ, ydj1-h34q, and ydj1-g315d strains were compared at t = 0 and after a 10-min chase period, defects in P2 processing and α-factor secretion were evident (Fig. 4). At t = 0, all strains contained both the P1 and P2 forms of pro-α-factor. During the 10-min chase incubation, YDJ1 processed and exported a large portion of pro-α-factor (Fig. 4, lane 1 versus lane 4). However, in the case of the mutant strains, little P1 was processed to P2 and very little α-factor export was observed. As observed for the ydj1Δ strain, at t = 0, a relatively small
media of the ydj1-g315d strain after the 10 min of chase, the processing defect in this mutant appeared somewhat less severe than that observed in the ydj1-h34q strain. This result is consistent with the observation made in halo and mating assays (Fig. 1), where the ydj1-g315d strain was capable of secreting more α-factor and mating more efficiently than the ydj1Δ strain. Apparently, mutation of the J-domain has a more severe effect on Ydj1p function than mutation of the polypeptide binding domain.

**Axl1p Expression from a High Copy Plasmid Does Not Complement Defects in α-Factor Secretion**—Next, we examined whether defects in processing of the P2 intermediate of pro-α-factor observed in ydj1Δ strains could be overcome by overexpression of Axl1p from a high copy plasmid (Fig. 5A). To accomplish this we compared the size of halos formed around axl1Δ, YDJ1, ydj1Δ, and ydj1-g315d strains to those formed by identical strains that were transformed with a 2-μm YEP352 plasmid that harbors a fully functional copy of HA tagged AXL1 (26). The size of the halo around the axl1Δ strain was markedly smaller than the one that surrounded the WT-type strain (Fig. 5A). Transformation of the axl1Δ strain with YEP352-HA-AXL1 restored α-factor secretion to levels observed in WT-type strains. The halo that surrounded the ydj1Δ strain was of similar size to the one that surrounded axl1Δ (Fig. 5A), but transformation with YEP352-HA-AXL1 did not detectably influence halo formation. Halos around ydj1-g315d were slightly larger than those around the ydj1Δ colony. However, the introduction of YEP352-HA-AXL1 again had no effect on α-factor secretion. An identical analysis that involved overexpression of a non-HA tagged version of Axl1p yielded similar results (data not shown). Thus, introduction of a high copy plasmid to drive the overexpression of Axl1p in ydj1Δ strains was insufficient to suppress defects in α-factor secretion.
Mutations in YDJ1 Cause Defects in Axl1p Biogenesis—The inability of Axl1p overexpression to suppress the defects in a-factor secretion observed in ydj1 strains suggests two possible scenarios as to why pro-a-factor processing is defective. The first is that mutation of YDJ1 causes a defect in the a-factor processing pathway prior to the Axl1p-dependent processing step. If this were the case, then Axl1p overexpression would not be expected to complement the pro-a-factor processing defect we observe. Second, ydj1 strains may not be able to support the expression of active Axl1p and this defect may limit the ability of YEP352-HA-AXL1 to suppress the defects in pro-a-factor processing.

To address these issues, Western blots of cell extracts from YDJ1, ydj1Δ, and ydj1-g315d strains were probed with anti-HA-antibody to examine the level of expression of HA-Axl1p from YEP352-HA-AXL1. The striking result from this analysis was that HA-Axl1p could not be detected in the ydj1Δ strain. In contrast, Axl1p was detected at comparable levels in the YDJ1 and ydj1-g315d strains (Fig. 5B). When these studies were extended in pulse-chase experiments, HA-Axl1p could not be detected in ydj1Δ (data not shown). In addition, we observed that the half-life of HA-Axl1p was greatly reduced in the ydj1-g315d strain (Fig. 5C).

In control experiments, we examined the half-life of a number of other endogenous yeast proteins to determine whether loss of Ydj1 function caused a general defect in protein folding. We observed that the steady state levels of proteins such as Ste6p, Dpm1p, Cyp6p, Alpp, Vma1p, Vma2p, and Pgkp were not influenced in the ydj1Δ or ydj1-g315d strain (data not shown).

Similar results have been reported in studies that examined the heterologous expression of proteins such as luciferase (46), GFP (46), v-SRC (47), and the glucocorticoid receptor (17, 18). Thus, formation of stable Axl1p molecules appears to have specific features that require the presence of fully functional Ydj1 in the cell.

Two important conclusions are drawn from these data on the steady state levels of Axl1p and the measurement of its half-life. First, the ydj1Δ strain appears unable to support levels of Axl1p expression that are sufficient to promote the efficient processing of pro-a-factor. Second, Ydj1 action appears to be required in order for Axl1p to fold into a stable conformation.

A perplexing observation from these studies was that the steady state levels of Axl1p were elevated in the ydj1-g315d strain, but mating efficiency and the half-life of newly synthesized Axl1p were both markedly reduced (Figs. 1 and 5). The simplest interpretation of these results is that newly synthesized Axl1p has three fates in the ydj1-g315d strain. One fate of Axl1p is to fold properly and support the low level of a-factor processing observed. The second fate is to become misfolded and be rapidly degraded. A third fate, which appears to predominate would be to misfold and accumulate in a stable, but less active or inactive form.

Fig. 3. Mutant ydj1 strains exhibit defects in pro-a-factor processing. A, the amino acid sequence and processing sites for pro-a-factor that is encoded by MFA1. Exhibited in this schematic is the P1 processing intermediate of pro-a-factor that has been modified with farnesyl and carboxymethylated, but has an unprocessed amino-terminal extension. Cleavage of pro-a-factor by Ste24p between residues 7 and 8 generates the P2 form. Cleavage of pro-a-factor by Axl1p between residues 21 and 22 generates mature a-factor (M). B, pulse-chase and immunoprecipitation analysis of intracellular (lanes 1–4) and extracellular (lanes 6–9) forms of pro-a-factor that were generated by YDJ1 (GM202) and ydj1Δ (GM201) strains. The indicated strains were grown to mid-log phase at 30 °C and labeled for 5 min with [35S]-cysteine. A chase mix containing excess unlabeled methionine/cysteine and cycloheximide was then added, and the cells were aliquoted equally into separate polypropylene tubes. These mixtures were then incubated at 30 °C for the indicated times to allow for the pro-a-factor processing and secretion to occur. Then, an antibody that recognizes a region within the a-factor that was present intracellularly (lanes 1–4) or extracellularly (lanes 5–8). Immunoprecipitated material was analyzed on Tricine SDS-PAGE and visualized by autoradiography. Positions where the P1, P2, and mature forms of a-factor migrate are denoted on the left side of the panel.

Fig. 4. Mutations in the J-domain and polypeptide binding domain of YDJ1 reduce intracellular processing of pro-a-factor. Pulse-chase and immunoprecipitation analysis of intracellular (lanes 1 and 2) and extracellular (lanes 3 and 4) forms of pro-a-factor generated by strains with the genotype of YDJ1 (GM202), ydj1Δ (GM201), ydj1-h34q (GM203), and ydj1-g315d (GM204). Yeast strains were grown at 30 °C to mid-log phase and labeled for 5 min with [35S]-cysteine. A chase mix containing excess unlabeled methionine/cysteine and cycloheximide was then added, and cells were split into separate polypropylene tubes. Immediately (lanes 1 and 3) or following a 10-min chase (lanes 2 and 4), the intracellular and extracellular forms of pro-a-factor were immunoprecipitated from cell lysates or from material bound to the respective tubes (see “Materials and Methods” for details). Immunoprecipitated material was then analyzed by Tricine SDS-PAGE and visualized by autoradiography. Positions where the P1, P2, and mature forms of a-factor migrate on gels are denoted on the left side of each panel.
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In YDJ1 strains the presence of YEP352-HA-AXL1 increased AXL1 mRNA levels by an average of around 10-fold. To our surprise, AXL1 mRNA levels were 80% and 45% lower in the ydj1Δ and ydj1-g315d strains. Qualitatively similar reductions in levels of AXL1 mRNA were observed in respective ydj1 strains that did not harbor YEP352-HA-AXL1 (data not shown).

These data demonstrate that Ydj1p function is somehow required to promote the accumulation of AXL1 mRNA and that abnormalities in this process contribute to the defects in pro-α-factor processing observed in ydj1 strains. In the ydj1Δ strain, it appears that reduction of AXL1 mRNA is the major reason why P2 is processed with very low efficiency. However, in the ydj1-g315d strains, defects in AXL1 mRNA accumulation and folding of nascent Axl1p both contribute to the mating defects observed.

Axl1p Action in Bud Site Selection Appears Normal in ydj1 Strains—Axl1p is a multifunctional enzyme whose activity is required for the axial patterning of bud sites as well as pro-α-factor processing (26, 45). Therefore, we examined whether loss of Ydj1p function had an effect on Axl1p action in bud site selection. We determined the pattern of bud scars formed by YDJ1, ydj1Δ, and ydj1-g315d strains and compared it to that of a ydj1Δ strain (Fig. 6). YDJ1, ydj1Δ, and ydj1-g315d strains exhibited nearly identical patterns of bud site selection with about 75% of bud scars being arranged in an axial pattern. This pattern is typical of haploid yeast with fully functional Axl1p activity to support normal bud site selection. This result suggests that yeast has differential requirements for Axl1p activity in pro-α-factor processing and bud site selection.

**DISCUSSION**

We demonstrate that the action of the Hsp40 protein Ydj1p is required for the efficient mating of MATa yeast strains. Ydj1p functions in the yeast mating pathway by promoting the biogenesis of Axl1p. In the absence of functional Ydj1p, Axl1p activity was reduced and this led to the intracellular accumulation of the P2 processing intermediate of pro-α-factor. The inability of ydj1 strains to secrete normal quantities of α-factor reduced mating efficiency. Two mechanisms appear to contribute to loss of Axl1p function in ydj1 strains. First, AXL1 mRNA levels were dramatically reduced in the ydj1Δ strain. Second, data from pulse-chase experiments demonstrated that the half-life of newly synthesized Axl1p is also reduced in ydj1 strains. These data suggest that Ydj1p action is required for the normal folding/assembly of Axl1p. In the absence of Ydj1p, a portion of Axl1p appears to misfold and is rapidly degraded.

**FIG. 5. Assay of Axl1 biogenesis in ydj1 mutants.** A, effects of Axl1p overexpression on α-factor secretion ydj1 strains. Secretion of α-factor was measured by halo assay in strains harboring a high copy expression plasmid YEP352 that harbors an HA-tagged version of AXL1 (+HA-AXL1), which has been shown to be fully functional (26) or an empty vector. Strains harboring pHA-AXL1 were: ydj1Δ (GMY281), YDJ1 (GMY276), ydj1Δ (GMY271), and ydj1-g315d (GMY279). Strains harboring the empty vector were: axl1Δ (GMY283), YDJ1 (GMY277), ydj1Δ (GMY270), and ydj1-g315d (GMY280). Equal amounts (5.0 OD) of the indicated strains were spotted onto a lawn of the MATa tester strain XAH8-2C. To allow for zones of growth inhibition to form around the respective strains that harbor different forms of YDJ1, plates were incubated at 30 °C for 48 h. B, Western blot analysis of steady-state HA-Axl1p levels in lysates made from strains with YDJ1 (GMY276), ydj1Δ (GMY262), and ydj1-g315d (GMY279) genotypes. The band marked with an arrow denotes HA-Axl1p. C, pulse-chase analysis of HA-Axl1p synthesis and turnover in YDJ1 and ydj1-g315d strains. Spheroplasts were labeled for 15 min with [35S]methionine and lysed after the indicated chase time. HA-Axl1p was then immunoprecipitated with anti-HA antiserum, and the products of this reaction were analyzed by SDS-PAGE and fluorography (see "Materials and Methods" for details). The band marked with the arrow denotes HA-Axl1p. The band marked with * corresponds to a radiolabeled product that interacts with HA-antibody in a nonspecific manner. The lane annotated with a (−) contains the products of an immunoprecipitation that was carried with a YDJ1 yeast strain that did not contain the HA-Axl1p expression plasmid. D, analysis of AXL1 mRNA levels. Levels of AXL1 mRNA were determined by Northern blot and then normalized to ACT1 mRNA levels. Valves expressed are the average of three experiments ± standard deviation and are normalized to the ratio of AXL1 to ACT1 mRNA detected in the YDJ1 strain that harbored the HA-AXL1 expression plasmid.

**FIG. 6. The influence of mutations in ydj1 bud site selection.** Analysis of bud site selection in ydj1 mutants. Strains with the genotypes of YDJ1 (GMY276), ydj1-g315d (GMY214), ydj1Δ (GMY200), and axl1Δ (MS19863) were grown to mid-log phase and then suspended for 5 min in 0.5 mg/ml calcifluor. Cells were then washed and examined by light microscopy with a Hoescht filter (magnification, X1000) to observe the orientation of bud scars on individual cells. Axial, bipolar, and random distribution of bud scars was then scored. In the schematic, bud scars are represented by the small circles and patterns are shown for their axial, random, or bipolar distribution. The numbers exhibited in the table represent the percentage of the total number of cells counted that had bud scars in the indicated patterns. The total number of cells counted for the respective strains were 125 for YDJ1, 108 for ydj1-g315d, 160 for ydj1Δ, and 96 for axl1Δ.
degraded. Thus, reductions in AXL1 mRNA levels and the instability of newly synthesized Axl1p both contribute to defective pro-a-factor processing.

The cell requires Axl1p function to promote pro-a-factor processing and bud site selection. We observed that bud site selection was normal in ydj1Δ strains, whereas pro-a-factor processing was severely compromised. These data demonstrate that functional Axl1p is present in ydj1Δ strains and that cells can compensate for loss of Ydj1p to generate reduced levels of this protease. In addition, these data suggest that the threshold level of Axl1p activity that is required for bud site selection is lower than that for pro-a-factor processing.

Why do mutations in YDJ1 lead to reduced levels of AXL1 mRNA? We suggest three possible mechanisms to explain this observation. First, Ydj1p may be required for the transcription of the AXL1 gene. However, levels of AXL1 mRNA transcribed from both the AXL1 promoter and the ADH promoter were reduced when YDJ1 was mutated. Thus, it does not appear that Ydj1p is specifically required for expression of proteins from the AXL1 promoter. Furthermore, published studies suggest that mutations in YDJ1 do not give rise to pleiotropic defects in mRNA synthesis (9). A second scenario is that Ydj1p may act with Hsp70 to promote the proper folding and/or stabilization of the AXL1 message. A recent finding that demonstrates Hsp70 to form binary complexes with mRNA and functions to regulate mRNA stability (48) supports this supposition. Alternatively, Ydj1p action may be required to promote the proper folding of a RNA-binding protein that regulates AXL1 mRNA stability. No matter what the mechanism for the reduction in AXL1 mRNA levels, the data we report are the first to suggest a function for Ydj1p in mRNA metabolism.

Axl1p is a large 138-kDa protein that contains a domain responsible for its proteolytic activity and other functionally independent domains that enable it to facilitate bud site selection (26, 45). Since mutations in Ydj1p decrease the half-life of Axl1p, it appears that this multiple domain protein requires the action of the Hsp70/Hsp40 chaperone system to promote its folding. This is an important observation because the dependence of cellular protein folding on the hsp70/Hsp40 chaperone system is not clear (6, 49, 50), and it defines Axl1p as a cellular protein that requires the action of this chaperone system. In addition, these data are consistent with current models for in vivo protein folding, which suggest that small single domain proteins fold independent of the Hsp70/Hsp40 system (51, 52). In contrast, large multiple domain proteins appear to require the co-translational action of Hsp70/Hsp40 chaperone proteins to promote intramolecular protein:protein interactions between subdomains that are required for proper assembly (40, 52). Ydj1p may function in a similar manner to promote Axl1p folding.

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