Targeting of Aminopeptidase I to the Yeast Vacuole Is Mediated by Ssa1p, a Cytosolic Member of the 70-kDa Stress Protein Family*

Received for publication, April 25, 2000, and in revised form, July 18, 2000
Published, JBC Papers in Press, July 19, 2000, DOI 10.1074/jbc.M003514200

Eduardo Silles‡‡, María J. Mazón‡, Kris Gevaert**, Marc Goethals, Joel Vandekerckhove‡‡, Regina Leber‡‡ and Ignacio V. Sandoval†††

From the Instituto de Investigaciones Biomédicas Albeto Sala, Consejo Superior Investigaciones Científicas-Universidad Autónoma of Madrid, 28029 Madrid, Spain, the Flanders Interuniversity Institute for Biotechnology, Department of Biochemistry, Universiteit Gent, Ledeganckstraat 35, B-9000 Gent, Belgium, and the Centro de Biología Molecular Severo Ochoa, Consejo Superior Investigaciones Científicas-Facultad de Ciencias, Universidad Autónoma of Madrid, 28049 Madrid, Spain

The two cytosolic members of the highly conserved 70-kDa stress protein family, Ssa1p and Ssa2p, were specifically retained by the prepro-NH2 extension of the vacuolar aminopeptidase I precursor (pAPI) conjugated to agarose (Sulfolink). A temperature-sensitive mutant strain a1-45 (ssa1+ ssa2 ssa3 ssa4), when incubated at the restrictive temperature, was able to assemble the API precursor into dodecamers, but failed to pack pAPI into vesicles and to convert it into mature API (mAPI), a process that occurs in the vacuole. Altogether these results indicate that Ssa1p mediates the targeting of pAPI to the vacuole.

The yeast vacuolar protein aminopeptidase I (API)1 is synthesized as a cytosolic precursor and transported to the vacuole by a cotranslational mechanism (1, 2). Genetic studies indicate that this pathway uses many of the molecular components of the degradative autophagy pathway (3, 4). API transport is made specific and saturable by an unknown receptor that appears to recognize specific transport motifs in the prepro-NH2 extension of the pAPI (5–7).

There is evidence that, soon after its synthesis, pAPI is assembled into homododecamers (8), which become associated with spherical particles (termed Cvt complex) and are delivered to the vacuole by a vesicle-mediated mechanism (9, 10). Depending on the environmental conditions, the cell uses different membrane bound structures to sequester the Cvt complex and deliver pAPI to the vacuole. It has been proposed that, during vegetative growth, the Cvt complex is selectively wrapped by a double membrane sac (10) or translocated to a prevacuolar compartment (5), whereas in cells subjected to starvation conditions the Cvt complex is taken up by an autophagosome (10).

While searching for proteins interacting with the prepro-NH2 extension of pAPI, the segment that mediates the transport of the protein precursor to the vacuole (5, 11), we have observed its specific in vitro interaction with Ssa1p and Ssa2p, two highly homologous members of the cytosolic hsp70 family that are constitutively expressed during logarithmic growth and have been involved in protein folding and translocation (12–14). To study the role of Ssa1p in the targeting of pAPI to the vacuole, we analyzed this process in a mutant strain carrying a temperature-sensitive allele of SSA1 and inactivated alleles of the SSA2, SSA3, and SSA4 genes. We show here that targeting of the pAPI to the vacuole is mediated by Ssa1p under both growing and nitrogen starvation conditions.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media—To generate the yeast strains used in this work we introduced mutations in each of the four SSA genes in the W303-1b genetic background. Gene disruptions of SSA2, SSA3, and SSA4 were performed by the short flanking homology technique (15). ssa2::LEU2 and ssa3::TRP1 disruption cassettes were amplified by PCR using genomic DNA of strain a1-45 (12) as template. SSA4 was replaced by the dominant-resistant module KmrMX, which confers resistance to Geneticin (16). The PCR fragments contained in their 3′ and 5′ ends a minimum of 45 base pairs homologous to the flanking regions of the target gene, thus allowing for homologous recombination at their genomic loci. Disruption cassettes were consecutively transformed into W303-1b cells (17), and disruption of each gene was verified by analytical PCR using specific oligonucleotides for the 5′ and 3′ regions of the corresponding gene. The resultant strain, deleted for SSA2, SSA3, and SSA4, was named ESY170 (see Table I). To obtain strain ESY216 (ssa1+ ssa2 ssa3 ssa4) a 1.7-kilobase pair Kpn1-Sph1 fragment of the ssa1 temperature-sensitive allele in strain a1-45, containing mutation Pro417 to Leu (12), was amplified and cloned into the integrative vector pRS306 (URA3) (18), digested at the unique ClaI site internal to the cloned fragment, and transformed into strain ESY170. Transformants being both Ura- and temperature-sensitive for growth were counterselected in fluorotic acid-containing plates and analyzed again for temperature sensitivity. Correct integration and presence of the Pro147→Leu mutation were confirmed by PCR and sequencing. Strain ESY228 was constructed by disrupting the API gene using the HIS3 marker as described previously (5). ESY228 was used to overexpress the API gene carried in a 2-µm plasmid under the control of the GAL1–10 promoter (5).

The genotypes of all the strains used in these studies are listed in Table I. The pGAL-API has been described previously (5, 11).

Yeast cells were grown in synthetic minimal medium: 0.67% yeast nitrogen base, 0.006% glucose (SD), 2% raffinose (SRaf), or 2% galactose.
(SGal) and the appropriate auxotrophic requirements. Synthetic minimal medium containing 2% glucose and 0.17% yeast nitrogen base without amino acids and ammonium sulfate (SD-N) was used for nitrogen starvation experiments.

Standard techniques for yeast strain propagation and genetic manipulation were as described (19). Yeast transformation was performed by the method of Ito et al. (20) and DNA manipulations as described (21).

**Purification of Ssa1p/Ssa2p by Affinity Chromatography—** 60 ml of SD medium containing the appropriate requirements were inoculated with W303-1b cells and grown overnight at 30 °C. Cells were harvested in the logarithmic phase by centrifugation, resuspended in 2 ml of yeast nitrogen base without amino acids and ammonium sulfate prepared to 0.17%, 0.4% NH₄Cl, 0.3% glucose, incubated for 30 min at 30 °C and labeled for 1 h at 30 °C with 1 mCi of [³⁵S]methionine/cysteine (specific activity > 1000 Ci/mmol; Promix, Amersham Pharmacia Biotech). The cells were then washed with cold phosphate-buffered saline, 0.15 M NaCl (PNC buffer), disrupted with glass beads in 1 ml of the same buffer, phenylmethylsulfonyl fluoride added to 1 μM, and cell debris removed by centrifugation twice for 10 min at 2000 × g. The resulting extract was centrifuged for 30 min at 150,000 × g in a TL100.3 rotor (Beckman) and the supernatant (S150) made 2% in Triton X-100, whereas the pellet (P150) was resuspended in 1 ml of PNC buffer, 2% Triton X-100 incubated for 30 min at 4 °C and then centrifuged for 30 min at 150,000 × g.

The 43-amino acid-long MEEQREILEQLKTLQMLTVEPSKNQIANEEKKENENSWC-COOH and MEEQRECLEQKLKTLQM-LTVEPSKNQIANEEKKENENSWC-COOH peptides from the prepro-NH₂-terminus of the pAPI (prepro-peptide) were coupled to agarose (Sulfolink) (Pierce) at a ratio of 5 mg/ml gel, according to the manufacturer’s instructions (Pierce). The S150 and P150 fractions were mixed with 1 volume of PNC buffer and incubated overnight with the prepro-peptide conjugated to agarose (Sulfolink). The resin was then washed three times for 15 min with PNC buffer, 0.5% Triton X-100, once for 15 min with phosphate-buffered saline, 1 x NaCl, 0.5% Triton X-100, and then quickly with water. Aliquots from the yeast crude extract, the S150 and P150 fractions, the fourth wash, and the washed resin were boiled in Laemmli buffer, resolved by SDS-12% PAGE, and the electrophoretograms analyzed for protein both by Coomassie Blue staining and autoradiography (Fig. 1A).

**Results**

**Analysis of API Dodecamers by Sedimentation Velocity Centrifugation—** a1+a2344ape1 cells carrying the 2-μg pGAL-API were grown in SRA medium overnight to 1 OD₆₀₀ and then shifted to SGA medium for 4 h at 24 or 37 °C. The cells were incubated the last 15 min in SGA medium with or without 20 μM NaN₃, harvested by low speed at the corresponding temperatures, and immediately frozen in liquid nitrogen. Crude cell extracts prepared with glass beads in cold 50 mM Tris-HCl, 5 mM MgCl₂, 20 mM Pipes, pH 6.8. Unlysed spheroplasts were removed by centrifugation for 2 min at 500 × g. Lysates were treated at 4 °C for 30 min with 50 μg/ml proteinase K (Life Technologies, Inc.) in the absence and presence of 0.2% Triton X-100. Digestion of the protease was stopped with a mixture of protease inhibitors (5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and pepstatin, aprotinin, and leupeptin (5 μg/ml each) and the protein precipitated with trichloroacetic acid using 100 μg/ml bovine serum albumin as carrier, and API immunoprecipitated as described above and analyzed by SDS-10% PAGE and autoradiography.

**TABLE I**

| Strain       | Name used in text | Genotype                                                                 | Source |
|--------------|-------------------|---------------------------------------------------------------------------|--------|
| a145         | MAT a his3-11, 15 leu2-3, 112 trp1-Δ1 lys2 ura3-52 ssa1* sao2 : LEU2 sas3 : TRP1 ssa2 : LYS2 | Ref. 12 |
| W303-1b      | Wild type         | MAT a ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100               | Ref. 17 |
| ESY170       | A1a234            | MAT a ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 Ssa2 : LEU2 ssa3 : TRP1 | This study |
| ESY16        | a1+a234           | W303-1b; MAT a ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 Ssa1 : LEU2 ssa2 : TRP1 | This study |
| ESY228       | a1+a234Δape1      | W303-1b; MAT a ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 Ssa1 : LEU2 ssa2 : TRP1 | This study |

**Protein Transport from Cytoplasm to Vacuole**
peptidyl affinity columns (data not shown), thus indicating that the two chaperones recognized a motif contained in the primary structure of the peptide (see “Discussion”).

**Targeting of the pAPI to the Vacuole by the Cvt Pathway Is Mediated by Ssa1p**—To examine whether Ssa1p was actually involved in the targeting of the pAPI to the vacuole, we studied the processing of the pAPI by a1tsa234 cells. These cells were developed by introducing the disrupted SSA2, SSA3, and SSA4 genes as well as a ssa1ts allele from a1-45 cells (12) in the genetic background of W303-1b. After their shift to the nonpermissive temperature, the a1-45 cells have been shown to stop growing immediately as well as to undergo defects in the translocation and processing of several ER and mitochondrial protein precursors (12).

Processing of the pAPI into the mature form was first studied in a1tsa234 cells under vegetative conditions (Fig. 2). The cells were pulse-labeled for 10 min with [35S]methionine/cysteine, extracted, fractionated, incubated with prepro(API)-peptidyl-affinity columns (data not shown), thus indicating that the two chaperones recognized a motif contained in the primary structure of the peptide (see “Discussion”).

**Fig. 1.** A, retention of a 70-kDa peptide by prepro-API-peptidyl-affinity chromatography. Growing W303-1b cells were labeled with 1 mCi of [35S]methionine/cysteine, extracted, fractionated, incubated with prepro-API-Sulfolin affinity resin, and the resin washed and eluted as described under “Experimental Procedures.” The proteins were resolved by SDS-PAGE, stained with Coomassie Blue (A, left) and analyzed by autoradiography (A, right): crude extract (lane 1), S150 fraction (lane 2), P150 fraction (lane 3), proteins from the S150 (lanes 4 and 6) and P150 fractions (lanes 5 and 7) retained by the resin and eluted with 1 M NaCl (lanes 4 and 5) or by boiling in Laemli buffer (lanes 6 and 7). B, characterization of the in-gel-digested 70-kDa polypeptide as Ssa1p/ Ssa2p by mass spectrometry analysis. B, left, linear delayed extraction MALDI mass spectrometry spectrum of fraction 7 after RP-HPLC separation of the trypsin Ssa1p and Ssa2p digests; B, right, MALDI-PSD spectrum of the peptide with a mass of 1424.63 Da contained in fraction 7 from RP-HPLC and having the sequence NH2-FKEEDEKESQR-COOH from the yeast Ssa1p and Ssa2p proteins. C, common and unique peptides sequenced from Ssa1p and Ssa2p using MALDI-PSD.

To further investigate if the interaction of the Ssa1p/Ssa2p pair with the prepro-NH2 extension of the pAPI required a structural motif contained in the primary or secondary structure of the peptide, the Ile residue in position 7 was substituted by a Gly. This substitution has been shown to disrupt the α-helix conformation of the peptide into a random coil and to inhibit the transport of the pAPI to the vacuole (7). We observed that the introduction of Gly in position 7 did not suppress the retention of the Ssa1p and Ssa2p by prepro(API)-peptidyl affinity columns (data not shown), thus indicating that the two chaperones recognized a motif contained in the primary structure of the peptide (see “Discussion”).

**Assembly of the pAPI into Dodecamers Does Not Require Ssa1p**—The observation that newly synthesized pAPI molecules are assembled into homododecamers in the cytoplasm and transported in this form into the vacuole (8) prompted us to study if Ssa1p was required for their assembly. For this purpose, a1tsa234Δape1 cells carrying the pGAL-API plasmid were induced with galactose for 4 h at both 24 and 37 °C and crude extracts fractionated by sedimentation velocity centrifugation.
in glycerol gradients as described (8). Western analysis of the gradient fractions using an anti-API specific antibody showed that at both temperatures pAPI migrated with the mobility expected for dodecamers (Fig. 4), thus excluding a role for Ssa1p and the other family members in their assembly.

**pAPI Remains Unprotected in the Cytoplasm of a1 tsa234 Cells at the Restrictive Temperature**—To further define the role of Ssa1p/Ssa2p in the targeting of pAPI to the vacuole, we investigated whether pAPI molecules were retained or not in the cytoplasm at the permissive and restrictive temperature. This is important since Ssa1p/Ssa2p are localized in the cytoplasm and it has been reported in a recent study that all the pAPI in the cell is within vesicles in a \( \Delta \text{ssa1} \) mutant (25). For this purpose, the sensitivity of newly synthesized pAPI molecules to proteinase K was studied in a1 tsa234 spheroplasts pulsed for 10 min with \([35\text{S}]\) methionine/cysteine and chased for the indicated times at the corresponding temperature. Processing of cell extracts and analysis of the API species were performed by immunoprecipitation and autoradiography as described under “Experimental Procedures.”

**Analysis of the protease protection of CPY in a parallel experiment run under the conditions described for pAPI** showed that, as expected, after 2 min of chase the CPY precursor (pCPY) transported through the secretory pathway was converted into iAPI (i.e. intermediate API) and mAPI. Moreover, in the absence of detergent, the majority of pAPI and iAPI was digested by proteinase K into the comparatively protease-resistant mAPI, whereas in its presence digestion was complete (Fig. 5A). It should be noted that no significant vacuolar disruption, that would have interfered with the protease protection assay, occurred during lysis of spheroplasts, as shown by the absence of mAPI in the immunoprecipitates obtained from spheroplasts chased for 2 min at the permissive temperature. When the experiment was performed with lysates from a1 tsa234 spheroplasts incubated at the restrictive temperature, we observed that 4 h after its synthesis the majority of pAPI remain unprocessed, the small amount of mAPI detected probably being generated by minor vacuolar breakage during the disruption of the spheroplasts by osmotic lysis. Moreover, all the pAPI was digested by the protease in the absence of detergent (Fig. 5A).

Analysis of the protease protection of CPY in a parallel experiment run under the conditions described for pAPI showed that, as expected, after 2 min of chase the CPY precursor (pCPY) transported through the secretory pathway was
Protein Transport from Cytoplasm to Vacuole

Protected from proteinase K in the absence of detergent, whereas it was digested into the protease-resistant mature form (mCPY) when detergent was added. Furthermore, after 4 h of chase, CPY was observed to migrate with the size corresponding to mCPY and to be resistant to the protease both in the absence or presence of detergent (Fig. 5B). These results were consistent with the entrance of pCPY in the secretory pathway concomitant with its synthesis and reassessed the validity of the protease protection assay as a valid technique to study the retention of pAPI in the cytoplasm.

Altogether these results indicated that a significant amount of the pAPI synthesized and chased at the permissive temperature remained unprotected in the cytoplasm long after its synthesis and that at the restrictive temperature in the absence of a functional Ssa1p protein, all the precursor retained in the cytoplasm was unprotected.

**DISCUSSION**

The results of studies on the transport of API to the vacuole are compatible with two models of transport that are not mutually exclusive. In the first model, API assembled into tödecamers was selectively wrapped by a double membrane sac that eventually fuses with the vacuole to unload the protein packed in the inner vesicles into the vacuolar lumen (9). The second model proposes that the precursor is translocated through the membrane of a transport intermediate or the vacuolar membrane (5).

We show here that Ssa1p and Ssa2p, the two constitutive chaperones involved in the folding, oligomerization, and targeting of proteins from the cytosol to such diverse organelles as ER (14, 26, 27), mitochondria (28), and nucleus (29), interact specifically in vitro with the peptide that mimics the prepro-NH$_2$ extension of pAPI, an extension that is necessary and sufficient for the targeting of pAPI to the vacuole (11).

The predominance of the 70-kDa peptides among the few proteins retained by the prepro(API)-peptidyl affinity column stresses the specificity of the interaction between the Ssa1p/Ssa2p and the prepro-NH$_2$ extension of pAPI. Nevertheless, when considering that chaperones often bind to exposed hydrophobic patches on incompletely folded proteins and that most of the peptide mimicking the pre-part and the pre-(Gly$^7$) peptide are unfolded, the possibility that their in vitro interaction could be unspecific should be considered. With regard to this, it is important to note that the mean hydrophobicity per residue of nonpolar face in the pre-part (MEEQREGLEQLKKTLQ) is only 0.28 (7) and that the pro-part (MLTVEPSKNNQIANEKEKKKENENS) is even less hydrophobic. These observations make it unlikely that the interaction of the prepro-NH$_2$ extension with Ssa1p/Ssa2p is an artifact provoked by the physicochemical properties of the peptide.

We have shown that, in aqueous solution, only 24% of the peptide mimicking the pre-part and 1.7–2.4% of the pre-(Gly$^7$) peptide fold into an α-helix (7). However, Ssa1p/Ssa2p are retained with similar efficiency by the prepro- and pre-(Gly$^7$) pro-affinity columns, suggesting that either the motifs recognized by the chaperones are contained in the primary structure of the pre-part or in the pro-part. In addition, the recovery of the pAPI extracted from a$^{14}$a234 cells grown at 24 and 37 °C as a dodecamer upon centrifugation in glycerol gradients indicates that Ssa1p is not involved in its oligomerization.

The inhibition of the conversion of pAPI into mAPI in a$^{14}$a234 cells incubated at the restrictive temperature under either vegetative or nitrogen starvation conditions indicates that Ssa1p mediates the transport of pAPI to the vacuole under both conditions. This result is in contrast with recent observations by Satyanarayana et al. (25) who have reported that Ssa1p is not involved in the transport of pAPI to the vacuole under nitrogen starvation conditions favoring autophagocytosis.

The possibility that Ssa1p may play a role in engaging the properly folded precursor with the capture/transport machinery that operate in its transport to the vacuole should be considered. Wrapping of pAPI complexes by double membrane sacs might only occur after their interaction with the cytoplasmic chaperone, whereas if pAPI is translocated through the membranes of transport vesicles/vacuole the chaperone could interact with a specific membrane receptor. The demonstration that a significant amount of newly synthesized pAPI remains unprotected in the cytoplasm 4 h after its synthesis, at the permissive temperature, as shown by the protease protection assay, agrees with the results of previous studies (2) and seems to discard the existence of membrane barriers between pAPI and Ssa1p that could prevent their interaction, as indicated in a recent study (25). Furthermore, the complete digestion of the pAPI synthesized and chased for 4 h at the restrictive temperature, by proteinase K in the absence of detergent, indicates that the inactivation of Ssa1p inhibits the pAPI protection. This observation strongly suggests that the chaperone mediates the capture/downloading of pAPI into Cvt vesicles.

**Acknowledgments**—We thank Drs. E. Craig and G. Høgenauer for kindly supplying the a1-45 yeast strain and the anti-CPY antibody, respectively. We are also indebted to Dr. F. Portillo for helpful discussions.

**REFERENCES**

1. Klionsky, D. J., Cuevas, R., and Yaver, D. S. (1992) J. Cell Biol. 119, 287–299
2. Scott, S. V., and Klionsky, D. J. (1995) J. Cell Biol. 131, 1727–1735
3. Scott, S. V., Hefner-Gravink, A., Morano, K. A., Noda, T., Ohsumi, Y., and Klionsky, D. J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 12304–12308
4. Harding, T. M., Hefner-Gravink, A., Thumm, M., and Klionsky, D. J. (1996) J. Biol. Chem. 271, 17621–17624
5. Seguí-Real, B., Martínez, M., and Sandoval, I. V. (1995) EMBO J. 14, 5476–5484
6. Oda, M. N., Scott, S. V., Hefner-Gravink, A., Caffarelli, A. D., and Klionsky, D. J. (1996) J. Cell Biol. 132, 999–1010
7. Martínez, E., Jiménez, M. A., Seguí-Real, B., Vandekerckhove, J., and
Protein Transport from Cytoplasm to Vacuole

Sandoval, I. V. (1997) _J. Mol. Biol._ **267**, 1124–1138
8. Kim, J., Scott, S. V., Oda, M. N., and Klionsky, D. J. (1997) _J. Cell Biol._ **137**, 609–618
9. Scott, S. V., Baba, M., Ohsumi, Y., and Klionsky, D. J. (1997) _J. Cell Biol._ **138**, 37–44
10. Baba, M., Osumi, M., Scott, S. V., Klionsky, D. J., and Ohsumi, Y. (1997) _J. Cell Biol._ **139**, 1687–1695
11. Martinez, E., Seguí-Real, B., Silles, E., Mazon, M. J., and Sandoval, I. V. (1999) _Mol. Microbiol._ **33**, 52–62
12. Becker, J., Walter, W., Yan, W., and Craig, E. A. (1996) _Mol. Cell. Biol._ **16**, 4378–4386
13. Unno, K., Kishido, T., Hosaka, M., and Okada, S. (1997) _Biol. Pharmacol. Bull._ **20**, 1240–1244
14. Kim, S., Schilke, B., Craig, E. A., and Horwich, A. L. (1998) _Proc. Natl. Acad. Sci. U. S. A._ **95**, 12860–12865
15. Baudin, A., Ozier-Kalogeropoulos, O., Denouel, A., Lacroute, F., and Cullin, C. (1993) _Nucleic Acids Res._ **21**, 3229–3330
16. Wach, A., Brach, A., Pohmann, R., and Philipsen, P. (1994) _Yeast_ **10**, 1793–1808
17. Thomas, B. J., and Rothstein, R. (1989) _Cell_ **56**, 619–630
18. Sikorski, R. S., and Hieter, P. (1989) _Genetics_ **122**, 19–27
19. Sherman, F., Fink, G. R., and Hicks, J. B. (1986) _Laboratory Course Manual for Methods in Yeast Genetics_, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
20. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) _J. Bacteriol._ **153**, 163–168
21. Sambrook, J. E., Fritsch, E. F., and Maniatis, T. (1989) _Molecular Cloning: A Laboratory Manual_, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
22. Gevaert, K., Demol, H., Sklyarova, T., Vandekerckhove, J., and Heutheave, T. (1998) _Electrophoresis_ **19**, 909–917
23. Mann, M., and Wilm, M. (1994) _Anal. Chem._ **66**, 4390–4399
24. Griffin, P. R., MacCoss, M. J., Eng, J. K., Blevins, R. A., Aaronsen, J. S., and Yates, J. R., 3rd. (1995) _Rapid Commun. Mass Spectrom._ **9**, 1546–1551
25. Satyanarayana, C., Schroder-Kohne, S., Craig, E. A., Schu, P. V., and Horst, M. (2000) _FEBS Lett._ **470**, 232–238
26. Arnold, C. E., and Wittrup, K. D. (1994) _J. Biol. Chem._ **269**, 30412–30418
27. McClellan, A. J., Endres, J. B., Vogel, J. P., Palazzi, D., Rose, M. D., and Brodsky, J. J. (1998) _Mol. Biol. Cell_ **9**, 3533–3545
28. Endo, T., Mitsui, S., Nakai, M., and Roise, D. (1996) _J. Biol. Chem._ **271**, 4161–4167
29. Liu, Y., Liang, S., and Tartakoff, A. M. (1996) _EMBO J._ **15**, 6750–6757