An Artificial Transmembrane Segment Directs SecA, SecB, and Electrochemical Potential-dependent Translocation of a Long Amino-terminal Tail*

(Received for publication, August 13, 1998, and in revised form, December 7, 1998)

Jonathan L. McMurry and Debra A. Kendall‡

From the Department of Molecular and Cell Biology, The University of Connecticut, Storrs, Connecticut 06269

Membrane integration and assembly of integral membrane proteins are complex processes. Integral membrane proteins have been classified according to their transmembrane topography and mode of membrane translocation (1). Type I consists of proteins with a cleavable signal peptide and a translocated amino terminus. In *Escherichia coli*, the signal peptide engages the Sec translocation machinery, which is responsible for the translocation of the amino-terminal periplasmic segment across the inner membrane. However, many integral membrane proteins are synthesized without signal peptides. These segments, although poorly understood, have been reported to be independent of the protein secretion machinery. In contrast, here we describe alkaline phosphatase mutants containing artificial transmembrane segments that demonstrate that translocation of a long N-tail across the membrane is dependent upon SecA, SecB, and the electrochemical potential in the absence of a signal peptide. The corresponding mutants containing signal peptides also use the secretion machinery but are less sensitive to inhibition of its components. We present evidence that inhibition of SecA by sodium azide is incomplete even at high concentrations of inhibitor, which suggests why SecA-dependent translocation may not have been detected in other systems. Furthermore, by varying the charge around the transmembrane segment, we find that in the absence of a signal peptide, the orientation of the membrane-bound alkaline phosphatase is dictated by the positive inside rule. However, the presence of a signal peptide is an overriding factor in membrane orientation and renders all mutants in an N_out-C_in orientation.

Many integral membrane proteins contain an amino-terminal segment, often referred to as an N-tail, that is translocated across a membrane. In many cases, translocation of the N-tail is initiated by a cleavable, amino-terminal signal peptide. For N-tail proteins lacking a signal peptide, translocation is initiated by a transmembrane segment that is carboxyl to the translocated segment. The mechanism of membrane translocation of these segments, although poorly understood, has been reported to be independent of the protein secretion machinery. In contrast, here we describe alkaline phosphatase mutants containing artificial transmembrane segments that demonstrate that translocation of a long N-tail across the membrane is dependent upon SecA, SecB, and the electrochemical potential in the absence of a signal peptide. The corresponding mutants containing signal peptides also use the secretion machinery but are less sensitive to inhibition of its components. We present evidence that inhibition of SecA by sodium azide is incomplete even at high concentrations of inhibitor, which suggests why SecA-dependent translocation may not have been detected in other systems. Furthermore, by varying the charge around the transmembrane segment, we find that in the absence of a signal peptide, the orientation of the membrane-bound alkaline phosphatase is dictated by the positive inside rule. However, the presence of a signal peptide is an overriding factor in membrane orientation and renders all mutants in an N_out-C_in orientation.

This paper is available on line at http://www.jbc.org

* This work was supported in part by National Institutes of Health Grant GM37639 (to D. A. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Molecular and Cell Biology, Box U-44, The University of Connecticut, Storrs, CT 06269. E-mail: kendall@uconnvm.uconn.edu.

6776 This paper is available on line at http://www.jbc.org
III relative to type I proteins.

In contrast to previous findings, we describe alkaline phosphatase mutants containing artificial transmembrane segments that demonstrate that translocation of a long amino-terminal segment across the membrane is SecA-dependent, SecB-dependent, and electrochemical potential-dependent, regardless of the presence or absence of a signal peptide. Mutants lacking signal peptides obey the positive inside rule. Those with more positive regions flanking the amino terminus of the transmembrane segment orient in an N\textsubscript{ins}-C\textsubscript{out} manner. By varying the charge in the regions flanking the transmembrane segment to make the carboxyl flanking region more positive than the amino flanking region, a reversion of orientation occurs that results in an N\textsubscript{int}-C\textsubscript{out} orientation in which a long amino-terminal segment is translocated. Surprisingly, mutants containing a signal peptide have an N\textsubscript{int}-C\textsubscript{out} orientation regardless of flanking charge. Further, these mutants demonstrate that when signal peptide-mediated translocation is compromised by SecA inhibition, transmembrane segment-mediated translocation also does not occur.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Media**—E. coli AW1043 (\textsc{lac gal u gal K \textsc{delena-ara}) phoA-E15 pro C::Tn5} was used for the generation and propagation of mutant forms of alkaline phosphatase and for translocation and membrane association analyses. For general propagation of AW1043, LB medium (9) was used. E. coli CK1953 (MC4100 secB::Tn5; gift from C. A. Kumamoto) was used for SecB-dependent translocation analysis. CK1953 was propagated in M63 medium (10). All media were supplemented with 50 \text{µg/ml kanamycin} and 250 \text{µg/ml ampicillin.}

**DNA Manipulations and Mutagenesis**—Mutants were generated from a previously described construct, CN-LA2 (8, 11), by insertions. In all mutants, the hydrophobic segment of the amino-terminal segment across the membrane is SecA-dependent, regardless of the presence or absence of a signal peptide. The final to the TMS indicates whether the wild type sequence or an insertion of 5 lysines flanks the transmembrane sequence.

**Electrophoresis and Quantitation of Proteins**—Immunoprecipitated proteins were separated by electrophoresis on 7.5% or 10% Laemmli polyacrylamide gels (17). Patterns were visualized by autoradiography and quantified by phosphorimage analysis using a Bio-Rad phosphorimager and Molecular Analyst version 1.4.1 software.

**RESULTS**

To examine the requirements for stable membrane integration and orientation, an artificial transmembrane segment was inserted into E. coli alkaline phosphatase. Position 278 was chosen for insertion of the transmembrane segment because it is in a loosely folded region removed from the core and the catalytic site of the enzyme (18, 19) and can accommodate large insertions. In all mutants, the hydrophobic segment of the transmembrane region consists of 21 amino acids, 5 leucines, and 16 alanines. This composition was chosen because previous studies (8) suggested that it offered a balance between being stably membrane-associated yet not so highly hydrophobic that effects due to tandem charges would be obscured. To analyze the role of charged residues within the regions flanking the transmembrane segment, the degree of charge and the charge balance of the region flanking the termini of the transmembrane segment were varied. On the amino-terminal side of the transmembrane segment, the charge was varied from 0 to +5 using various combinations of lysine and glycine residues. In the region immediately flanking the carboxyl terminus of the transmembrane segment, the charge was the wild type charge of +0.5 or +5.5, the latter generated by the insertion of five lysine residues. Parallel constructs were made in which the amino-terminal signal peptide was deleted. Fig. 1 shows the amino acid sequences of the mutants.

To ascertain the ability of the inserted sequences to function as transmembrane segments, NaOH extractions were performed by the method of Russel and Model (14). The extent of membrane association of the signal peptide-containing mutants is shown in Fig. 2A. All of these mutants show membrane association, and little difference was observed as a result of

---

1 The abbreviations used are: CCCP, carbonyl cyanide 3-chlorophenylhydrazone; CHAPS, 3-[3-cholamidopropyl]dimethylammoniomio]-1-propane-sulfonate; TMS, transmembrane segment.
varying the charge surrounding the transmembrane segment. Membrane association is further confirmed by cell fractionation (Fig. 2B). By treating spheroplasts with proteinase K, the transmembrane topography of these mutants was determined. Unlike the tightly folded native alkaline phosphatase, which is transported across the membrane to the periplasm and is resistant to proteolysis, the periplasmic segments of membrane-bound alkaline phosphatase mutants are very sensitive to proteolysis. Conversely, the fraction of the mutant that remains in the cytoplasm is protected from proteolysis. Due to the position of the transmembrane segment at approximately two-thirds the length of the protein away from the amino terminus, the membrane orientation can be determined by the size of the fragment protected. If the protein inserts in an N<sub>out</sub>-C<sub>in</sub> orientation, the carboxyl-terminal one-third of the protein will be protected. If the protein inserts in a C<sub>in</sub>-N<sub>out</sub> orientation, the amino-terminal two-thirds of the protein will be protected. Treatment of the spheroplasts with 0.2% CHAPS to solubilize the membrane results in proteolysis of the two types of membrane integrated mutants. The schematic at the bottom provides a model for the orientation of the mutants.

Addition of CHAPS resulted in the loss of this protected fragment, consistent with its cytoplasmic location. It is remarkable that clustered positive charges are translocated across the membrane in mutants containing five lysines (SP5TMW) and three lysines (SP3TMW) immediately amino-terminal to the transmembrane segment. The positive inside rule (7) would predict that the amino termini of the transmembrane segment in these constructs would not be translocated because they are more positively charged than the carboxyl termini. However, in this context, the presence of a wild type signal sequence seems to override the positive inside rule and dictate that the orientation of the transmembrane span be N<sub>out</sub>-C<sub>in</sub>.

The extent of membrane association of mutants with deleted signal peptides is shown using NaOH extraction (Fig. 3A) and cell fractionation (Fig. 3B). Like the signal peptide-containing mutants, all deleted signal peptide mutants demonstrate membrane association. To determine the orientation of these mutants, protease protection analysis was performed (Fig. 3C). In the absence of a signal peptide, the orientation of the protein is

---

**Fig. 2.** Membrane association, translocation, and orientation of signal sequence containing mutants. A, NaOH extraction to verify membrane association. Cells containing plasmids encoding the indicated mutants were labeled with [35S]methionine for 30 s and chased with excess cold methionine for 30 s prior to extraction in 0.1 M NaOH. The percentage of total alkaline phosphatase associated with the membrane fraction is plotted on the ordinate. B, cell fractionation. Lanes W, whole cell fraction; lanes M, membrane fraction; lanes C, cytoplasmic fraction. C, protease protection analysis to determine translocation and orientation. Cells were labeled as described, converted to spheroplasts, and subjected to protease digestion. Addition of proteinase K and CHAPS are indicated by plus signs. The positions of mature form, a degradation product thereof, and the protected fragment corresponding to the carboxyl-terminal one-third of the protein that contains the TMS are indicated by m, d, and C1/3, respectively. Molecular weight markers N2/3+TM and C1/3+TM were used for comparison. N2/3+TM and C1/3+TM are expressed fragments that include the amino-terminal two-thirds of the protein and the TMS or the carboxyl-terminal one-third and the TMS, respectively, as would result from proteolysis of the two types of membrane integrated mutants. The schematic at the bottom provides a model for the orientation of the mutants.
dependent upon the charge state of the regions flanking the transmembrane segment. Mutants with amino-terminal flanking regions that are more positive than the carboxyl-terminal flanking regions (DS5TMW and DS3TMW) exhibited an N-in-C-out orientation as indicated by protected fragments corresponding to the amino-terminal two thirds of the protein (N2/3). No fragments corresponding to the carboxyl-terminal one third of the protein (C1/3) were evident in the DS5TMW or DS3TMW mutants, indicating that no significant fraction of the expressed protein is in the opposite orientation of N-out-C-in. This suggests that in the absence of the signal peptide, a transmembrane segment with amino-terminal positive charges behaves like a signal anchor, i.e., it functions to initiate translocation of the carboxyl-terminal one-third of the protein. In contrast, the mutants with a more positive carboxyl flanking region (DS0TMW and DS0TM5) exhibited the opposite orientation of N-out-C-in as evidenced by the presence of a fragment corresponding to the carboxyl-terminal one-third of the protein plus the transmembrane segment. Hence they contain a translocated amino-terminal segment of approximately 288 residues that is accessible to proteolysis. As with the signal peptide-containing mutants, addition of CHAPS resulted in the loss of all protected fragments. Thus, in the absence of a signal peptide, the charge balance surrounding the transmembrane segment influences orientation, and the positive inside rule is followed, even though it necessitates translocation of a long N-tail. The fraction of full-length protein remaining in the presence of protease indicates that some of the population of labeled protein is not fully translocated. Thus the transmembrane segment is less efficient at initiating translocation than...
methionine, conversion to spheroid for 1 min prior to labeling with 

The DS0TM5 mutant was chosen to investigate the mechanism of translocation of long amino-terminal segments. A concentration course of sodium azide treatment was used to ascertain the involvement of SecA. In this experiment inhibition of translocation was demonstrated by a decrease in the amount of protected fragment (C1/3) and a concomitant increase in the amount of full-length protein that was protected from proteolysis with increasing azide concentration. As shown in Fig. 4, sodium azide treatment blocked the translocation of the amino-terminal segment of DS0TM5 as demonstrated by the near absence of the protected small fragment at high azide concentrations. Across the concentration course from low azide concentrations to high, the fraction of total labeled protein that is represented in the small fragment decreases, and the fraction of full-length, fully protected protein increases to about 100% at 10 mM, indicating that SecA-mediated transport is inhibited.

The SecA dependence of amino-terminal segment translocation is also evident from azide treatment of mutant SP0TM5 (Fig. 5). There are two possible initiators of translocation in this mutant, the signal peptide and the transmembrane segment. The presence of the mature form in the samples not treated with protease indicates that signal-peptide-mediated translocation has occurred, because the signal peptide is cleaved upon translocation. If translocation via the signal sequence is inhibited, there is no transmembrane segment-mediated translocation. Were transmembrane segment-mediated translocation SecA-independent, one would expect the precursor form to be susceptible to proteolysis, because it too would be translocated. Even if the signal peptide itself remained associated with the membrane due to its hydrophobicity, the bulk of the amino-terminal segment should be translocated and protease sensitive. This experiment further indicates that the signal peptide is more resistant to compromise of the Sec machinery by sodium azide than the transmembrane segment; substantial levels of signal peptide-mediated translocation readily occur at concentrations of azide at which no transmembrane segment-mediated translocation occurs for both the DS0TM5 and SP0TM5 mutants.

Because only about 30% of the expressed SP0TM5 is in precursor form even at high azide concentrations, a pulse-chase analysis following azide treatment was performed. We found that following treatment with 10 mM azide and a 30-s pulse labeling followed by a 30-s cold methionine chase that 74% of the labeled protein was in the precursor form compared with 6% in the untreated control (data not shown). This suggests that a larger population was inhibited in the azide treatment experiment described above (Fig. 5) but that much of the precursor chased through to the mature form during the time taken for protease treatment.

The SecB dependence of SP0TM5 and DS0TM5 mutants was assayed by protease protection analysis of translocation in the SecB null strain CK1953 (Fig. 6). Two previously described signal peptide-containing proteins were used as controls (20). WT-MCS(dl), a modified form of wild type alkaline phosphatase, is SecB-independent, and WT-K5L5 is SecB-dependent. Both proteins exhibited this behavior under the conditions used in the present study. SP0TM5 is readily translocated in the absence of SecB because most of the population of labeled protein is present in the mature form in the sample not treated with protease. The mature form is susceptible to proteolysis, giving rise to the C1/3 fragment, further indicating that the amino-terminal segment has been translocated even in the absence of SecB. Only a small fraction of the SP0TM5 population is not translocated and accumulates as precursor. In contrast, DS0TM5 is translocated poorly in the SecB null strain. Virtually all of the labeled protein is protected from proteolysis, indicating that DS0TM5 is largely untranslated and is thereby very sensitive to the loss of SecB. This is consistent with the notion that dependence on SecB is more pronounced for proteins that are translocated slowly and post-translationally (21).
Dissipation of the electrochemical potential by CCCP also inhibits translocation of the amino-terminal segment (Fig. 7). Treatment with 100 mM CCCP greatly affects the efficiency of translocation in DS0TM5 as evidenced by the near absence of a protected fragment and a concomitant increase in the amount of full-length protected protein. SP0TM5 is less affected; the amount of protected fragment present under CCCP treatment is comparable with that of the untreated parallel sample. Thus, translocation of DS0TM5 is virtually entirely inhibited at 100 mM CCCP, whereas that of SP0TM5 is somewhat less inhibited, suggesting that transmembrane segment mediated translocation is more sensitive to loss of the electrochemical potential than signal peptide-mediated translocation. These results parallel the sensitivities of DS0TM5 and SP0TM5 to azide treatment. This trend was previously observed for several signal peptide (22, 23) and SecB-dependent (20) mutants and points to the intriguing possibility that the requirement for SecA and the electrochemical gradient are directly related.

**DISCUSSION**

Mutants that do not have a signal peptide obey the positive inside rule (7), which stipulates that the orientation of a transmembrane segment is determined by the flanking charge, with the more positively charged flanking region remaining in the cytoplasm. As demonstrated in Fig. 3C, mutants with deleted signal peptides have an N_{in}-C_{out} orientation when the more positively charged flank is at the amino terminus of the transmembrane segment. However, mutants with reduced amino-terminal charge or increased carboxyl-terminal charge exhibit an N_{out}-C_{in} orientation.

In contrast, the presence of a wild type signal peptide is an overriding factor in membrane orientation. Regardless of amino-terminal flanking charge, all signal peptide-containing mutants have an N_{out}-C_{in} orientation (Fig. 2C). **Prima facie**, the positive inside rule would predict that the orientation of the mutants would be determined by the charge balance surround-
N-Tail Translocation

ing the transmembrane segment, as it does correctly in the deleted signal peptide mutants. However, the signal peptide seems to dictate assembly, when necessary initiating the translocation of as many as five lysines at the amino terminus of the transmembrane segment with little effect upon extent of membrane integration. Perhaps the reason that translocation of a high density of basic residues occurs in this system and not others (24) is because of the considerable length of polypeptide between the signal peptide and the transmembrane segment. Upon translocation initiation by the signal peptide, the long N-tail may be translocated before the cluster of basic residues is encountered, and at that point the system is poised strongly in favor of the passage of the basic residues and completing translocation.

DS0TM5 also gives rise to a translocated N-tail of approximately 288 amino acids. The energy required to translocate such a long hydrophilic segment cannot be explained by membrane partitioning. Although it is reasonable that very short hydrophilic segments may be translocated as a result of spontaneous insertion of a nearby transmembrane segment (25), the free energy barrier for translocating longer segments is too high to be overcome by the free energy gained through insertion of a transmembrane segment. Thus, the SecA dependence of N-tail translocation may result from the need for active translocation.

In the reported examples of SecA-independent N-tail translocation that utilize sodium azide as a SecA inhibitor, the concentration of sodium azide used was typically 2–3 mM. In our study, mutant DS0TM5 is largely translocated at 2 mM azide (Fig. 4); significant inhibition of translocation is not observed until higher azide concentration. Treatment of cells with sodium azide can be expected to be leaky; the concentration of azide used may not be sufficient to completely inhibit all SecA in these experiments. Under such conditions, sequences with high affinities for SecA will be preferentially translocated. Such a possibility has been suggested to account for differences in the sensitivity of alkaline phosphatase signal peptide mutants to azide treatment (21, 22) and underscores the potential difficulties in using proteins that differ from the protein whose transport is being studied as reporters for the loss of SecA function. Similarly, MalF, an integral membrane protein, was reported to be SecA-independent using azide treatment (26) but later shown to be SecA-dependent by Traxler and Murphy (27).

It has been proposed that the Sec machinery has an amino-to-carboxyl directionality (6). The reported examples of SecA independence of N-tail translocation would seem to confirm this because the transmembrane segment-mediated translocation has the opposite directionality and apparently does not utilize the Sec machinery. However, our results indicate that amino-terminal segment translocation is SecA-dependent and can be initiated by a transmembrane segment that ultimately resides in an N-term-C-term orientation. In our system, there are no other segments between the amino terminus and the transmembrane segment that even remotely resemble a signal sequence and that might be expected to fulfill this role even transiently. Therefore, it is interesting to speculate how the interaction of a transmembrane segment with SecA occurs. The interaction may be relatively nonspecific, involving hydrophobic surfaces and perhaps the flanking charged region. These features could be contributed by the amino acid side chain irrespective of the directionality of the peptide bonds. Such interactions involving overall amino acid side chain properties have been demonstrated for other systems including β-endorphin (28) using peptides composed of D rather than L amino acids. Alternatively, SecA may interact with polypeptides exclusively in the amino-to-carboxyl direction with the final orientation of the transmembrane region being subsequently driven by the balance of charged flanking residues. However, such a model would require that the transmembrane segment undergo a reversion of orientation at some point during the translocation process.

The SecA-dependent translocation of an artificially produced amino-terminal tail suggests that it is very likely the case that many N-tails are translocated in the same fashion. Further, it is possible that many sequences shorter than the one described here are SecA-dependent as well but that translocation of those tails has been observed under insufficiently impaired SecA conditions. Future studies will need to consider the affinity of SecA for various signal sequences and transmembrane segments. If truly SecA-independent translocation does occur, it is probable that the partitioning of transmembrane segments into the membrane would provide only enough energy to translocate very short N-tails.

Mutant DS0TM5 is considerably more reliant on the presence of SecB for translocation than is SP0TM5. It may be the case that SecB is required to maintain the amino-terminal two-thirds of the protein in an unfolded, translocation competent state until the transmembrane segment can be synthesized and then recognized by the translocation machinery. The folding state of the amino-terminal domain of a type III protein has been shown to affect translocation (29). In that study, rapidly folding domains inserted into the amino-terminal segment hindered or altogether prevented translocation of the amino-terminal segment. Thus, the involvement of SecB in translocation of membrane proteins may be a common phenomenon.

In contrast to previous reports of amino-terminal tail translocation that is independent of the translocation machinery, we show that the amino-terminal tail in our study requires SecA and SecB for efficient membrane translocation. Moreover, the lack of a signal peptide in our type III membrane protein confers greater susceptibility to compromises in transport machinery function and electrochemical gradient disruption.

Acknowledgments—We thank Jinoh Kim and Sharay Rush for helpful discussions of these studies.

REFERENCES

1. von Heijne, G., and Gavel, Y. (1988) Eur. J. Biochem. 174, 671–678
2. Wallin, E., and von Heijne, G. (1995) Protein Eng. 8, 693–698
3. Can, G., and Dalbey, R. E. (1994) EMBO J. 13, 4661–4669
4. Whitley, P., Gafvelin, G., and von Heijne, G. (1995) J. Biol. Chem. 270, 29831–29835
5. Cao, G., Kuhn, A., and Dalbey, R. E. (1995) EMBO J. 14, 866–875
6. Dalbey, R. E., Kuhn, A., and von Heijne, G. (1995) Trends Cell Biol. 5, 380–383
7. von Heijne, G. (1996) EMBO J. 15, 3021–3027
8. Chen, H., and Kendall, D. A. (1995) J. Biol. Chem. 270, 14115–14122
9. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., p. A.1, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
10. Miller, J. H. (1972) Experiments in Molecular Genetics, p. 431, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
11. Kendall, D. A., and Kaiser, E. T. (1988) J. Biol. Chem. 263, 7261–7265
12. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
13. Kendall, D. A., Bock, S. C., and Kaiser, E. T. (1986) Nature 321, 706–708
14. Russel, M., and Model, P. (1982) Cell 28, 177–184
15. Oliver, D. B., Cabrili, R. J., Dolan, K. M., and Jarosik, G. P. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 8227–8231
16. Kuhn, A., and Wickner, W. (1985) J. Biol. Chem. 260, 15914–15918
17. Lasemlli, U. K. (1970) Nature 227, 680–685
18. Kim, E. E., and Wycoff, H. W. (1991) Clin. Chem. Acta 186, 175–188
19. Kim, E. E., and Wycoff, H. W. (1991) J. Mol. Biol. 218, 449–464
20. Kim, J., and Kendall, D. A. (1998) J. Bacteriol. 180, 1396–1401
21. Franetic, O., and Kumaomoto, C. A. (1996) J. Bacteriol. 178, 5954–5959
22. Rusch, S. L., Chen, H., Izard, J. W., and Kendall, D. A. (1994) J. Cell Biol. 125, 209–217
23. Izard, J. W., Rusch, S. L., and Kendall, D. A. (1996) J. Biol. Chem. 271, 21579–21582
24. von Heijne, G. (1994) Annu. Rev. Biophys. Biomol. Struct. 23, 167–192
25. Engelman, D. M., and Steitz, T. A. (1981) Cell 23, 411–422
26. McGovern, K., and Beckwith, J. (1991) J. Biol. Chem. 266, 20870–20876
27. Traxler, B., and Murphy, C. (1996) J. Biol. Chem. 271, 12394–12400
28. Blanc, J. P., and Kaiser, E. T. (1984) J. Biol. Chem. 259, 9549–9556
29. Denzer, A. J., Nabholz, C. E., and Speiss, M. (1995) EMBO J. 14, 6311–6317