Vol. 193, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 28, 1993 Pages 228-234

SEC-Y PROTEIN IS LOCALIZED IN BOTH THE CYTOPLASMIC AND THYLAKOID MEMBRANES IN THE CYANOBACTERIUM *Synechococcus* PCC7942

Masato Nakai‡, Daigo Sugita‡, Tatsuo Omata† and Toshiya Endo‡*

‡ Department of Chemistry, Faculty of Science, and †Department of Applied Biological Sciences, School of Agricultural Sciences, Nogoya University, Chikusa-ku, Nagoya 464-01, Japan

Received April 13, 1993

SUMMARY: Members of the SecY protein family mediate protein export in bacterial cells. Southern analyses showed that *secY* is likely a single copy gene in the cyanobacterium *Synechococcus* PCC7942. Then the subcellular location of the cyanobacterial SecY protein was determined; i) antiserum raised against a fusion protein between the SecY fragment and maltose binding protein were used for immunoblotting of the membrane fractions, and ii) a modified SecY protein carrying the c-Myc peptide tag was expressed in the cyanobacterial cells, and the subcellular distribution of the SecY-c-Myc fusion protein was analyzed with the anti-c-Myc antibodies. The obtained results suggest that the SecY protein is localized in the thylakoid membrane as well as the cytoplasmic membrane; the SecY protein probably mediates protein translocation across both the cytoplasmic and thylakoid membranes in *Synechococcus* PCC7942.

The SecY protein has been found to be one of the essential components of the protein translocation (export) machinery of the cytoplasmic membrane in the gram-negative bacterium, *Escherichia coli* (1). We have cloned a *secY* gene homologue from the genome of the cyanobacterium *Synechococcus* PCC7942 (2); the cyanobacterial *secY* gene is located at the promoter distal region of the putative *spc* operon, like the *secY* genes found in the other organisms (3, 4). Cyanobacteria, which are gram-negative bacteria but perform oxygenic photosynthesis, have not only the typical structure of cell envelopes consisting of the outer and inner cytoplasmic membranes but also thylakoid membranes in the cytoplasm. The thylakoid membrane has no obvious physical connection with the cytoplasmic membrane (5). Since proteins with transmembrane destinations are synthesized in the cytoplasm, the SecY protein in *Synechococcus* PCC7942 may mediate protein translocation across the cytoplasmic membrane and/or the thylakoid membrane. By using two independent immunolocalization methods, we now demonstrate that the SecY protein is located in both the cytoplasmic and thylakoid membranes in the *Synechococcus* PCC7942 cells.

* To whom correspondence should be addressed.

0006-291X/93 $4.00

MATERIALS AND METHODS

Strains and Culture Conditions. *Synechococcus* PCC7942 (R2-SPc) was grown at 30°C in BG11 medium (6) as described by Omata *et al.* (7). Two *E. coli* strains, TG1 (8) and TB1 (New England Biolabs), were used for propagation of DNA constructs in plasmid vectors, and cultivated according to the published procedure (8).

Southern Analyses. Chromosomal DNA was extracted and purified from *Synechococcus* PCC7942 cells according to the published procedure (9). Southern hybridization was performed as described previously (8) using the 0.9 kbp *Sal*I fragment derived from the *secY* gene as a probe.

Subcellular Fractionation and Immunoblotting. Cell walls, cytoplasmic membranes, and thylakoid membranes were prepared from *Synechococcus* PCC7942 cells as described previously (10). Since polypeptide compositions of the three fractions are distinct one another, cross contaminations among the fractions were estimated by densitometric scanning of the protein bands of each fraction, which were stained with Coomassie Brilliant Blue. Protein samples were solubilized with sample buffer containing 60 mM Tris-HCl, pH 6.8, 2 % SDS, 5 % glycerol, 5 % ß-mercaptoethanol, and 0.002 % bromophenol blue at 37°C for 5 minutes, subjected to 12 % SDS polyacrylamide gel electrophoresis, and transferred to Immobilon PVDF membrane filters (Milipore). The filters were treated with the anti-MBP/ΔSecY(see below) or anti-c-Myc (Oncogene Science) antibodies, and the enhanced chemiluminescence method with ECL detection kit (Amersham) was used for protein detection. Affinity purification of the anti-MBP/ΔSecY antibodies was carried out with the purified MBP/ΔSecY protein according to the procedure of Johnson *et al.* (11).

Expression of the MBP/ΔSecY Fusion Protein in *E. coli*, and Production of the Antisera. The *secY* gene fragment corresponding to the amino acid residues 343-439, was ligated to pMAL-c expression vector (New England Biolabs). Then the MBP/ΔSecY fusion protein was purified from the *E. coli* transformants (TB1 strain) harboring the above construct. Antisera against the purified fusion protein were obtained from the rabbits which had been injected initially with 0.3 mg of the protein emulsified in complete Freund's adjuvant and then with 0.3 mg of the protein for the boost.

Construction and Expression of the *secY* / c-*myc* Fusion Gene. The entire coding region of the *secY* gene was inserted into pTrc99A, an *E. coli* expression vector (Pharmacia) containing the *trc* promoter sequence and the *lacI*^{q} repressor gene. Then the oligonucleotides corresponding to the human c-Myc peptide tag (12) were inserted at the end of the *secY* gene and an *in frame* fusion gene between *secY* and the c-*myc* tag was generated. The *Nsi*I / *Sca*I fragment containing this expression unit was ligated with the *Sal*I/ *Eco*RI fragment of pUC303, a cyanobacterium-*E. coli* shuttle vector (13). The resultant plasmid pDS-secY/c-myc was transformed into *Synechococcus* PCC7942 cells as described previously (7). As control plasmids, pDS-secY carrying the authentic *secY* gene without the c-*myc* tag and pUC303 were also separately transformed into cyanobacterial cells. The transformants were then selected and cultivated in BG11 medium containing 10 µg / ml of streptomycin. Expression from the *trc* promoter was induced by addition of 2 mM isopropyl-ß-D-thiogalactopyranoside (IPTG).

RESULTS

Southern Analyses of the *secY* Gene in *Synechococcus* PCC7942. In a previous study we have cloned a *secY* gene homologue from the genome of *Synechococcus* PCC7942 (2). In order to examine whether the cloned *secY* is a single copy gene in the organism or not, we performed Southern analyses using the cloned *secY* as a DNA probe. When the 0.9-kbp *Sal*I fragment derived from the *secY* gene was hybridized to the total *Synechococcus* PCC7942 DNA (Fig. 1), it hybridized only to the 0.9-kbp *Sal*I fragment from which it was derived, and to the 9-kbp *Hind*III and the 6.3-kbp *Eco*RI fragments which overlapped the *Sal*I fragment of the chromosomal DNA; the restriction map shown in Fig. 1A has been constructed on the basis of restriction analyses of the genomic DNA inserts of the λDASH II clones containing the *secY* gene (2). No other strong hybridization signal was detected even if stringency for hybridization

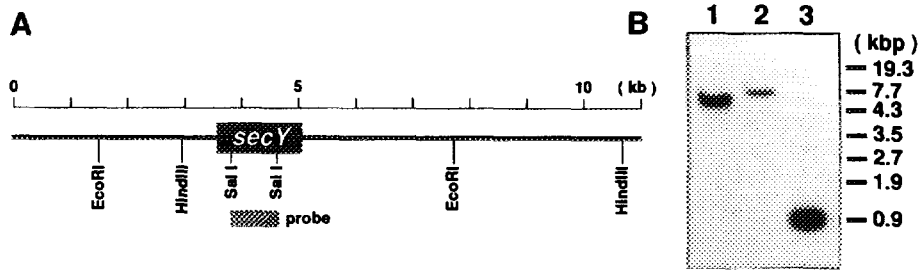

Figure 1. (A) A restriction map around the *secY* gene in the genome of *Synechococcus* PCC7942, and (B) an autoradiograph of Southern blot of *Synechococcus* PCC7942 probed with the 0.9 kbp *Sal*I fragment derived from the *secY* gene as shown in (A); the *Eco*RI (lane 1), *Hind*III (lane 2), and *Sal*I (lane 3) digests were run on a 0.8 % agarose gel.

was lowered to some extent (data not shown), and therefore, *Synechococcus* PCC7942 most likely contains a single copy of a *secY* homologue gene.

Preparation of the Anti-MBP/ΔSecY Serum and Immunological Detection of the SecY Protein. We cloned a fragment of the *Synechococcus* PCC7942 *secY* gene into an *E. coli* expression vector pMAL-c (New England Biolabs) to make a fusion gene encoding a hybrid protein (MBP/ΔSecY) consisting of the amino terminal 380 residues of the *E. coli* maltose-binding protein (MBP) and the carboxyl terminal 97 residues of the SecY protein. The overexpressed hybrid protein formed an inclusion body in the *E. coli* cells harboring the fusion gene and was subjected to further purification (data not shown). We injected the purified fusion protein into rabbits to raise antibodies.

We utilized antiserum against the MBP/ΔSecY fusion protein to assess the subcellular distribution of the *secY* gene product in *Synechococcus* PCC7942 by immunoblotting. The cyanobacterial cells were subfractionated into the cell wall (including the outer membrane) fraction (Fig. 2, lanes 1), the cytoplasmic membrane fraction (Fig. 2, lanes 2), and the thylakoid membrane fraction (Fig. 2, lanes 3). As shown in Fig. 2A, virtually no cross-contamination was observed when these fractions were analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie Brilliant Blue staining; less than 6% of cytoplasmic membranes cofractionated with thylakoid membranes and less than 5% of thylakoid membranes with cytoplasmic membranes. The anti-MBP/ΔSecY antiserum, but not the preimmune serum, reacted primarily with 37 kDa proteins in both the cytoplasmic and thylakoid membrane fractions, and bound weakly to 30 kDa and 63 kDa proteins of the cytoplasmic membrane and to 30 kDa protein of the thylakoid membrane (Fig. 2B and 2C).

The anti-MBP/ΔSecY antibodies were further affinity-purified against the MBP/ΔSecY protein immobilized on Immobilon PVDF membranes and used for immunoblotting. As shown in Fig. 3A, the 37 kDa proteins in both the cytoplasmic and thylakoid membranes, and the 31 kDa protein in the thylakoid membrane were detected. When the affinity-purified antibodies were conversely depleted of the anti-MBP/ΔSecY antibodies by preincubation with the cell lysate of *E. coli* expressing the MBP/ΔSecY fusion protein prior to immunoblotting, only the signal from the 37 kDa protein disappeared (Fig. 3C). Preincubation of the anti-MBP/ΔSecY antibodies with the lysate of the *E. coli* cells expressing MBP did not affect the signals from the

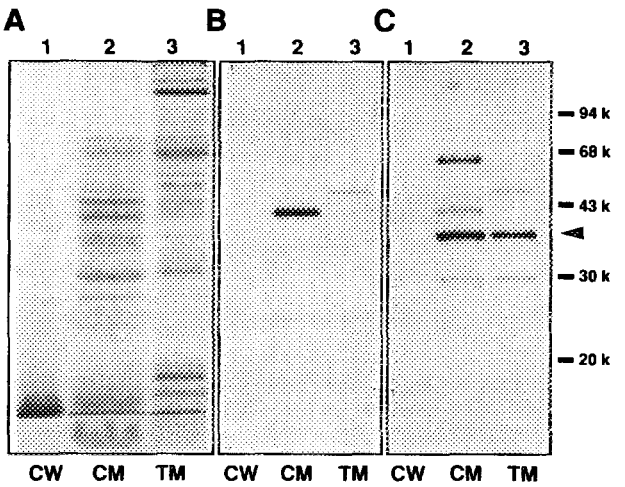

Figure 2. Immunodetection of the SecY proteins in the cell wall, cytoplasmic membrane and thylakoid membrane fractions prepared from *Synechococcus* PCC7942. Proteins of the cell wall fraction (20 μg, lanes 1 indicated as CW), the cytoplasmic membrane fraction (10 μg, lanes 2 indicated as CM), or the thylakoid membrane fraction (10 μg, lanes 3 indicated as TM) were separated by 12 % SDS polyacrylamide gel electrophoresis, followed by Coomassie Brilliant Blue staining (A), or immunoblotting with the preimmune serum (B) or the anti-MBP-ΔSecY antiserum (C). The 37 kDa proteins are denoted with the arrowhead.

37 kDa and 31 kDa proteins at all (Fig. 3B). Thus it is concluded that the 37 kDa proteins in the cytoplasmic and thylakoid membranes are specifically recognized by the antibodies that recognize the SecY moiety of the MBP/ΔSecY fusion protein. The apparent molecular size 37 kDa differs from the value (47,150) calculated from the primary structure of the SecY protein (2). However, this difference is likely due to the extreme hydrophobic nature of the cyanobacterial SecY protein since an anomalous migration behavior was previously observed for the *E. coli* SecY protein in the SDS-PAGE analysis (14).

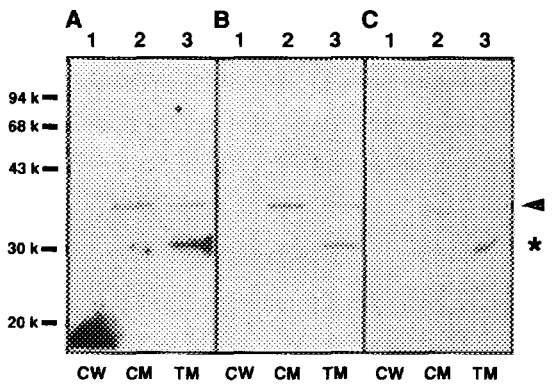

Figure 3. Determination of the antigenicity of the 37 kDa protein detected with the anti-MBP/ΔSecY serum. Immunoblotting was performed as described in the legend of Fig. 2, except for the use of affinity-purified anti-MBP/ΔSecY antibodies (A), or of anti-MBP/ΔSecY antibodies pretreated with the *E. coli* cell extracts containing MBP (B) or MBP/ΔSecY (C). The 37 kDa proteins are denoted with the arrowhead. The signals with the asterisk probably arise due to the artefact of chemiluminescence detection.

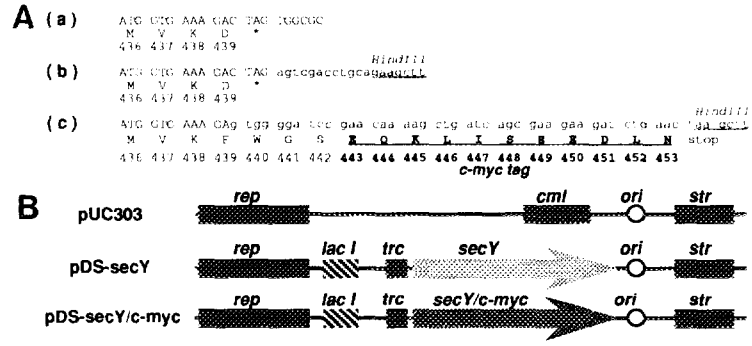

Figure 4. Construction of the *secY*/c-*myc* fusion gene. (A) A region around the 3' end of the *secY* gene (a) was modified as shown in (b) to be cloned into the pUC303, or altered and fused to the sequence encoding the human c-Myc peptide tag (c). (B) Schematic representation of the plasmids used for expression of the *secY* or *secY*/c-*myc* gene in *Synechococcus* PCC7942. Abbreviations: *str*, the streptomycin-resistance gene; *cml*, the chloramphenicol-resistance gene; *rep*, the autonomously replicating sequence derived from *Synechococcus*; *ori*, the replicating origin derived from *E. coli*; *trc*, the *trc* promoter sequence; and *lacI*, the *lacI*^{q} repressor gene.

Subcellular Localization of the c-Myc Epitope-Tagged SecY Protein in *Synechococcus* PCC7942. As a second approach to assess the subcellular localization of the SecY protein, we utilized the epitope tag method of immunodetection (12). We made a DNA construct encoding a fusion protein (SecY/c-Myc) in which the carboxyl-terminal Asp-439 of the SecY protein was replaced by the epitope tag of the 15 amino acid residues derived from the human c-Myc protein (Fig. 4A, (c)). The fusion gene was inserted into the cyanobacterium-*E. coli* shuttle vector, pUC303 (13), with the *E. coli trc* promoter and the *lacI*^{q} repressor gene (Fig. 4B). The transformants were grown in the presence or absence of 2 mM IPTG, an inducer for the expression from the *trc* promoter, subfractionated, and analyzed for subcellular localization of the SecY/c-Myc protein by immunoblotting with the monoclonal antibody recognizing the c-Myc epitope. As shown in Fig. 5, lanes 5-8, 38 kDa proteins were detected in both the cytoplasmic and thylakoid membranes only when the cells were grown in the presence of the inducer. The 38 kDa protein was not observed in the cells carrying the plasmid-borne *secY* gene without the c-*myc* tag, when detected with the anti-SecY/c-Myc antibody (Fig. 4 and Fig. 5, lanes 1-4). These results indicate that the 38 kDa protein associated with the cytoplasmic and thylakoid membranes represents the SecY/c-Myc fusion protein.

DISCUSSION

In the present study we have demonstrated that the SecY protein is localized in both the cytoplasmic and thylakoid membranes in *Synechococcus* PCC7942. This conclusion is drawn from the results of the two independent immunological analyses. First, the antibodies against the MBP/ΔSecY fusion protein reacted with the 37 kDa proteins in the cytoplasmic and thylakoid membrane fractions. This observation could be challenged by the argument that the antibodies against the MBP/ΔSecY fusion protein might have recognized a second SecY protein homologue, if any, in addition to the SecY protein; the detection of the 37 kDa proteins in the

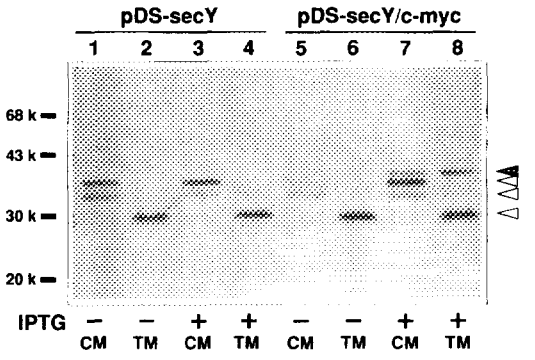

Figure 5. Immunological detection of the SecY/c-Myc proteins. Plasmid pDS-secY (lanes 1-4) and pDS-secY/c-myc (lanes 5-8) were separately transformed into the wild-type *Synechococcus* cells, and the transformed cells were cultivated in the absence (lanes 1, 2, 5, and 6) or presence (lanes 3, 4, 7, and 8) of 1 mM IPTG. Cytoplasmic membranes (CM, lanes 1, 3, 5, and 7) and thylakoid membranes (TM, lanes 2, 4, 6, and 8) were analyzed by immunoblotting with the monoclonal antibodies against the c-Myc peptide tag. Closed arrowhead, the SecY/c-Myc protein; open arrowheads, unidentified cross-reactive proteins.

cytoplasmic and thylakoid membranes might have merely reflected that both membranes had structurally related, but distinct SecY proteins. However this seems rather unlikely since the Southern analyses using the secY gene as a DNA probe strongly suggested that *Synechococcus* PCC7942 contains a single copy of a *secY* homologue gene.

Second, the monoclonal antibody against the c-Myc epitope reacted with the 38 kDa proteins in the cytoplasmic and thylakoid membrane fractions prepared from the cells with the plasmid-borne SecY/c-Myc fusion protein. This does not fit the possibility mentioned above, either, that there may be two related SecY proteins in *Synechococcus* PCC7942. Conversely, the results of the immunological analysis with the anti-MBP/ΔSecY fusion protein make the potential artefact of the epitope tag method unlikely that the subcellular location of the fusion protein carrying the c-Myc epitope may not reflect that of the authentic SecY protein.

Thus, although each of the two methods has its pitfalls, the same answer yielded by the two independent approaches makes the conclusion convincing. Since the SecY protein has been found to be a membrane protein that is indispensable for protein translocation across the cytoplasmic membrane in *E. coli* (15), the same SecY protein appears to constitute protein translocation machineries in the two distinct membranes, the cytoplasmic and thylakoid membranes, in *Synechococcus* PCC7942. Studies to examine if the SecY protein indeed mediates protein translocation across both the cytoplasmic and thylakoid membranes are currently in progress.

Acknowledgments : We thank Dr. H. Tokuda (University of Tokyo) for variable suggestions for immunological detection of the SecY protein. Oligonucleotide synthesis at the Center for Gene Research of Nagoya University is gratefully acknowledged. This work was supported by a grant from the Human Frontier Science Program Organization, a grant for "Biodesign Research Program" from The Institute of Physical and Chemical Research (RIKEN)

and a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1. Ito, K. (1992) Mol. Microbiol. 6, 2423-2428.
2. Nakai, M., Tanaka, A., Omata, T. and Endo, T. (1992) Biochim. Biophys. Acta 1171, 113-116.
3. Cerretti, D. P., Dean, D., Davis, G. R., Bedwell, D. M. and Nomura, M. (1983) Nucleic Acids Res., 11, 2599-2616.
4. Nakamura, K., Nakamura, A., Takamatsu, H.., Yoshikawa, H. and Yamane, K. (1990) J. Biochem. 107, 603-607.
5. Golecki, J. R. and Drews, G. (1982) in The Biology of Cyanobacteria (N. G. Carr and B. A. Whitton, Eds.), pp. 125-141. Blackwell, Oxford.
6. Stanier, R. Y., Kunisawa, R., Mandel, M. and Cohen-Bazire, G. (1971) Bacteriol. Rev., 35, 171-205.
7. Omata, T., Carlson, T. J., Ogawa, T.,and Pierce, J. (1990) Plant Physiol., 93, 305-311.
8. Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Second Edition. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.
9. Williams, J. G. K. (1988) Methods Enzymol. 167, 766-778.
10. Omata, T. and Murata, N. (1983) Plant Cell Physiol. 24, 1101-1112.
11. Johnson, L. M., Snyder, M., Chang, L. M. S., Davis, R. W. and Campbell, J. L. (1985) Cell 43, 369-377.
12. Munro, S. and Pelham, H. R. B. (1987) Cell 48, 899-907.
13. Kuhlemeier, C. J., Thomas, A. A. M., van der Ende, A., van Leen, R. W., Borrias, W. E., van den Hondel, C. A. M. J. J. and van Arkel, G. A. (1983) Plasmid 10, 156-163.
14. Ito, K. (1984) Mol. Gen. Genet. 197, 204-208.
15. Nishiyama, K., Kabuyama, Y., Akimaru, J., Matsuyama, S., Tokuda, H. and Mizushima, S. (1991) Biochim. Biophys. Acta 1065, 89-97.