Isolation and Characterization of a cDNA Clone Encoding a Cognate 70-kDa Heat Shock Protein of the Chloroplast Envelope*

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The translocation of proteins into the endoplasmic reticulum, the mitochondrion, and the chloroplast has recently been shown to involve homologues of the highly conserved 70-kDa heat shock protein (HSP70) family. In this study, we have isolated and sequenced a full-length cDNA clone encoding a cognate 70-kDa heat shock protein of the spinach chloroplast envelope (SCE70). The cDNA insert is 2,535 base pairs long and codes for 653 amino acid residues of a protein with a predicted molecular mass of 71,731 daltons. The deduced amino acid sequence shows a high degree of homology with HSP70 proteins from other organisms. Southern genomic and RNA analyses reveal different hybridization patterns than that observed for a heat-inducible 70-kDa protein gene. The protein synthesized from the SCE70 cDNA insert co-migrates with a 70-kDa polypeptide of the chloroplast envelope following sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Western blot analysis and import studies indicate that SCE70 is associated with the chloroplast outer envelope. The import data suggest that SCE70 is targeted to the envelope membrane via a pathway different from other plastidic precursors but similar to that recently reported for outer envelope proteins SOE1 and OM14.

The chloroplast envelope plays a major role in a number of important biochemical processes, among them protein import. Most chloroplast proteins are synthesized as precursors and are then imported from the cytosol via a post-translational mechanism. The mechanism by which precursor proteins translocate the envelope membrane involves a number of as yet unidentified components. Recent genetic and biochemical studies have revealed that 70-kDa heat shock proteins (HSP70) are involved in protein import (for recent reviews, see Refs. 1–3). Various members of the HSP70 family appear to be required for facilitating transport of proteins into the endoplasmic reticulum (4, 5), the mitochondrion (6), and the chloroplast (7). Characteristics commonly shared by HSP70 proteins are the ability to tightly bind to ATP and the possession of weak ATPase activities. In addition, these HSP70 homologues are often found associated with membranes and protein aggregates, characteristics that lead to the proposal that they bind or interact with precursor proteins in a folding/unfolding capacity to maintain an import-competent conformation required for translocation (for review, see Ref. 1).

In recent studies by Marshall et al. (8) and Amir-Shapira et al. (9), HSP70 components or homologues have been found to exist within the chloroplast compartment as well as in the chloroplast envelope. Waegemann and co-workers (7) further showed that two HSP70-related cytoplasmic factors found in leaf extracts are necessary for post-translational import of chlorophyll a/b-binding protein precursors into the chloroplast. The presence of these cytosolic factors renders the chlorophyll a/b-binding protein precursors more susceptible to trypsin treatment, suggesting a change in conformation, probably through unfolding of the precursors. In this report we have identified and characterized a full-length cDNA clone for a spinach HSP70-related chloroplast envelope protein (SCE70). The SCE70 gene appears to be constitutively expressed and exhibits no significant changes in steady-state mRNA levels during heat-shock treatment. This characteristic is in contrast to a second spinach 70-kDa cDNA clone which exhibits heat-inducible expression similar to that observed for some members of the HSP70 family. Western blot analysis and in vitro import studies revealed that SCE70 is targeted on the outside of the chloroplast envelope and is targeted to the membrane by a mechanism different from other imported polypeptides, but perhaps the same as two other outer envelope proteins recently reported by Salomon et al. (10) and Li et al. (11).

MATERIALS AND METHODS

Antibody Preparation—The polyclonal antiserum used in the identification of cDNA clones was raised in rabbits against total pea chloroplast envelope proteins. Chloroplast envelopes (both outer and inner membranes) were isolated as described by Cline et al. (12) from 9–11-day-old pea seedlings (Progress No. 9) grown at 21°C under fluorescent lighting with a 16:8 light:dark photoperiod. The immunization of rabbits and purification of the antiserum was carried out according to Chua et al. (13).

Identification of cDNA Clones for the 70-kDa Heat Shock-related Protein, SCE70—A spinach leaf cDNA library constructed in the phage vector Agt11 (14) was a generous gift from R. Zielinski (University of Illinois). The cDNA library was immunoscreened essentially as described by Hunyh et al. (15) with the modifications outlined in Hoffman et al. (16). Immunopositive recombinant phage were purified and the cDNA inserts were retrieved by EcoRI digestion. The cDNA inserts were subsequently subcloned into the transcription...
plasmid pGEM4 (Promega) for further analysis.

**Nucleotide Sequence Analysis**—The nucleotide sequence of the SCE70 cDNA clone was determined by the dideoxynucleotide chain termination method (17) with modifications described by Biggin et al. (18). Sequencing was carried out using double-stranded DNA templates (19).

**Protein Analysis Procedures**—Chloroplast envelope membranes isolated from pea and spinach were analyzed by denaturing SDS-polyacrylamide gel electrophoresis (20). Protein gels were electrophoretically transferred onto nitrocellulose filters (21) and analyzed by immunoblotting. Protein transfers were probed and processed as described by Hoffman et al. (16). Primary antibody reactions were detected using the avidin-biotin detection system purchased from Vector Laboratories (Burlingame, CA).

**Blot Hybridization Analysis**—Genomic DNA was prepared from spinach leaves according to Watson and Thompson (22) or Rogers and Bendich (23). Phage DNA was isolated from small liquid lysates or plate lysates (24). The DNAs were digested with various restriction endonucleases and separated by agarose gel electrophoresis. The gels were then transferred onto nitrocellulose sheets and subjected to Southern blot analysis.

Total plant cell RNA was isolated from spinach leaves as described by Cashmore (25). RNA samples were separated on formaldehyde gels, transferred onto nitrocellulose, and hybridized according to Maniatis et al. (24). Equal amounts of RNA were used for comparative purposes. The samples were seen to be qualitatively similar with respect to the integrity of the cytosolic rRNA by staining the gels. Autoradiograms and negative photographic copies of the RNA gels were scanned by LKB Ultrascan XLB laser densitometer and normalized. Densitometer data were analyzed using the LKB Gel Scan Software (version 2.1).

**In Vitro Transcription and Translation**—Transcription plasmids were linearized at the appropriate restriction site 3′ to the gene construct and repurified by extraction with phenol/chloroform/isoamyl alcohol (24:24:1) before in vitro transcription. The linearized templates were transcribed in *vitro* using SP6 RNA polymerase (26) in the presence of unmethylated cap analog (Pharmacia LKB Biotechnology Inc.). The transcripts were translated in a wheat germ system containing 35S radiolabeled methionine (New England Biolabs; Amersham) or Tran35S-label (ICN). The wheat germ extract was prepared according to Erickson and Blobel (27) except that the flocculation step was omitted.

**Protein Import into Chloroplasts and Subfractionation**—Intact chloroplasts were purified from pea seedlings as described by Bartlett et al. (28) or Cline et al. (29). The growth conditions were identical to that described earlier. The *in vitro* import assays were assembled in 0.3-ml volumes as described by Bartlett et al. (28). The reaction mixtures typically contained an equivalent of 100 μg of chlorophyll; 35S radiolabeled translation products were detected by SDS-polyacrylamide gel electrophoresis (20). After electrophoresis, the gels were prepared for fluorography using ENHANCE (Du Pont-New England Nuclear) and exposed to Kodak XAR x-ray film.

**RESULTS**

**Isolation of SCE70 cDNA Clones**—The polyclonal antibodies used to isolate the cDNA clone for the 70-kDa heat shock-related chloroplast envelope protein (SCE70) were raised against total pea chloroplast envelope proteins. The strong cross-reactivity observed for pea and spinach indicates a high degree of immunological homology between the respective envelope proteins, allowing us to use the antibodies to search DNA libraries made from heterologous plant sources (data not shown). The spinach cDNA expression library was screened with the antiserum and the immunopositive plaques were further purified. From approximately 2.5 × 10^6 phage plaques examined we obtained 114 positive clones representing different types of chloroplast envelope proteins. To select cDNA clones that encoded a specific protein band, the individual purified recombinant phage were used to generate recombinant antigens which were in turn used to purify cDNA clone-specific antibodies from the polyclonal preparation. The affinity purified antibodies were then used to probe a Western blot of total envelope protein. Over 80% of the selected positive clones encoded an envelope protein with a relative molecular mass of approximately 70 kDa. Similar results were obtained during immunoscreening of a tomato cDNA expression library. Over 90% of the tomato positive clones encoded a 70-kDa protein. The cDNA clone-specific affinity purified antibodies cross-reacted predominantly with the 70-kDa protein of the chloroplast envelope (Fig. 1A, lane 1). Cross-reactions were very low in fractions containing purified total stromal and thylakoid proteins (Fig. 1A, lanes 2 and 3). Restriction enzyme analysis was used initially to sort out the different types of cDNA inserts and their relative molecular sizes. The largest cDNA insert of approximately 2500 base pairs was subcloned as two EcoRI fragments into pGEM4 for further analysis (pSCE 70–1 and pSCE 70–2; these two clones will be referred to collectively as pSCE70 unless stated otherwise). Restriction endonuclease analysis and mapping of the 2.5-kilobase pair cDNA fragment as well as the shorter cDNA inserts confirmed that the two EcoRI fragments were genuinely linked (data not shown). The sequencing data of shorter clones and the *in vitro* synthesis experiments presented below further confirm this linkage. Another cDNA insert that occurred only once among the 114 positive clones was subcloned into pGEM4 for comparative analysis. This cDNA fragment (pHSE70) encoded a second type of 70-kDa heat shock-related protein. The length of this partial cDNA insert was approximately 1600 base pairs.

![Fig. 1. Immunoblot analysis of the SCE70 chloroplast envelope protein.](image-url)

A, cDNA clone specific affinity purified antibodies against SCE70 (lanes 1–3) and HSE70 (lanes 4–6) were reacted to mixed envelope (lanes 1 and 4), thylakoid (lanes 2 and 5), and stromal (lanes 3 and 6) fractions. B, antibodies against total envelope (lanes 1 and 2) or the SCE70 carboxyl terminus (lanes 3 and 4) were reacted to total envelope proteins. Lanes 1 and 3 represent untreated total envelopes and lanes 2 and 4 contain envelope protein profiles treated with thermolysin prior to purification. In lanes 5–7, SCE70 carboxyl-specific antiserum was used to probe a blot of mixed envelope, stromal and thylakoid subfractions, respectively. SCE70 carboxyl-specific antibodies also reacted to overexpressed SCE70 (lane 8). C, SCE70 carboxyl-specific antiserum was reacted to mixed envelope (lane 1), inner envelope (lane 2), and outer envelope (lane 3) fractions. The same blot was subsequently reacted to monospecific antibodies raised against the 37-kDa inner envelope membrane protein as a control.

K. Ko, unpublished results.
Nucleotide Sequence Analysis—The DNA sequence of the 2500-base pair cDNA fragment (pSCE70) was determined in both directions and the resulting nucleotide and deduced amino acid sequences are shown in Fig. 2. The cDNA fragment encoded an open reading frame of 1959 nucleotides plus 377 and 199 nucleotides of 5’- and 3’-untranslated sequences, respectively. The exact total length of the cDNA clone was 2535 nucleotides, compared with a mRNA size of 2500 nucleotides, estimated by agarose gel electrophoresis and blot analysis.

The initiating methionine codon was assigned to nucleotides 378–381 which results in a protein of 653 amino acids. We confirmed the position of the initiation codon using various deletions and in vitro expression systems and more recently in *Escherichia coli* overexpression vectors. The predicted protein sequence was calculated to have a molecular mass of 71,731 Da and a pI of 5.13. These calculated values are in close agreement with those recently observed for cotton HSP70 proteins (32). More than 20 isoforms, mostly within an acidic pI range (3.5–5.6) and a relative molecular mass range of 61,000–72,000 Da, were detected using two-dimensional gel electrophoresis and silver staining. A minor set of proteins at M, 72,000 with pI values of 5.1 were also observed.

We are currently examining the relationship between SCE70 and the various HSP70 isoforms found in plants. The hydropathy profile generated for the SCE70 protein sequence is shown in Fig. 3. The plot did not show any clear dominant characteristics (i.e., hydrophobic or hydrophilic) which could have given us a clue to its location in the chloroplast envelope membrane. This characteristic is reflected in the amino acid composition, 48.6% for nonpolar and 47.7% for polar residues.

Comparison of the *sce70* nucleotide and deduced amino acid sequences to entries in the GenBank revealed that the cDNA clone encodes a heat shock 70-kDa protein. The *sce70* nucleotide sequence exhibits a high degree of homology to the DNA sequences of HSP70 proteins from other organisms. The highest percentage of homology at the nucleotide level found to plant *hap70* at 80%, such as petunia, tomato, or soybean (33–35). Homologies greater than 70% were calculated with genes for mouse and rat HSP70 cognate proteins. The gene sequences for the SSA1 and SSA2 proteins of *Saccharomyces cerevisiae* exhibit homologies of 74 and 70% to SCE70, respectively (36). Slightly lower percentages of homology were found with genes for the human BiP protein (immunoglobin heavy chain-binding protein) and glucose-regulated proteins of human and Chinese hamster (68 and 67%, respectively).

The degree of homology between SCE70- and HSP70-related proteins was even higher for the amino acid sequence (greater than 80%). A significantly high degree of homology was also found with the *E. coli* HSP70 counterpart, DNAK (37). The percentage of homology was calculated to be >65% at the nucleotide level and >45% at the protein level. However, the level of exact homology to DNAK was not as high as that reported for the mitochondrial HSP70 proteins SSCI from *S. cerevisiae* (38) and SSP1 from *Schizosaccharomyces pombe* (39) (57.8 and 56%, respectively). The higher level of homology to yeast SSA1 and SSA2, cytoplasmic HSP70 forms, suggests that SCE70 is most likely not an internal chloroplast protein but an external form. The localization and import experiments presented below confirm this hypothesis.

Although the highest degree of homology was found with HSP70-related proteins, SCE70 also exhibited low levels of homology to other proteins (data not shown). Of particular interest are matches to myosin heavy chains, glycoprotein precursors, and RNA polymerases, as well as a variety of receptors. Such similarities were also found with 11 chaperonin 60 protein sequences recently reported in a review by Ellis (40). The biological significance of these similarities remains to be elucidated.

In a recent published report, a Ca2+/calmodulin (CaM)-binding sequence was found to be highly conserved among several HSP70 type proteins (41). This highly conserved domain was also found within the SCE70 protein sequence. The Ca2+/CaM-binding sequence was found to be highly conserved among various HSP70 type proteins (41). This highly conserved CaM-binding domain in SCE70 exhibits 76% exact amino acid homology to the mouse HSP70 Ca2+/CaM-binding sequence.

All HSP70 type proteins have been found to bind ATP with high affinity (42) and most are involved in functions thought to require the hydrolysis of ATP. The NH2-terminal ATP-binding domain has been isolated from bovine heat shock cognate protein and characterized by x-ray crystallography (43). The *E. coli* counterpart, DNAK, is also capable of phosphorylating threonine residues either on itself or on other proteins such as glutamine tRNA synthetase and threonyl tRNA synthetase (44). We scanned the SCE70 protein sequence for possible ATP-binding domains or a protein kinase catalytic domain. Two areas of SCE70 appear to contain some of the prerequisite residues for a possible ATP-binding site (overlined in Fig. 2). The first ATP-binding site is located at the extreme NH2-terminal end of SCE70 (residues 9–20). This
obtained by hybridization of sce70 transcripts onto nitrocellulose. The genomic blot was hybridized to a number of DNA fragments giving rise to a different pattern than the sce70 hybridization. These results indicate that the sce70 gene is clearly distinct from the heat-inducible 70-kDa gene, hse70. Heat-inducible hsp70 genes have previously been reported for petunia, maize, and soybean (33, 35, 50).

Expression Characteristics of sce70 versus hse70—Northern blot analysis of total RNA from spinach leaves revealed that the cDNA probe hybridized to mRNA of approximately 2500 nucleotides (Fig. 5). The cDNA hybridization probes utilized were the same ones used in the analysis of genomic DNA described above. The amount of sce70 transcripts was severalfold less than the highly abundant transcripts of rbcS and cab.

Analysis of total RNA prepared from plants given light and dark treatments revealed that the steady-state level of sce70 mRNA is not significantly influenced by light (Fig. 5, set 1). Likewise transcript levels for hse70 also did not appear to be affected by light (Fig. 5, set 2). The sce70 mRNA level appears to remain constant despite dark treatment for 3 days, whereas steady-state mRNA levels of the positive light-regulated rbcS and rca genes decreased to a very low amount (Fig. 5, set 3 and panel marked RBCS). The hse70 probe gave very low signals in both light and dark treated plants. To avoid complications due to circadian effects on transcription, leaves were harvested and RNA extracted between 11:00 and 13:00 h when RNA levels for photosynthetic genes are near their peak (51).

The effects of cold and heat shock on the steady-state mRNA levels of both 70-kDa genes were also analyzed (Fig. 5, lanes labeled HEAT). Neither cold nor heat shock affected the expression of sce70 in a dramatic fashion (Fig. 5, set 1). The sce70 mRNA remains constant relative to the control samples (ambient or room temperature), whereas hse70 shows the third possible ATP-binding region is at the extreme carboxyl-terminal end (residues 619–642) (Fig. 2). This region appears to contain an ATP-binding domain similar to that found in protein kinases (GXGXXG . . . K) (48). Although these are relatively weak similarities, a search of the databases with the 75 carboxyl-terminal amino acids did turn up some matches with ATPases. However, the significance of this domain remains to be determined.

Genomic DNA Analysis of sce70 versus hse70—Total spinach genomic DNA was digested to completion with restriction endonucleases, subjected to agarose gel electrophoresis, and transferred onto nitrocellulose. The genomic blot was hybridized using high stringency conditions with a probe constructed from 3’-translated and untranslated sequences of pSCE70. The carboxyl-terminal quarter of hsp70 genes diverge significantly (49), therefore DNA probes constructed from these regions are made specific for a particular gene. A computer search indicates that the degree of homology between carboxyl-terminal regions is generally lower. Two DNA fragments hybridized strongly with the probe (Fig. 4, lane 1). The estimated molecular sizes of the EcoRI fragments were 8 and 1 kilobase pair. The weak hybridizations observed with other DNA fragments indicate some cross-reactivity to related genes. We also carried out a Southern genomic analysis of the second 70-kDa cDNA clone (pHSE70) for comparison (Fig. 4, lane 2). This heat shock-inducible 70-kDa cDNA clone reacted to a number of DNA fragments giving rise to a different pattern than the sce70 hybridization. These results indicate that the sce70 gene is clearly distinct from the heat-inducible 70-kDa gene, hse70. Heat-inducible hsp70 genes have previously been reported for petunia, maize, and soybean (33, 35, 50).
a dramatic elevation in the steady-state mRNA level subsequent to a 1-h heat shock (42 °C) treatment (Fig. 5, set 2). The hse70 gene, however, did not appear to respond to a cold shock treatment of the same duration. These results suggest that sce70 is constitutively expressed in terms of light and temperature, whereas the second 70-kDa gene (hse70) is induced or regulated by elevated temperatures.

As a result of the RNA experiments, a Southern cross-hybridization analysis between pSCE70 and the heat-inducible 70-kDa cDNA clone (pHSE70) was performed to determine the relatedness of the two different cDNA inserts (Fig. 6). The hybridization clearly showed cross-reaction of pSCE70 to the heat-inducible 70-kDa cDNA clone, indicating that pSCE70 possesses DNA sequences that are homologous to pHSE70. However, the level of cross-hybridization was relatively weak compared to the control, indicating that the two cDNA clones were indeed different. Recent nucleotide sequencing results confirm this conclusion.

**Localization of the SCE70 Protein**—The localization of the SCE70 protein in the chloroplast envelope was determined by subfractionation and Western blot analysis (Fig. 1, A and B). The immunoaffinity monospecific antibodies purified using SCE70 recombinant antigen cross-reacted with a 70-kDa protein predominantly in the envelope profile (Fig. 1A, lane 1). Very little cross-reactivity was observed with stromal or thylakoid proteins (Fig. 1A, lanes 2 and 3), indicating that SCE70 is located in the chloroplast envelope. The 70-kDa SCE70 protein appears to be relatively abundant and may represent a major 70-kDa band. Immunoaffinity purified antibodies to HSE70 recombinant antigen cross-reacted with a minor band in the envelope (Fig. 1A, lane 4). These results indicate that there may be more than one HSP70 related species in the envelope which complicates interpretation of the results. Since HSP70 type proteins are highly conserved and antibodies generated to the NH2-terminal portion will cross-react with any related polypeptide, we generated monospecific antibodies to the carboxyl end of SCE70 where amino acid sequences diverge between HSP70s. This carboxyl-specific antibody also cross-reacted with a 70-kDa band predominantly in the envelope fraction (Fig. 1B, lanes 5–7). As a control the carboxyl antisera was shown to cross-react with SCE70 overexpressed in bacteria (Fig. 1, lane 8).

Treatment of chloroplast with thermolysin prior to envelope isolation resulted in a significant reduction of a 70-kDa immunoreactive band (Fig. 1B, lanes 1–4). Thermolysin treatment significantly depressed the cross-reactivity of a 70-kDa envelope antigen with the monospecific antibody to the carboxyl end of SCE70 (Fig. 1B, lanes 3–4), suggesting that SCE70 is exposed on the cytoplasmic side of the chloroplast envelope. A minor 70-kDa band remains intact after thermolysin treatment. Further purification of the envelope into outer and inner membranes shows that SCE70 is present in both fractions (Fig. 1C, lanes 1–3). The blot was subsequently reacted to a monospecific antibody of the 37-kDa inner membrane protein (52) to confirm inner and outer envelope subfractions. Interestingly, the highest concentration is found in the inner membrane fraction. Undoubtedly cross-contamination will contribute to the abundance found in the inner membrane fraction, but it is also possible that SCE70 is associated with or predominantly located in the contact sites, which would copurify with inner membranes. Further experimentation is required to resolve the precise location of SCE70.

**Import Studies of SCE70**—In vitro translation of sce70 mRNA made by utilizing the SP6 promoter of pGEM4 resulted in a protein with a relative molecular mass of 70 kDa as determined by SDS-polyacrylamide gel electrophoresis (Fig. 7, lane marked TR). The in vitro synthesis data confirm that the cDNA insert encoded a full-length version of sce70. The in vitro translation product comigrated with the 70-kDa polypeptide of the chloroplast envelope (Fig. 7, lanes E and TR). These results indicate that SCE70 is synthesized and targeted to the envelope membrane without the processing observed with other chloroplast-destined precursors including the phosphate translocator (55) and the 37-kDa inner envelope polypeptide (52). The in vitro import experiments discussed in the next section confirm this observation.

Radiolabeled SCE70 precursors were used directly for assaying import into chloroplasts, after which intact plastids were re-isolated as described under "Materials and Methods." Thermolysin treatment of re-isolated intact chloroplasts was employed to determine the location of the imported products. Proteins exposed on the outside face of the chloroplast envelope would be susceptible to this protease. The import data for SCE70 are presented in Fig. 8. The SCE70 protein was targeted to the chloroplast and was susceptible to protease treatment, indicating that SCE70 is located on the outside face of the envelope (Fig. 8, lanes 1 and 2). Control import experiments using radiolabeled cytoplasmic proteins such as pyruvate kinase and chloroplast proteins without transit peptides (e.g. the 33-kDa oxygen evolving protein) (54), do not import nor do they bind to the chloroplast (data not shown). The same characteristics were also demonstrated for RBCS.

![Fig. 6. Cross-hybridization between sce70 and hse70. A minigel of the cDNA clones pSCE70 and pHSE70 digested with EcoRI is presented in lanes 1 and 2, respectively. The corresponding autoradiograph is shown in lanes 3 and 4 in the reverse order to lanes 1 and 2. The sce70 cDNA probe cross-reacted to hse70 as indicated by the arrowhead. The arrow labeled V indicates hybridization with the plasmid vector DNA band.](image)

![Fig. 7. In vitro transcription and translation analysis of the SCE70 cDNA clone. Coomassie Blue-stained gel is shown on the left. The lanes marked S, E, and T represent stromal, envelope, and thylakoid fractions, respectively. The translation profile of sce70 is shown in the lane marked TR of the autoradiograph. The molecular weight markers are indicated on the left in kilodaltons.](image)
by Friedman and Keegstra (55). The affinity SCE70 possesses for chloroplasts is therefore genuine and not a nonspecific reaction. Recent import studies with NH₂- and COOH-terminal deletions of SCE70 confirm the specificity of its targeting. The results of these experiments will be reported separately.

The suborganellar location of imported SCE70 was first determined by crude subfractionation (Fig. 8A, lanes 3–5). Imported SCE70 was found predominantly in the membranous fractions, i.e. crude envelope and thylakoid membranes. Very little radioactivity was detected in the stromal fraction. Contamination of thylakoid membranes with envelopes especially in a crude fraction was expected. Thermolysin treatment of chloroplasts prior to subfractionation abolished the radiolabeled SCE70 band (Fig. 8A, lanes 6–8), indicating that SCE70 is externally located, consistent with the immunological results, and that the band in the crude thylakoid fraction was due to envelope contamination. The mixed envelope fractions were further purified using sucrose gradients as described under "Materials and Methods." The imported SCE70 proteins were located in the envelope fraction (Fig. 8A, lane 9), which is consistent with the above observations.

Imported SCE70 co-migrated with the authentic 70-kDa envelope polypeptide and the in vitro translation product, strongly indicating that this protein did not contain a cleavable NH₂-terminal transit peptide (data not shown). This characteristic is similar to the two outer membrane proteins recently reported by Salomon et al. (10) and Li et al. (11).

The translocation characteristics of SCE70 also appear to be similar to the 6.7-kDa SOEl (10) and the 14-kDa OM14 outer envelope proteins (11), but different from precursors destined for internal plastidic sites. Import of SCE70 to the outer envelope membrane was not significantly altered by ATP depletion or protease pretreatment of the chloroplast (Fig. 8B, lanes 3 and 4 and 5 and 6, respectively). Nigericin pretreatment to deplete ATP levels required for import did not prevent targeting to the chloroplast. Additional apyrase treatment also did not result in any observable changes in import characteristics (data not shown). Removal of protein receptors by thermolysin also did not interfere with targeting. Therefore like the 6.7-kDa SOEl and the 14-kDa OM14 proteins, SCE70 translocates through a mechanism different than other chloroplast proteins.

**DISCUSSION**

We have identified a full-length cDNA clone encoding a cognate heat shock-related 70-kDa protein of the spinach chloroplast outer envelope (SCE70). The nucleotide and amino acid sequences exhibit extensive homology with HSP 70 proteins or homologues from a variety of organisms (70% or greater). As expected, the SCE70 cDNA sequence was most conserved when compared to a plant HSP70 sequence such as petunia. A large proportion of the immuno-selected positive clones encoded see70, suggesting that the transcript is present at a level higher than other envelope genes. RNA blot analysis confirms that the see70 mRNA was more abundant than other envelope genes. Northern analysis also revealed that see70 is expressed constitutively in terms of control by different temperature and light parameters. This characteristic is in contrast to the second isolated 70-kDa gene, hse70, where expression was inducible by heat shock. Despite the different Southern genomic DNA patterns exhibited by see70 and hse70, cross-hybridization between the two cDNA clones indicated some homology at the DNA level. In conclusion, SCE70 is a constitutively expressed or cognate heat shock-related protein of the spinach chloroplast envelope that is distinct from the heat-inducible HSE70 protein.

Results of the immunological analyses together with the import assays provide evidence that SCE70 is a chloroplast outer envelope protein. The monospecific antibodies purified against the recombinant antigen derived from the SCE70 cDNA clone as well as the carboxyl end monospecific antibodies cross-react strongly with a 70-kDa polypeptide present only in the envelope fractions. SCE70 appears to be distributed throughout the surface of the chloroplast since it was present in both inner and outer membrane fractions. see70 appears to encode an abundant 70-kDa protein band distinct from the minor 70-kDa polypeptide reported by Marshall et al. (8) or encoded by hse70. Unlike the 70-kDa HSP70 homologue observed by Marshall et al. (8), SCE70 appears to be externally located on the outer envelope membrane accessible to proteases. These observations are consistent with the import results. The imported protein is targeted to the envelope and is sensitive to thermolysin. Control import assays with pyruvate kinase, a similar size cytosolic protein, and 53-kDa OEE1 without a transit peptide, do not show binding or import indicating that SCE70 is targeting specifically to the envelope. We recently confirmed this specificity by delineating an envelope targeting sequence. These results will be reported separately. The translocation characteristics of SCE70 appear to be distinct from other chloroplast precursors, but similar to SOEl and OM14. Like these two outer envelope proteins, SCE70 does not appear to contain a cleavable transit sequence. The imported protein co-migrates with the authentic 70-kDa band and the in vitro translation product. Targeting to the chloroplast envelope does not appear to require ATP or proteinchromosomes. The see70 cDNA clone will allow us to further study the translocation pathway of this outer envelope protein.

The constitutive nature of SCE70 coincides with recent findings by Marshall et al. (8). Three non-heat inducible HSP 70 homologues were identified in pea chloroplasts, one of which is located in the outer envelope. Waegemann and co-workers (7) were able to demonstrate that precursor chlorophyll a/b-binding protein requires two cytoplasmic factors present in leaf extracts to facilitate import. One component can be mimicked by purified HSC70 (a constitutive or cognate form of HSP70) and the other component requires ATP. Recent work on protein transport into mitochondria and the endoplasmic reticulum (4, 6) has resulted in the proposal that...
HSP70s are necessary components for facilitating import. These proteins are believed to utilize the energy of ATP hydrolysis to maintain precursor proteins in a transport competent conformation in the cytoplasm. For example, immunoglobulin heavy chain binding protein (BiP) has been shown to bind with high affinity to the hydrophobic surfaces of newly synthesized proteins that are incompletely assembled or incorrectly folded. ATP is believed to be utilized in the refolding or folding of these proteins. Correctly folded poly-peptides can then be secreted from the endoplasmic reticulum (56). The E. coli counterpart, dnaK gene product, has been shown to be necessary for phage DNA replication. DNAK also appears to be dependent on ATP for its involvement in protein unfolding and disassembly during the initiation of replication (57). The SCE70 cDNA clone may represent such a component of the outer chloroplast envelope membrane. The high degree of homology to HSP70 proteins such as the human BiP and the yeast SSA1/2 suggests that SCE70 likely plays a similar role in the transport of proteins across the envelope membrane and into the chloroplast. Other HSP70 homologues, such as those in the stromal compartment, may have similar functions during assembly of protein subunits/complexes or are possibly involved in unfolding and folding of chloroplast proteins synthesized inside the organelle from chloroplast DNA-encoded genes.

There are a number of ways in which SCE70 may be regulated. Analysis of the deduced amino acid sequence revealed a number of potential protein kinase phosphorylation sites. The biological significance of these sites, however, remains to be tested. A more definitive regulatory mechanism of SCE70 is found in the highly conserved Ca2+/CaM-binding domain. This domain is highly conserved among HSP70 proteins from different organisms indicating that it is necessary for some essential functional, structural, or regulatory role (41). Stevenson and Calderwood (41) hypothesized that the Ca2+/CaM-binding site functions in the regulation of HSP70 in a manner similar to that of myosin light chain kinase (MLCK) found in the smooth and skeletal muscle. The mechanism of MLCK regulation takes advantage of a pseudosubstrate domain which is lodged in the MLCK catalytic site, keeping MLCK in an inactive state. When Ca2+/CaM binds near the pseudosubstrate site, the pseudosubstrate is displaced and the MLCK becomes an active kinase. A similar mechanism for the regulation of HSP70 proteins could be speculated. In plants, various stimuli such as heat shock or even light cause a rise in cellular Ca2+ levels which bind to CaM, forming the ubiquitous Ca2+/CaM complexes. These Ca2+/CaM complexes would then bind to the HSP70 proteins, resulting in alterations that affect their function. Dissociation of the Ca2+ complexes would occur when Ca2+ homeostasis had been re-established, thereby reversing or restoring its function. Further studies of the effect of Ca2+ on the activity of SCE70 will have to be done to confirm calcium's regulatory role on SCE70.

The availability of a full-length SCE70 cDNA provides an approach to study the function of this chloroplast envelope protein in terms of the possible regulatory role it exerts on incoming protein as well as its possible contribution to the mechanism of protein translocation. In addition, we are examining the signals or factors involved in the specific targeting of SCE70 to the chloroplast envelope.

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