Insertion of OEP14 into the Outer Envelope Membrane Is Mediated by Proteinaceous Components of Chloroplasts

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Most chloroplastic outer envelope membrane proteins are synthesized in the cytosol at their mature size without a cleavable targeting signal. Their insertion into the outer membrane is insensitive to thermolysin pretreatment of chloroplasts and does not require ATP. The insertion has been assumed to be mediated by a spontaneous mechanism or by interaction solely with the lipid components of the outer membrane. However, we show here that insertion of an outer membrane protein requires some trypsin-sensitive and some \textit{N}-ethylmaleimide–sensitive components of chloroplasts. Association and insertion of the outer membrane protein are saturable and compete with the import of another outer membrane protein. These data suggest that import of chloroplastic outer membrane proteins occurs at specific proteinaceous sites on chloroplasts.

INTRODUCTION

Most chloroplastic proteins are encoded by the nuclear genome and synthesized in the cytosol. Nuclear-encoded chloroplastic proteins can be divided into roughly two groups on the basis of the presence or absence of cleavable targeting signals. Proteins in the first group are synthesized as higher molecular weight precursors with \textit{N}-terminal extensions called transit peptides. Import of these precursor proteins into chloroplasts requires ATP and some thermolysin-sensitive receptor proteins on the chloroplastic surface (Cline et al., 1985; Olsen et al., 1989; Theg et al., 1989). This group of proteins includes all proteins destined for the interior of chloroplasts and at least one protein destined for the outer envelope membrane (Schnell et al., 1994; Tranel et al., 1995). The second group of proteins consists of most of the outer membrane proteins identified so far. These outer membrane proteins are synthesized at their mature size in the cytosol without a cleavable transit peptide (Salomon et al., 1990; Li et al., 1991; Ko et al., 1992; Fischer et al., 1994; Kessler et al., 1994; Seedorf et al., 1995; Bolter et al., 1999). The import mechanism for the first group of proteins has been studied extensively, and many protein components of the import machinery have been identified (Chen and Schnell, 1999; Keegstra and Cline, 1999; May and Soll, 1999). In contrast, very little is known about how the outer membrane proteins in the second group are targeted and inserted into the outer membrane.

Two unique characteristics mark the import of these outer membrane proteins. For almost all of these proteins, thermolysin pretreatment of chloroplasts and ATP removal have no effect on insertion of the proteins into the outer membrane (Salomon et al., 1990; Li et al., 1991; Ko et al., 1992; Fischer et al., 1994). Because these results suggest that insertion of these proteins does not require any surface-exposed chloroplastic proteins or energy, their insertion has generally been assumed to be accomplished by a spontaneous mechanism or through interaction with the lipid components of the outer membrane (Bruce, 1998; Keegstra and Cline, 1999).

However, the hypotheses of spontaneous insertion or interaction with lipids both have their problems. Among the outer membrane proteins that do not require thermolysin-sensitive components and ATP for insertion, outer membrane–targeting signals have been identified from the mature protein regions of OEP14 and COM70 (Wu and Ko, 1993; Li and Chen, 1996). Signals from both of these proteins are necessary and sufficient to specifically target the proteins themselves and other passenger proteins to the outer membrane. For OEP14, even though its outer membrane–targeting signal resembles an endoplasmic reticulum–targeting signal peptide in appearance, the signal is nevertheless specific for the chloroplastic outer membrane and does not direct protein insertion into the microsomal membrane (Li and Chen, 1996) or mitochondria (Li et al., 1991). Whether a spontaneous insertion mechanism can achieve this kind of specificity is doubtful. As for the hypothesis of interaction with lipids, the import of OEP14 is completely normal in chloroplasts deficient in the major...
chloroplast-specific outer membrane lipid digalactosyl diacyl-glycerol (DGD) (Chen and Li, 1998). Of course, lipids other than DGD or some thermolysin-resistant chloroplastic proteins, or both, could be required for the targeting and insertion of these outer membrane proteins.

To understand the targeting and insertion mechanism of the outer membrane proteins, we used OEP14 as a model protein and analyzed the requirements of OEP14 insertion into the outer membrane. We show here that trypsin-sensitive and N-ethylmaleimide (NEM)-sensitive protein components are required for the insertion of OEP14. Association and insertion of OEP14 are saturable and compete with the import of other outer membrane proteins. These data suggest that specific proteinaceous sites mediate the import of OEP14.

RESULTS

Recombinant OEP14-His$_6$ Is Soluble and Import Competent

To identify components required for OEP14 targeting and insertion into the chloroplastic outer membrane, we overexpressed OEP14 in *Escherichia coli* with a C-terminal tag consisting of six consecutive histidine residues (His$_6$) to facilitate purification. We refer to this recombinant protein as OEP14-His$_6$. As shown in Figure 1, OEP14-His$_6$ was soluble and could be purified to apparent homogeneity by affinity chromatography (Figure 1, lane 5). Antibodies generated by using OEP14-His$_6$ specifically recognized a protein in the chloroplastic outer membrane with the same molecular weight and topology as endogenous OEP14 (data not shown).

We have shown previously that the targeting/insertion signal of OEP14 for the outer membrane is located at the N-terminal first 30 amino acids (Li and Chen, 1996). OEP14 is also anchored in the outer membrane by this N-terminal portion, with the N terminus facing the intermembrane space of the envelope and the C-terminal portion of the protein being exposed in the cytosol. When treated with thermolysin after insertion, OEP14 is cleaved to a specific 4-kD membrane-protected fragment corresponding to the N-terminal membrane anchor (Li and Chen, 1996). Throughout this article, we refer to the amount of full-length OEP14 observed on chloroplasts after an import reaction and before thermolysin treatments as “total association.” This includes two groups of OEP14 molecules: those bound to the surface of chloroplasts and those that have truly inserted into the outer membrane. We use the amount of the 4-kD membrane-protected fragment after thermolysin digestion to represent the amount of OEP14 that has truly inserted into the outer membrane.

To check the import competency of the purified OEP14-His$_6$, we used OEP14-His$_6$ in an in vitro protein import experiment with isolated pea chloroplasts. As shown in Figure 2, OEP14-His$_6$ could associate and insert into chloroplasts as well as did in vitro–translated OEP14. The molecular mass of OEP14-His$_6$ is slightly greater than that of the in vitro–translated OEP14 because of the C-terminal His$_6$ tag (cf. lanes 1 and 4). However, the thermolysin-resistant fragments generated after insertion (lanes 3 and 6) were the same size for OEP14-His$_6$ and OEP14. This again confirmed that the 4-kD membrane-protected fragment represents the N-terminal portion of OEP14. For reasons not clear to us, OEP14, especially OEP14-His$_6$, migrates more slowly in gels when samples contain chloroplasts (cf. lane 1 with lane 2 and lane 4 with lane 5).

Cytosolic Extracts Did Not Increase the Import Efficiency of OEP14-His$_6$

Most nuclear-encoded chloroplastic precursor proteins are insoluble when overexpressed in *E. coli* (Cline et al., 1993). These overexpressed precursor proteins can be made import-competent if they are denatured by guanidinium chloride or urea before use in import reactions. Under denatured conditions, the proteins are import-competent without any addition of cytosolic proteins (Cline et al., 1993; Perry and Keegstra, 1994; Schnell et al., 1994). However, the denaturation treatments may alter the conformation of the precursor
Figure 2. OEP14-His<sub>6</sub> Can Associate and Insert into the Chloroplastic Outer Membrane.

Import of in vivo–labeled OEP14-His<sub>6</sub> (lanes 1 to 3) and in vitro–translated OEP14 (lanes 4 to 6) into chloroplasts. Lanes 1 and 4, proteins used for the import reactions; lanes 2 and 5, reisolated chloroplasts after the import reactions; and lanes 3 and 6, same as lanes 2 and 5, except that the chloroplasts were further treated with thermolysin after import, as indicated by (+) below the lanes. Molecular masses of marker proteins are labeled at left.

protein such that any cytosolic factor requirement that normally exists is bypassed. Because overexpressed OEP14-His<sub>6</sub> is soluble, it should be a good substrate to investigate whether any cytosolic factor is required for targeting or insertion of OEP14 into chloroplasts. We therefore tested whether adding various cytosolic extracts could increase the import efficiency of OEP14-His<sub>6</sub>. As shown in Figure 3, we found that adding wheat germ extract (lanes 2 and 7), rabbit reticulocyte lysate (lanes 3 and 8), or soluble extracts from young pea seedlings (lanes 4 and 9) had no significant and consistent effect on the association or insertion of OEP14-His<sub>6</sub> into chloroplasts. We sometimes observed an increase in import when reticulocyte lysate was added (lanes 3 and 8); however, the same effect could be achieved by adding hemoglobin alone (lanes 5 and 10).

Import of OEP14-His<sub>6</sub> Requires Some Trypsin-Sensitive Chloroplastic Envelope Proteins

To investigate whether chloroplastic components were required for the import of OEP14, we treated chloroplasts with various proteases or protein/lipid modification agents to see whether any of the treatments would affect the import of OEP14. Although the import of interior-targeted precursor proteins is reduced by pretreating chloroplasts with as little as 5 µg/mL thermolysin (Cline et al., 1985), OEP14 inserts normally into chloroplasts that have been pretreated with 200 µg/mL thermolysin (Li et al., 1991). As shown in Figure 4A, the same result was obtained with OEP14-His<sub>6</sub>. Association and insertion of OEP14-His<sub>6</sub> into chloroplasts was not affected, even when chloroplasts were pretreated with 600 µg/mL thermolysin.

In contrast to its resistance to thermolysin pretreatment, insertion of OEP14-His<sub>6</sub> into chloroplasts was very sensitive to trypsin pretreatment of chloroplasts. Pretreatment of chloroplasts with 20 to 100 µg/mL trypsin reduced the amount of insertion by 40 to 60% (Figures 4B and 4C). Association did not seem to be affected by the pretreatments (Figure 4B, lanes 2 to 5). These results indicate that some trypsin-sensitive chloroplastic components were required for the insertion of OEP14-His<sub>6</sub> into the outer membrane. The fact that the amount of OEP14-His<sub>6</sub> associated with chloroplasts remained unchanged in trypsin-pretreated chloroplasts also indicated that the trypsin used to pretreat chloroplasts had been sufficiently quenched after the pretreatments.

Trypsin is known to be able to penetrate the chloroplastic outer membrane and access the intermembrane space (Jackson et al., 1998). To check the possibility that the trypsin pretreatment might have compromised the structural integrity of the envelope membranes, we checked the integrity of some outer and inner membrane proteins after the trypsin pretreatments. As shown in Figure 4D, three integral inner membrane proteins—Tic110 (Jackson et al., 1998), the phosphate translocator (Knight and Gray, 1995), and IEP21 (Kouranov et al., 1998)—remained unaffected under the trypsin treatment conditions used. The integral outer membrane protein Toc75 was very sensitive to the trypsin pretreatments. Because Toc75 has been proposed to function as the protein translocation channel at the outer membrane (Hinnah et al., 1997), perhaps part of the protein is accessible from the aqueous solution and therefore is susceptible to trypsin digestion. We then used OEP14 itself as another marker for integral outer membrane proteins. Protein import experiments were performed with OEP14-His<sub>6</sub>, and the chloroplasts were then treated with 20 to 100 µg/mL trypsin. As shown in Figure 4E, as with thermolysin, trypsin could remove only the part of OEP14 exposed in the cytosol; the N-terminal membrane anchor remained inaccessible (Figure 4E, arrow). This protection is the result of insertion in
the outer membrane, not of any intrinsic protease resistance of OEP14, because free OEP14-His$_{6}$ was very sensitive to trypsin digestion (Figure 4E, lane 7). These results suggest that the inner membrane and at least part of the outer membrane remained intact after the trypsin treatments.

**Import of OEP14-His$_{6}$ Requires Some NEM-Sensitive Chloroplastic Envelope Proteins**

We also tested the effect of the protein sulfhydryl alkylating agent NEM on the import of OEP14. As shown in Figure 5, pretreatment of chloroplasts with 0.5 to 8 mM NEM had little effect on the amount of OEP14-His$_{6}$ associated with chloroplasts (Figure 5A, lanes 2 to 6). However, only ~60% of the OEP14-His$_{6}$ associated with chloroplasts could insert into the outer membrane (Figures 5A, lanes 7 to 12, and 5D). This inhibition in insertion was prevented if DTT was present during the NEM pretreatment (Figure 5B, lane 7), but DTT alone did not affect OEP14 import (Figure 5B, lane 8). These results suggest that sulfhydryl groups on certain chloroplastic proteins are important for the insertion of OEP14. We also tested another sulfhydryl modification agent, fluorescein-5-maleimide, and obtained the same results as we did using NEM (data not shown).

**Association and Insertion of OEP14 into the Outer Membrane Are Saturable**

The results with the trypsin and NEM pretreatments suggested that specific proteinaceous components were required for insertion of OEP14 into the chloroplastic outer membrane. If specific proteinaceous sites are involved, we might expect that association or even insertion of OEP14 would be saturable if inserted OEP14 stays at the insertion site for some time and does not diffuse to its final destination immediately. As shown in Figure 6, association and insertion of OEP14-His$_{6}$ were indeed saturable. The amounts of OEP14-His$_{6}$ associated and inserted into the chloroplasts reached a plateau at ~6 μM. This concentration is similar to
the saturation concentration observed for thylakoid membrane proteins (Cline et al., 1993).

**OEP14-His<sub>6</sub> Competes with the Import of OEP14 and Another Outer Membrane Protein**

Additional evidence for the involvement of specific sites for OEP14 import is that OEP14 import is self-competing and also competes with the import of other outer membrane proteins if these proteins share the same import pathway. As shown in Figure 7A, when increasing concentrations of unlabeled OEP14-His<sub>6</sub> were added to the import reactions, the amounts of OEP14 associated and inserted into the outer membrane decreased. Competition occurred when the competitor was present at concentrations in the micromolar range, which are the same concentrations observed for the saturation of OEP14 import (Figure 6) and similar to the concentrations observed for competition among proteins targeted into the interior of chloroplasts through the general import apparatus (Tranel et al., 1995). The competition seemed to occur at the binding step because association and insertion were inhibited to a similar extent (Figure 7C).

We also investigated whether OEP14-His<sub>6</sub> could compete with the import of another outer membrane protein, atToc34 (Li and Chen, 1997). As shown in Figure 7B, OEP14-His<sub>6</sub> also competed with association and insertion of atToc34. The extent of competition was similar to that observed for OEP14 (Figure 7C).

**DISCUSSION**

We have shown that insertion of OEP14 into the chloroplastic outer membrane is sensitive to trypsin and NEM pretreatments of chloroplasts. Saturation and competition experiments also suggest that specific sites on chloroplasts mediate the import of OEP14. These results discount the long-held assumption that insertion of OEP14 into the outer membrane is mediated by a spontaneous mechanism or solely by interaction with lipid bilayers (Bruce, 1998; Keegstra and Cline, 1999).

Targeting and specific insertion of proteins by interacting with lipids is an attractive hypothesis for chloroplastic membrane proteins such as OEP14. Chloroplastic membranes contain several unique lipids, such as DGD, MGD, and sulfolipid (Douce and Joyard, 1990). Although it is appealing to hypothesize that OEP14 can recognize some of these lipids and that the lipids assure the specific insertion of OEP14 into the outer membrane, our data with duramycin did not lend support for OEP14 interaction with MGD. However, we hesitate to stress this negative result, especially when the mechanism of duramycin action is not clear and the effect, if any, of duramycin on intact chloroplasts is not known. Nevertheless, mutant chloroplasts deficient in the other major outer membrane lipid, DGD, are completely normal in...
importing OEP14 but defective in importing interior-targeted precursor proteins (Chen and Li, 1998). Nonetheless, other than the trypsin-sensitive and NEM-sensitive protein components we identified, lipids such as MGD and sulfolipid possibly also play a role in the import of OEP14. Investigations are under way using coimmunoprecipitation and cross-linking to identify components associated with OEP14 during its import process.

Thermolysin has been used as a reliable protease to probe the surface of chloroplasts. This protease removes only surface-exposed proteins and seems unable to cross the outer membrane of chloroplasts (Cline et al., 1984). Trypsin, on the other hand, can cross the outer membrane and digest proteins in the intermembrane space and the inner envelope membrane (Jackson et al., 1998). These two proteases also are specific for different amino acids. The fact that insertion of OEP14 is resistant to thermolysin pretreatment but sensitive to trypsin pretreatment indicates that OEP14 insertion requires some protein components with only a few surface-exposed residues that can be digested by trypsin but not by thermolysin. Perhaps OEP14 insertion also requires some proteins located on the side of the outer membrane facing the intermembrane space or even some soluble proteins in the intermembrane space. Our data indicate that isolated pure outer membrane vesicles are sufficient for OEP14 insertion (Y.-S. Su and H.-m. Li, unpublished data); therefore, the involvement of inner membrane proteins in OEP14 insertion seems unlikely.

It is not clear why NEM pretreatment blocked only 40% of OEP14 insertion. Under the same treatment conditions, import of the precursor to the small subunit of RuBp carboxylase (prSS), a stroma-targeted protein, was almost totally inhibited (data not shown). These results again support the notion that protein import into the outer membrane and protein import into the interior of chloroplasts use distinct pathways. Perhaps the NEM-sensitive component in the OEP14 import pathway still has 60% activity left after NEM modification, or possibly two components are cooperating to mediate OEP14 insertion. Perhaps after one of the components is modified by NEM, the other component can mediate insertion at only 60% efficiency. We also cannot exclude the possibility that NEM is exerting only a stereo-hindrance effect, that is, modifying a component that is in close contact with the component actually mediating insertion and therefore interfering with access of OEP14 to the insertion site.

Waegemann et al. (1990) showed that cytosolic factors were required for chloroplast import of an E. coli overexpressed precursor protein, even when the precursor protein has been denatured with urea. However, the same precursor protein and several other overexpressed chloroplastic precursor proteins were later shown to be fully import-competent without the addition of any other proteins, as long as the precursor proteins were denatured with urea plus DTT (Cline et al., 1993). In any event, because these precursor proteins have all been artificially denatured, they are unlikely to reflect the native conformations of the proteins in the cytosol. Whether protein targeting to chloroplasts requires any cytosolic factors in vivo remains unclear. The precursor of ferredoxin-NADP+ reductase has been successfully overexpressed in a soluble form. Adding pea cytosolic extracts, or urea denaturation, increased the efficiency of translocation of the precursor protein across the envelope, but these treatments had little effect on the binding of the precursor protein to the chloroplastic envelope (Ceccarelli et al., 1996). We did not observe any effect of various cytosolic extracts on the import of OEP14 to the outer membrane. Possibly the function of a cytosolic factor, if any, is to maintain the precursor proteins in a conformation more suitable for translocation through the double-membrane envelope. This translocation step is not required for OEP14. A cytosolic factor might also function to keep precursor proteins soluble in the cytosol. For a small protein such as OEP14, which contains no cysteine residues and can remain soluble even at high concentrations, such a cytosolic factor may not be necessary.

Five integral chloroplastic outer membrane proteins have been identified so far that clearly contain no cleavable transit peptides (Salomon et al., 1990; Li et al., 1991; Fischer et al., 1994; Kessler et al., 1994; Seedorf et al., 1995; Bolter et al., 1999). These proteins have been assumed to use a similar mechanism to insert into the outer membrane (Keegstra and Cline, 1999). However, of these five proteins, the import of OEP14, OEP6.7, and OEP21 is resistant to thermolysin pretreatment of chloroplasts and also does not require ATP (Salomon et al., 1990; Li et al., 1991; Bolter et al., 1999). The import of OEP24 is also resistant to thermolysin pretreat-
ment of chloroplasts, but its insertion into the outer membrane is stimulated by ATP (Fischer et al., 1994). The import of Toc34, on the other hand, is sensitive to thermolysin pre-treatment of chloroplasts and also requires ATP (Tsai et al., 1999). Perhaps, therefore, there are several different pathways for protein insertion into the outer membrane. However, we have observed competition between OEP14 and atToc34 as well as, under certain conditions, between OEP14 and the stroma-targeted prSS (S.-L. Tu and H.-m. Li, unpublished results). Therefore, these proteins probably use distinct but partially overlapped pathways. For example, each protein may have some unique components that it requires for its import, and the import pathways of different proteins may converge at various points during the import process.

METHODS

Expression and Purification of OEP14-His6

A DNA fragment encoding OEP14 was amplified by polymerase chain reaction (PCR) from the plasmid pSKG50.4 (Li et al., 1991) containing the OEP14 coding sequence. The primers used added an NdeI site at the N terminus and an EagI site at the C terminus of the fragment. The amplified fragment was subcloned into the NdeI and EagI sites of the plasmid pET22b (Novagen, Madison, WI), which added five amino acids from the multiple cloning sites and six histidine residues to the C terminus of OEP14. The resulting plasmid was called pET22b-OEP14. The sequences of the PCR-amplified region and the junctions were confirmed by sequencing. The plasmid pET22b-OEP14 was transformed into the Escherichia coli strain BL21(DE3)pLysS for overexpression of OEP14-His6.

Overexpression and labeling of OEP14-His6 were performed by inoculating 30 μL of an overnight culture of pET22b-OEP14 transformant into 3 mL of fresh Luria-Bertani medium containing 50 μg/mL ampicillin and further culturing at 37°C until the optical density at 600 nm reached ~0.6. The cells were pelleted, washed twice, and resuspended in 3 mL of EMEM medium without methionine (ICN, Costa Mesa, CA). The cells were further cultured for another 3 hr at 37°C, and isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 0.1 mM. Fifteen minutes after addition of IPTG, 200 μL (~2 μCi) of [35S]-methionine (Amersham Pharmacia, Piscataway, NJ) was added to the culture, and the cells were harvested 3 hr later. The cells were lysed and centrifuged at 20,000 g for 10 min. The supernatant was collected and incubated with TALON metal affinity resin (Clontech, Palo Alto, CA) containing various protease inhibitors (1 mM phenylmethylsulfonyl fluoride [PMSF], 1 μM leupeptin, and 1 μM pepstatin) at room temperature for 60 min. After washing the TALON resin three times with a washing buffer (50 mM NaH2PO4, 10 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.1% Triton X-100, and 5 mM imidazole) and once with the same buffer plus 30 mM imidazole, the bound OEP14-His6 was eluted with the same buffer plus 100 mM imidazole.

Chloroplast Pretreatments and Protein Import Reactions

Chloroplasts were isolated from 9- to 11-day-old pea (Pisum sativum cv Little Marvel) seedlings as described by Perry et al. (1991).
Thermolysin (Tsai et al., 1999) and N-ethylmaleimide (NEM; Friedman and Keegstra, 1989) pretreatments of chloroplasts were performed as described. Trypsin pretreatment of chloroplasts was performed by incubating chloroplasts (0.2 mg of chlorophyll mL⁻¹) with various concentrations of trypsin at 27°C in the dark for 1 hr. The treatment was terminated by adding soybean trypsin inhibitor (at sixfold the amount of trypsin used), PMSF (1 mM), pepstatin (1 μM), and leupeptin (1 μM). The treated chloroplasts were repurified through a 40% Percoll cushion, washed in import buffer (50 mM Hepes-KOH, pH 8.0, and 300 mM sorbitol), and used for import experiments. Trypsin inhibitor and PMSF were present in all buffers used after the pretreatment, including the final lysis step in SDS-PAGE sample buffer. Chloroplasts were pretreated with duramycin by incubating pretreatment, including the final lysis step in SDS-PAGE sample buffer. In vitro–translated OEP14 was synthesized through in vitro transcription (Perry et al., 1991) and in vitro translation with wheat germ extracts (Promega, Madison, WI) according to the manufacturer's specifications. Import of in vitro–translated proteins into chloroplasts was performed as described (Perry et al., 1991). Import of OEP14-His₆ into chloroplasts was performed with 5 μL of purified proteins in a 150-μL reaction containing 50 μL of chloroplasts (1 mg of chlorophyll mL⁻¹) at room temperature for 15 min. Saturation of OEP14-His₆ import was performed by incubating chloroplasts with various amounts of purified OEP14-His₆ under import conditions for 3 min. Import was terminated by reisolating chloroplasts through a 40% Percoll cushion. Competition was performed with in vitro–translated and ³⁵S-labeled OEP14 or atTOc34 with increasing amounts of unlabeled OEP14-His₆ in standard import reactions for 15 min at room temperature.

After import, chloroplasts were further treated with thermolysin as described (Smeeckens et al., 1986). Intact chloroplasts were reisolated after the protease post-treatments and analyzed by SDS-PAGE on 4 to 12% NuPAGE gels (Novex, San Diego, CA) with Mes running buffer. Quantification of samples was performed with the PhosphorImager SP (Molecular Dynamics, Sunnyvale, CA). Pictures of gels were taken from x-ray films generated by exposing the dried gels to Kodak MS film with a BioMax TranScreen-LE intensifying screen (Rochester, NY).

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