The Use of Chemical Cross-linking to Identify Proteins That Interact with a Mitochondrial Presequence*

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Amos S. Galkwad and Michael G. Cumsky‡

From the Department of Molecular Biology and Biochemistry, University of California, Irvine, California 92717

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Previous work has shown that when yeast mitochondria are incubated in the presence of the presequence peptide pL4(1-22), the peptide is imported and accumulates within the mitochondrial membranes, presumably at the import sites. If the extramitochondrial concentration of peptide is sufficiently high, enough peptide accumulates within the import sites to prevent the uptake of authentic precursor proteins. We have used chemical cross-linking to probe the interaction of this peptide with yeast mitochondrial proteins. We found that radio-labeled pL4(1-22) could be reproducibly cross-linked to a number of polypeptides. Interestingly, nearly all were membrane proteins. Several of the cross-linked proteins were located in the outer membrane, while others were located in the inner membrane. The interaction between the peptide and many of the cross-linked products was shown to be specific by two independent criteria. First, an excess of unlabeled peptide acted as a competitor in the cross-linking reaction, and, second, treatment of the peptide with the alkylating agent N-ethylmaleimide dramatically reduced its ability to form cross-links. Two of the cross-linked species corresponded to the outer membrane proteins, Mas70p and ISP42. Significantly, both of these proteins have previously been shown to play critical roles in mitochondrial protein import. While the role of the other cross-linked proteins in the import process remains to be determined, the results of this study demonstrate that our experimental approach may be useful in identifying components of the import machinery as well as proteins that interact with mitochondrial presequences.

Most mitochondrial proteins are encoded on nuclear DNA and translated in the cytosol. They must then be targeted to mitochondria, cross one or both mitochondrial membranes, and be correctly sorted into one of the four mitochondrial compartments. Despite considerable experimental effort, the molecular mechanism(s) underlying the import of proteins into mitochondria are only beginning to be understood. A detailed knowledge of this process is fundamental to our overall understanding of both mitochondrial biogenesis and protein transport into and across all biological membranes.

In the vast majority of cases, nuclear encoded mitochondrial proteins are initially synthesized with an NH2-terminal extension called a presequence or leader peptide. Over the last decade, the mitochondrial presequence and its role in protein import have been studied extensively. While presequences share no sequence homology, several structural features appear to be conserved. They (almost always) contain a high percentage of basic and hydroxylated amino acids, are devoid of acidic residues, and appear to form amphipathic structures (Roise and Schatz, 1988). Mitochondrial presequences are also known to mediate several of the early events in protein import. After the initial recognition and binding of the precursor protein by proteinaceous components at the mitochondrial surface, the translocation process begins by movement of the presequence into the matrix of the organelle (reviewed recently in Pfanner and Neupert (1990) and Glick and Schatz (1991)). This process, which requires the electrical component of the proton motive force (Δψ), is driven by the electrophoretic attraction of the positively charged presequence to the net negatively charged matrix (Martin et al., 1991). Translocation occurs at sites of contact between the two mitochondrial membranes (Pfanner et al., 1988; Pon et al., 1989), which appear to be composed of distinct translocation pores or channels within the individual bilayers (Glick et al., 1991; Pfanner et al., 1992). At present, the outer membrane translocation site has been partially characterized; it consists of at least two receptor-like proteins and several other membrane proteins that presumably form a channel within the bilayer (Kiebler et al., 1990; Sollner et al., 1992). On the other hand, very little is known about the inner membrane translocation site. While the nature of this site is actively being investigated in several laboratories, only a few proteins that are essential for transport across the inner membrane have thus far been identified (Maarse et al., 1992; Scherer et al., 1992; Emptage and Jensen, 1993).

It has been known for many years that the presequence targets proteins to mitochondria; presequences have been shown to be both necessary for mitochondrial delivery and sufficient to direct heterologous, non-mitochondrial proteins to the organelle (Hurt et al., 1984, 1985; Horwich et al., 1985). Yet, despite considerable experimental effort, it has still not become clear precisely how, on a molecular level, the information in the presequence is decoded. Furthermore, there is currently little evidence suggesting a direct interaction between the presequence and any mitochondrial protein, excepting only the processing peptide located in the matrix (Hawitschek et al., 1988; Jensen and Yaffe, 1988).

Do mitochondrial presequences interact with proteins of the translocation machineries within the mitochondrial membranes? In the present study, we have addressed this question by taking advantage of the unique properties of a synthetic presequence peptide, pL4(1-22) (Glaser and Cumsky, 1990a, 1990b). Using chemical cross-linking methods, we have identified several membrane proteins that specifically interact with this presequence. Two of the proteins, Mas70p and ISP42, are of particular note, because they correspond to previously identified components of the outer membrane translocation site. Several others, which are located within either the inner or the outer mitochondrial membrane, may represent previously uniden-
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dentified proteins that comprise part of the import machinery and/or facilitate the import process.

EXPERIMENTAL PROCEDURES

Synthetic Peptides—The synthesis, deprotection, and purification of the synthetic presequence peptide pL4(1-22) has been described previously (Glaser and Cumsky, 1990a). Each lot of peptide was tested for purity and biological activity by assaying its ability to block mitochondrial protein import over the concentration range of 5–20 µg.

Because covalent modification of any type has been shown to alter the biological activity of pL4(1-22) (Glaser and Cumsky, 1990a, 1990b), a radioiodinated derivative for use in cross-linking studies was synthesized by the following procedure. First, Fmoc-[35S]Met was synthesized in a reaction that contained 0.49 µmol (in acetone), 0.38 µmol [35S]Met, 0.38 mM Na2CO3. The reaction was carried out at room temperature with constant stirring for 12 h. The acetone was then evaporated, the pH value was adjusted to 6.5, and the mixture was extracted several times with ethyl acetate to separate Fmoc-[35S]Met from the unreacted Fmoc-[35S]Met. The purity of the product was also confirmed by thin layer chromatography.

The Fmoc-[35S]Met was coupled to the partially synthesized peptide (residues 2–22) in a single cycle using standard synthesis conditions (Atherton et al., 1981; Dryland and Sheppard, 1986). The labeled peptide was then deprotected by incubation for 8 h at room temperature in a mixture of 90% trifluoroacetic acid, 5% thioanisole, 3% ethanediol, 2% anisole (reagent R). It was then cleaved from the resin for 2 h at room temperature in a 20% solution of methanolic ammonia. The free deprotected peptide was dissolved in reagent R, precipitated by the addition of 10 volumes of anhydrous ethyl ether, pelletied, washed four additional times with ether, and lyophilized. The dried peptide was dissolved in anisole (reagent R). It was then cleaved from the resin for 2 h at room temperature in a 20% solution of methanolic ammonia. The free deprotected peptide was dissolved in reagent R, precipitated by the addition of 10 volumes of anhydrous ethyl ether, pelletied, washed four additional times with ether, and lyophilized. The dried peptide was dissolved in 0.1% trifluoroacetic acid and purified by reverse phase chromatography on a C18 column (Waters Associates). The peptide was then 20–35% acetonitrile gradient. The purified peptide, which eluted at 35% acetonitrile, was then concentrated in a speed vac.

The purity of the peptide was determined by SDS-polyacrylamide gel electrophoresis (PAGE) in the presence of 6 µg urea (12.5% polyacrylamide gel). The biological activity of the peptide was confirmed by its ability to block mitochondrial protein import (see “Results”).

In Vitro Import Reactions—Procedures for the in vitro transcription and translation of mitochondrial precursor proteins, preparation of yeast mitochondria, and in vitro import into yeast mitochondria have been described previously (Glaser et al., 1990; Miller and Cumsky, 1991, 1993). The high intramitochondrial concentration of pL4(1-22) prevented the uptake of authentic precursor proteins; it acted as a reversible inhibitor of mitochondrial protein import. Thus, we reasoned that chemical cross-linking of accumulated intramitochondrial peptide would be a viable means by which to examine the interaction between mitochondrial presequences and the import machinery. Because covalent modification of any type has been shown to destroy the biological activity of pL4(1-22) (Glaser and Cumsky, 1990a, 1990b), we synthesized a radioiodinated form of the peptide that contained [35S]Met at the NH2 terminus for use in cross-linking experiments (see “Experimental Procedures”). This peptide behaved identically to the unlabeled derivative in that it accumulated within the mitochondrial membranes (not shown) and reversibly blocked protein import at a concentration of 10–20 µM. As shown in Fig. 1, the labeled peptide was able to block import of the precursors to both subunit IV of cytochrome c oxidase (pIV) and the β subunit of the F1-ATPase (pF1β). It was, therefore, used in all subsequent cross-linking experiments.

To identify proteins that interact with pL4(1-22), we used EGS, a homobifunctional cross-linker that is membrane-permeable but can cross-link soluble proteins as well. Mitochondria were allowed to accumulate the labeled peptide for 15–30 min at room temperature, were incubated with the cross-linker as described under “Experimental Procedures,” and were then analyzed by SDS-PAGE. As shown in Fig. 2A, both major and minor cross-linked products were generated. Importantly,
were preincubated on ice in the presence of approximately 20 μM radiolabeled pU(1-22). After 5 min, 1.5 mM EGS, 10 mM final concentration of glycine to 20 mM, and the mitochondria were reisolated by centrifugation at 4 °C and processed for SDS-PAGE. Import reactions were analyzed by SDS-PAGE and fluorography; reactions involving pF,P were resolved on 15% gels. The position of representative marker proteins is indicated at the right; TM, total mitochondrial membranes. Cross-linked Products Reside in Both the Inner and Outer Mitochondrial Membranes—When mitochondria that had been treated with peptide and subsequently cross-linked were separated into soluble and membrane fractions, we found that the majority of the cross-linked products were membrane proteins (Fig. 5A). This result was consistent with our earlier observation that the vast majority of the imported peptide resided within the membranes (Glaser and Cumsky, 1990a, 1990b). To determine whether the products resided in the inner or outer membrane, total mitochondrial membranes were further separated by centrifugation on a sucrose density gradient. Fractions were then collected and analyzed by immunoblotting (to determine the presence of representative marker proteins) and by cross-linking to pL4(1-22). The results of the immunoblot analysis confirmed that this procedure resulted in a typical separation of the two membranes (Fig. 5C). The results of the cross-linking experiment are shown in Fig. 5B. Nearly all of the proteins that were strongly cross-linked to the peptide in intact mitochondria could also be cross-linked with the isolated membranes. We also found that these proteins did not reside exclusively in one of the two membranes; while a majority of the strongly cross-linked proteins that were competent with excess cold peptide (Fig. 2) fractionated with the outer membrane, at least one of this group clearly resided within the inner membrane (Fig. 5B). Moreover, recent results have shown that several of the less strongly cross-linked proteins, which are not clearly resolved in Figs. 2 and 5, are also inner membrane proteins (see "Discussion").

In addition to providing evidence that cross-linked products

2 A. S. Gaikwad, unpublished results.
All import reactions were analyzed by SDS-PAGE and fluorography on 15% gels. Addition of valinomycin or proteinase K treatment of the reaction respectively. The positions of the precursor indicated, the mitochondria were preincubated with various forms of the peptide pU(1-22); and 1.5

\[ \text{Fig. 3. NEM-inactivated pL4(1-22) does not block protein import into yeast mitochondria. Import reactions using 50 \mu g of mitochondria and 1.5 \times 10^6 \text{ radiolabeled precursor} (subunit Va of yeast cytochrome c oxidase, pVa) were performed as described in the legend to Fig. 1. Where indicated, the mitochondria were preincubated with various forms of the peptide pL4(1-22); pL4 and N-pL4 denote the native and NEM-treated forms of the peptide, respectively, while *pL4 and N-*pL4 denote the radiolabeled native and radiolabeled NEM-treated forms of the peptide, respectively. The positions of the precursor (pVA) and mature forms (Va) of subunit Va are indicated. Shown in lanes 13 and 14 are the results of a control import reaction performed in the presence of 2 mM NEM (inactivated with DTT, which was also present at a final concentration of 40 mM). All import reactions were analyzed by SDS-PAGE and fluorography on 15% gels. Addition of valinomycin or proteinase K treatment of the reaction mixture is also indicated.} \]

reside in both mitochondrial membranes, the results of the experiment presented in Fig. 5 are important for another reason. It has recently become clear that distinct translocation machineries reside within both the outer and inner mitochondrial membranes (Glick et al., 1991; Pfanner et al., 1992). It has also been established that specific import of precursor proteins can be achieved across isolated outer or inner membrane vesicles (Hwang et al., 1989; Mayer et al., 1993). The latter observation suggests that the respective translocation machineries of each membrane are largely intact in appropriate preparations of membrane vesicles. Our observation that essentially the same cross-linking profile is seen with either intact mitochondrial or membrane vesicles (Figs. 2 and 5) suggests that our vesicle preparations are clean. More significantly, the results shown in Fig. 5 underscore the fact that our cross-linking reactions are highly specific.

**Two Cross-linked Products Are Components of the Mitochondrial Import Machinery**—As a first step toward characterizing the proteins that interacted with pL4(1-22), we asked whether any of the cross-linked products corresponded to known mitochondrial proteins. To accomplish this, mitochondria were first incubated with the radiolabeled peptide and cross-linked using EGS as before. Aliquots of the cross-linked organelles were then solubilized and analyzed by immunoprecipitation using antisera that had been prepared against a number of different yeast mitochondrial proteins, including OMP45p, a 45-kDa outer membrane protein whose function is presently unclear (Yaffe et al., 1989); Mas70p, an outer membrane surface receptor (Hines and Schatz, 1993); ISP42p, a component of the outer membrane translocation site (Vestweber et al., 1989; Baker et al., 1990); p32, the 32-kDa product of the Mir1 gene, proposed to be an outer membrane import receptor (Murakami et al., 1990; Pain et al., 1990; Murakami et al., 1993); Mas6p, the product of the MAS6 gene, which is a 23-kDa integral inner membrane protein that may be involved in transport across the inner membrane (Emtage and Jensen, 1993); porin, a 29-kDa outer membrane protein that forms an anionic channel in the outer membrane (Dihanich, 1990; Pfanner et al., 1990), and AAC (the ADP/ATP carrier), an abundant 30-kDa inner membrane protein (Pfanner and Neupert, 1987) whose mammalian homolog was previously shown to be cross-linked to a precursor peptide (Meyer, 1990). Importantly, each of the antisera used has been shown to be able to immunoprecipitate its corresponding antigen in control experiments performed in both this and other laboratories (data not shown).

Most of the antisera failed to yield radiolabeled products when the immunoprecipitates were resolved by SDS-PAGE and fluorography. Included in this group were antibodies directed against OMP45 (Fig. 6A, lane 2), porin (Fig. 6B, lane 10), p32 (Fig. 6B, lane 11), Mas6p (Fig. 6B, lane 12), and AAC (Fig. 6B, lane 13). The results suggest that these proteins were not among those strongly cross-linked to pL4(1-22) under the conditions of our experiments. However, antisera against two of the proteins, ISP42 and Mas70p, immunoprecipitated distinct radiolabeled products of the appropriate size (Fig. 6, A, lanes 3 and 8, and B, lane 9), thereby suggesting that these proteins did interact with the peptide. For ISP42, we showed that immunoprecipitation could be significantly diminished if the radiolabeled peptide was chased with an excess of the cold derivative prior to cross-linking (Fig. 6A, lane 4). In contrast, if we used a nonspecific competitor for the chase (pATIII, see above), ISP42 could still be immunoprecipitated (Fig. 6A, lane 5). ISP42 could also be immunoprecipitated, albeit weakly, with antiserum prepared against total yeast mitochondrial outer membranes (Fig. 6, lane 1). For Mas70p, the antiserum precipitated two radiolabeled proteins, which, based upon electrophoretic mobility, appeared to be homologs of ISP42 and Mas70p (MOM38p and MOM72p, respectively) are part of a receptor complex in the mitochondrial outer membrane (Kiebler et al., 1990; Sollner et al., 1992) and

3 G. Shore, personal communication.

that, under certain conditions, the two proteins can be immunoprecipitated together (Kiebler et al., 1990). When taken together, the results of Fig. 6, A and B, strongly support the conclusion that ISP42 and Mas70p were cross-linked to pL4(1-22) and, therefore, must interact with the peptide under the conditions of our experiments.

**DISCUSSION**

The results presented here extend those of our earlier studies on the interaction of the presequence peptide pL4(1-22) with isolated yeast mitochondria. Consistent with our earlier work, we found that pL4(1-22) was efficiently taken up by mitochondria and, using chemical cross-linking as an assay, that it interacted with a number of mitochondrial membrane proteins. The interaction between pL4(1-22) and several of those proteins was found to be specific by two independent criteria. First, cross-linking to radiolabeled pL4(1-22) could be competed by an excess of the nonlabeled derivative. Second, pL4(1-22) that had been inactivated by NEM could not be cross-linked to any significant degree.

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**pL4(1-22) is primarily cross-linked to membrane proteins.** A, mitochondria (0.25 mg) were cross-linked to radiolabeled pL4(1-22) and then separated into total membrane and soluble fractions as described under "Experimental Procedures." Samples representing 50 µg of intact mitochondria (T) (pretreated with radiolabeled peptide and cross-linked as described in the legend to Fig. 2) or the total membrane (M) and soluble fractions (S) derived from approximately 50 µg of mitochondria were analyzed by SDS-PAGE and fluorography on 10% gels. The positions of representative molecular mass standards are indicated at the right. B, 50 µg of mitochondria were separated into outer and inner membrane vesicles using the procedure of Hwang et al. (1989). The pelleted membrane vesicles were resuspended in 75 µl of SH buffer and cross-linked to radiolabeled pL4(1-22) using the procedure described for mitochondria under "Experimental Procedures." Cross-linking reactions contained approximately 100 µg of membrane vesicles in 10 µl. After stopping the reactions with glycine, the samples were then processed and analyzed by SDS-PAGE and fluorography on a 10% gel. The lane designated M represents cross-linked products from 50 µg of intact mitochondria. The number under each lane represents the sucrose gradient fraction from which the vesicles were derived, numbering from the top to the bottom of the gradient. Fractions containing outer membrane (OM) or inner membrane (IM) vesicles were determined by immunoblotting samples from each fraction against known marker proteins (below). IDF, intermediate density fraction. The arrowheads denote the positions of the major cross-linked products that were shown to be comparable in Fig. 2. An arrowhead marking a protein in fraction 2 indicates that the protein was enriched in the outer membrane. The protein marked with the arrowhead in fraction 7 indicates that the product appeared to be enriched in the inner membrane. The arrowhead marking the protein in fraction 5 indicates that this product appeared essentially uniform throughout the gradient. The position of representative molecular mass standards is indicated at the right. C, 50 µg of total mitochondrial protein (M) or 50 µg of membrane vesicles derived from the indicated gradient fractions were fractionated by SDS-PAGE on 15% gel. Proteins from the gel were then transferred to nitrocellulose and analyzed by immunoblotting using antibodies prepared against the outer membrane protein ISP42 (Isp42) and the inner membrane protein subunit Va of cytochrome oxidase (Va).

**pL4(1-22) forms cross-links with ISP42 and Mas70p.** A, cross-linking or cross-linking/competition using 40 µg of mitochondria and radiolabeled pL4(1-22) was performed as described in the legend to Fig. 2. After stopping the reactions, the samples were subjected to immunoprecipitation as described (Glaser and Cumsky, 1990a). Antibodies used were raised against yeast mitochondrial outer membranes (MOM), OMP45 (45), or ISP42 (42) (see text). Competitor peptides were nonlabeled pL4(1-22) (S, specific) or pATIII (NS, nonspecific). Lane 6 contains 40 µg of cross-linked mitochondria. B, experiment is the same as in A except antibodies used were raised against ISP42 (42), Mas70p (70), porin (P), p32 (32), Mas6p (M6), or AAC (AAC) (see text). Lane 7 contains 40 µg of cross-linked mitochondria. ISP42 was included as a control, since the experiments shown in panels A and B were performed with different preparations of peptide and mitochondria.

**Fig. 5.** pL4(1-22) is primarily cross-linked to membrane proteins. A, mitochondria (0.25 mg) were cross-linked to radiolabeled pL4(1-22) and then separated into total membrane and soluble fractions as described under "Experimental Procedures." Samples representing 50 µg of intact mitochondria (T) (pretreated with radiolabeled peptide and cross-linked as described in the legend to Fig. 2) or the total membrane (M) and soluble fractions (S) derived from approximately 50 µg of mitochondria were analyzed by SDS-PAGE and fluorography on 10% gels. The positions of representative molecular mass standards are indicated at the right. B, 50 µg of mitochondria were separated into outer and inner membrane vesicles using the procedure of Hwang et al. (1989). The pelleted membrane vesicles were resuspended in 75 µl of SH buffer and cross-linked to radiolabeled pL4(1-22) using the procedure described for mitochondria under "Experimental Procedures." Cross-linking reactions contained approximately 100 µg of membrane vesicles in 10 µl. After stopping the reactions with glycine, the samples were then processed and analyzed by SDS-PAGE and fluorography on a 10% gel. The lane designated M represents cross-linked products from 50 µg of intact mitochondria. The number under each lane represents the sucrose gradient fraction from which the vesicles were derived, numbering from the top to the bottom of the gradient. Fractions containing outer membrane (OM) or inner membrane (IM) vesicles were determined by immunoblotting samples from each fraction against known marker proteins (below). IDF, intermediate density fraction. The arrowheads denote the positions of the major cross-linked products that were shown to be comparable in Fig. 2. An arrowhead marking a protein in fraction 2 indicates that the protein was enriched in the outer membrane. The protein marked with the arrowhead in fraction 7 indicates that the product appeared to be enriched in the inner membrane. The arrowhead marking the protein in fraction 5 indicates that this product appeared essentially uniform throughout the gradient. The position of representative molecular mass standards is indicated at the right. C, 50 µg of total mitochondrial protein (M) or 50 µg of membrane vesicles derived from the indicated gradient fractions were fractionated by SDS-PAGE on a 15% gel. Proteins from the gel were then transferred to nitrocellulose and analyzed by immunoblotting using antibodies prepared against the outer membrane protein ISP42 (Isp42) and the inner membrane protein subunit Va of cytochrome oxidase (Va).
The apparent molecular mass of several major cross-linked products fell into distinct size ranges: 30, 40–45, and 70 kDa. Therefore, we asked if those products corresponded to previously identified yeast mitochondrial membrane proteins. Using immunological techniques, we determined that the outer membrane proteins, porin, OMP45, and p32, and the inner membrane proteins, Mas6p and AAC, had not been cross-linked and, therefore, did not appear to strongly interact with pL4(1-22) under the conditions of our experiments. On the other hand, the outer membrane proteins, Mas70p and ISP42, had become cross-linked and, therefore, must somehow interact with this presequence peptide.

What is the nature of the interaction between pL4(1-22) and Mas70p/ISP42? Both of these proteins are often grouped within the general category of import receptors. Mas70p appears to function in the initial recognition and binding of precursor proteins to mitochondria, while ISP42 is thought to play a critical role in the recognition or uptake of bound precursors at the outer membrane translocation site (Pfanner and Neupert, 1988; Glick and Schatz, 1991). One possible interpretation of our data is that the function of ISP42 and Mas70p includes the ability to recognize, and therefore interact directly with, mitochondrial presequences. Alternatively, it is also possible that the interaction between pL4(1-22) and ISP42/Mas70p results from something more specific than the presequence peptide.

At present, the available data do not permit us to distinguish between the two interpretations. However, several observations suggest that the association between pL4(1-22) and the proteins to which it was strongly cross-linked, including ISP42 and Mas70p, results from something more specific than the accumulation of peptide within the translocation sites. First, pL4(1-22) was reproducibly cross-linked to a discrete set of proteins, even when a different cross-linking reagent was used. Second, several of the proteins cross-linked to pL4(1-22) were distinct from those observed in cross-linking studies performed in other laboratories (Gillespie, 1987; Vestweber et al., 1988; Söllner et al., 1992). Although we note that both ISP42 and Mas70p (or their N. crassa homologs, MOM38 and MOM72, respectively) have been cross-linked to intermediates spanning the outer and inner membrane translocation sites (Vestweber et al., 1989; Söllner et al., 1992). Finally, as shown in Fig. 5B, we were able to cross-link pL4(1-22) to the same set of proteins even after the mitochondria had been fractionated into preparations of outer and inner membrane vesicles. Considering the heterogeneous nature of these membrane preparations, we find it striking that such a high degree of cross-linking specificity was retained in the absence of mitochondrial integrity. We suggest that when taken together, these results provide additional support for the notion that the interaction between pL4(1-22) and yeast mitochondria is a highly precise one.

Could both ISP42 and Mas70p have the ability to specifically recognize and decode the information contained within mitochondrial presequences? Clearly, the answer to this question remains to be conclusively determined. It has long been thought that the so-called surface receptors (Mas70p and Mas20p in yeast and MOM72 and MOM19 in N. crassa) carried out this function, based primarily on the observations that the receptors mediate the initial interaction between precursors and mitochondria (Pfanner et al., 1988; Pfanner et al., 1988), and that precursors lacking a functional presequence fail to bind to mitochondria efficiently, if at all (Hurt et al., 1984, 1985). On the other hand, it now seems apparent that mitochondrial recognition and binding of precursor proteins is a complex process that may require several different mitochondrial components (Kiebler et al., 1990; Hines and Schatz, 1993), cytosolic factors (Murakami and Mori, 1990; Murakami et al., 1992), and portions of the presequence other than the presequence (Pfanner et al., 1987; Hines and Schatz, 1993). The precise role of ISP42 in the import process is even less well-defined, although ISP42 function is essential for both protein import and viability of yeast (Baker et al., 1990). Since surface receptors appear to enhance import efficiency and are not obligatory for uptake (Pfanner et al., 1989; Miller and Cumsky, 1991; Hines and Schatz, 1993), it is clear that mitochondria must contain an alternative receptor (or receptors) that first, accept precursors that are already bound to the outer membrane and second, can mediate recognition and binding of precursor proteins in the absence of surface receptor (Mas70p, Mas20p, MOM 19, MOM 72) function. It seems likely that such a receptor resides at the outer membrane translocation site; whether it is ISP42 remains to be demonstrated.

Currently, we are in the process of performing experiments that we hope will allow us to determine whether ISP42 and Mas70p recognize presequences directly or whether cross-linking occurred simply because of the proximity of pL4(1-22) to these proteins while it was within the translocation sites. We argue, however, that in either case, the experimental approach described here clearly provides a means to identify membrane proteins that may be fundamentally involved in the process of mitochondrial protein import. It is possible that one or more of the polypeptides that become strongly cross-linked to pL4(1-22) under the conditions of our experiments may represent previously uncharacterized proteins that comprise part of the translocation machinery or play some other role in the uptake of precursor proteins. Recent work in our laboratory has resulted in the purification of one of these proteins (the 75-kDa outer membrane protein, which we do not think is Mas70p) and the partial purification of several others (60, 40, and 30 kDa), which we are currently in the process of characterizing in more detail. We have also found that several less strongly cross-linked proteins can be identified on the basis of their immunological recognition by a complex antiserum generated against total mitochondrial inner membranes (a gift of G. Schatz). We are hopeful that the purification and characterization of these proteins will lead us to their corresponding structural genes and ultimately to the elucidation of what role, if any, they play in mitochondrial import.

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