growth. Furthermore, neither SAR gene expression nor resistance is inhibited when SA is co-applied with high concentrations of catechol (B. Vernooij, L. Friedrich, J. Ryals, unpublished results).
6. T. Delaney *et al.*, unpublished results.
7. G. Samuel, *Ann. Appl. Biol.* **21**, 90 (1934); C. Bennett, *J. Agric. Res.* **60**, 361 (1940).
8. *Arabidopsis thaliana* ecotype Col-0 was transformed [H. Huang and H. Ma, *Plant Mol. Biol. Rep.* **10**, 372 (1992)] with *Agrobacterium tumefaciens* strain CIB542 containing the cloned *nahG* gene under the control of an enhanced 35*S* promoter (*4*). Several independent homozygous transformed lines were identified and with the use of immunoblot analysis were shown to accumulate large amounts of salicylate hydroxylase protein. None of the transformed lines exhibited phenotypes apart from those described.
9. M. C. Whalen, R. W. Innes, A. F. Bent, B. J. Staskawicz, *Plant Cell* **3**, 49 (1991).
10. X. Dong, M. Mindrinos, K. Davis, F. Ausubel, *ibid.*, p. 61; T. Debener, H. Lehnackers, M. Arnold, J. Dangl, *Plant J.* **1**, 289 (1991).
11. B. J. Staskawicz, D. Dahlbeck, N. T. Keen, C. Napoli, *J. Bacteriol.* **169**, 5789 (1987); J. D. Dangl *et al.*, *Plant Cell* **4**, 1359 (1992); R. W. Innes *et al.*, *Plant J.* **4**, 813 (1993); S. R. Bisgrove, M. T. Simonich, N. M. Smith, A. Sattler, R. W. Innes, *Plant Cell* **6**, 927 (1994).
12. E. Koch and A. Slusarenko, *Plant Cell* **2**, 437 (1990); B. Mauch-Mani, K. Croft, A. Slusarenko, in Arabidopsis thaliana *as a Model for Plant-Pathogen Interactions*, K. Davis and R. Hammerschmidt, Eds. (American Phytopathological Society, St. Paul, MN, 1993), pp. 5–20; E. Holub, J. L. Beynon, I. R. Crute, *Mol. Plant-Microbe Interact.* **7**, 223 (1994).
13. Two-week-old *A. thaliana* plants of the indicated ecotype were inoculated with *P. parasitica* (race Wela or Noco) by spraying with a conidia suspension (approximately 1×10^5 spores per milliliter). Inoculated plants were incubated at 17°C under conditions of high humidity. Several plants from each treatment were randomly selected and stained with lactophenol–trypan blue for microscopic examination [R. C. Keogh, B. J. Deverall, S. McLeod, *Trans. Br. Mycol. Soc.* **74**, 329 (1980)]. Trypan blue stains fungal structures within the plant tissue and dead or damaged plant cells.
14. S. Uknes *et al.*, *Plant Cell* **4**, 645 (1992).
15. INA (Ciba-Geigy AG, Basel, Switzerland), formulated as a 25% active ingredient in a wettable powder carrier, was suspended in distilled water at 250 μg of INA per milliliter (325 μM) [J. P. Métraux *et al.*, in *Advances in Molecular Genetics of Plant-Microbe Interactions*, H. Hennecke and D. P. S. Verma, Eds. (Kluwer, Dordrecht, 1991), vol. 1, pp. 432–439]. This solution, or distilled water alone, was sprayed to the point of imminent runoff on *A. thaliana* plants as indicated. Acquired resistance from INA application is indistinguishable from that caused by biological agents, in that both cause the induction of the same suite of SAR genes (*16*) and the same spectrum of pathogen resistance [H. Kessmann *et al.*, *Annu. Rev. Phytopathol.* **32**, 439 (1994)].
16. E. R. Ward *et al.*, *Plant Cell* **3**, 1085 (1991); S. Uknes *et al.*, *Mol. Plant-Microbe Interact.* **6**, 692 (1993).
17. R. A. Dietrich, T. Delaney *et al.*, *Cell* **77**, 565 (1994); K. Broglie *et al.*, *Science* **254**, 1194 (1991).
18. D. Alexander *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 7327 (1993).
19. S. Huang *et al.*, *Science* **259**, 1742 (1993); R. Kamijo *et al.*, *ibid.* **263**, 1612 (1994); M. Kondo *et al.*, *ibid.* **262**, 1874 (1993); M. Noguchi *et al.*, *ibid.*, p. 1877; S. M. Russell *et al.*, *ibid.*, p. 1880.
20. We thank M.-D. Chilton, T. Brears, K. Lawton, U. Neuenschwander, and B. Lee for critically reviewing this manuscript. We also thank J. Watkins for preparing media and P. VanBourgondien Jr., S. Sips, and G. Crawford for assistance with plant culture.

26 May 1994; accepted 7 October 1994

The Role of Hsp70 in Conferring Unidirectionality on Protein Translocation into Mitochondria

Christian Ungermann, Walter Neupert,* Douglas M. Cyr

The entry of segments of preproteins of defined lengths into the matrix space of mitochondria was studied. The mitochondrial chaperone Hsp70 (mtHsp70) interacted with proteins emerging from the protein import channel and stabilized translocation intermediates across the membranes in an adenosine triphosphate–dependent fashion. The chaperone bound to the presequence and mature parts of preproteins. In the absence of mtHsp70 binding, preproteins with less than 30 to 40 residues in the matrix diffused out of mitochondria. Thus, protein translocation was reversible up to a late stage. The import channels in both mitochondrial membranes constitute a passive pore that interacts weakly with polypeptide chains entering the matrix.

How proteins are translocated across membranes during the formation of cellular structures is largely unknown (*1*, *2*). Because most proteins are synthesized in the cytosol, proteins destined for subcellular compartments must cross the boundary membranes of organelles. For mitochondria and chloroplasts, proteins must traverse more than one membrane (*3*, *4*). Components of protein translocation machinery and mechanisms for protein targeting have been identified (*1*, *3*), but how protein translocation is driven energetically and rendered unidirectional is still unclear.

In mitochondria, preproteins are transferred from the cytosol into the matrix in several steps (*3*). We investigated events that occur when the NH_2-terminal targeting signal of a mitochondrial precursor protein is translocated across the inner membrane and segments of it emerge in the matrix, and examined the components facilitating these reactions and the energy requirements of this process.

A series of chimeric precursor proteins that consisted of NH_2-terminal regions with various lengths of the *Neurospora crassa* F_o-adenosine triphosphatase (ATPase) subunit 9 precursor (pSu9) (*5*) fused in frame to murine dihydrofolate reductase (DHFR) was constructed (Fig. 1). The precursor has a 66–amino acid presequence with sites for cleavage by the matrix processing peptidase (MPP) at positions 35 and 66. Import of the different Su9-DHFR proteins into mitochondria was measured by monitoring the conversion of the larger precursor (p) to the smaller intermediate (i) and mature (m) forms (Fig. 1). This process was dependent on the membrane potential across the inner membrane, $\Delta\Psi$ (Fig. 1). A DHFR domain that is stabilized by a folate antagonist like methotrexate (Mtx) cannot be translocated across the mitochondrial membranes (*6*, *7*). In the absence of Mtx, the Su9-DHFR fusion proteins were completely imported into the matrix and processed by MPP to the m form (Fig. 1). Import of such fusion proteins into mitochondria in the presence of Mtx led to the formation of translocation intermediates that spanned both membranes, with the folded DHFR domain remaining on the mitochondrial surface. In the presence of Mtx, pSu9(1–86)-DHFR was imported and accumulated predominantly in the p form. A small quantity of the i form was observed after 10 min, and additional processing was seen after longer incubation periods (*8*). The first cleavage site on pSu9(1–86)-DHFR was not readily accessible to MPP in the matrix; and pSu9(1–94)-DHFR was processed to the i form more efficiently. This is in agreement with the observation that approximately 50 amino acid residues are required to span both outer and inner membranes (*7*). In the presence of Mtx, pSu9(1–112)-DHFR also entered the matrix far enough to allow processing from the p to the i form (Fig. 1) (*9*).

The i form of Su9(1–94)-DHFR did not accumulate in large amounts over time when import was arrested with Mtx (Fig. 1). Most of the iSu9(1–94)-DHFR formed was recovered in the supernatant of the import reaction (Fig. 1) (*9*). Its appearance in the supernatant was dependent on $\Delta\Psi$, time, and the addition of Mtx to reaction mixtures. In contrast, pSu9(1–86)-DHFR and iSu9(1–112)-DHFR were found primarily in association with mitochondria in the presence of Mtx (Fig. 1). Experiments with mitochondria from *N. crassa* yielded similar results (*8*).

When the DHFR domain on pSu9(1–94)-DHFR was stabilized by Mtx, the NH_2-terminus of this precursor was translocated

Institut für Physiologische Chemie der Universität München, Goethestr. 33, D-80336 München, Germany.

*To whom correspondence should be addressed.

into mitochondria where the presequence was processed; the i form produced cannot be maintained in the import apparatus and diffuses out. A similar observation was made with a fusion protein between precytochrome b2 and DHFR (*7*), which became processed by MPP and then dissociated from mitochondria in the presence of Mtx. In this case, however, temperatures of 30° to 37°C were required for most of this import intermediate to be released (*8*). The presence of longer NH_2-terminal segments in the matrix, such as the additional 18 residues in iSu9(1–112)-DHFR or the approximately 30 to 35 NH_2-terminal residues of pSu9(1–86)-DHFR, promoted stable association with the import machinery. When the chelators EDTA and *o*-phenanthroline (o-Phe) were used to inhibit MPP (*10*), amounts of pSu9(1–94)-DHFR in the supernatant were reduced, and it efficiently accumulated with mitochondria (Fig. 2). The p form of Su9(1–94)-DHFR was stably arrested spanning the membranes because the reactivation of MPP by addition of $MnCl_2$ resulted in efficient processing and release of the i form from mitochondria (*8*).

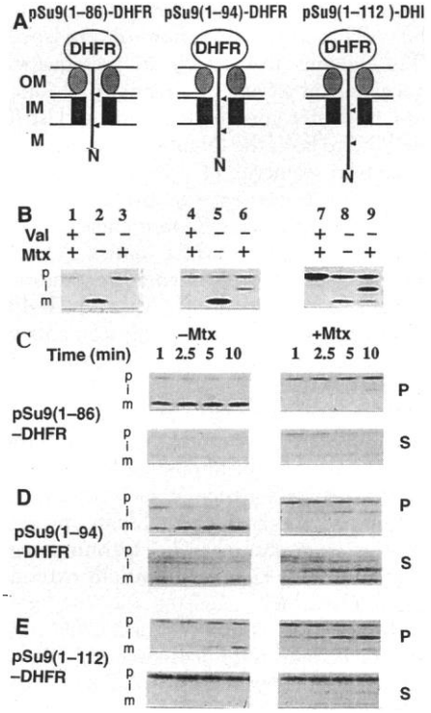

Fig. 1. Reverse translocation of import intermediates out of mitochondria. (**A**) Model of pSu9-DHFR fusion proteins arrested as import intermediates, which span both the mitochondrial outer (OM) and inner (IM) membrane, when methotrexate (Mtx) has stabilized the folded DHFR domain; M denotes the matrix. Standard cloning techniques were used to fuse regions of the precursor form of the *N. crassa* F_o-ATPase subunit 9 (pSu9) (*5*) to murine DHFR (*6*, *7*). Cleavage sites for MPP are represented by arrowheads. N, NH_2-terminus. (**B**) Association of different forms of ^{35}S-pSu9-DHFR fusion proteins with mitochondria in the presence and absence of Mtx (1 μM). Incubations of pSu9(1–86)-DHFR, pSu9(1–94)-DHFR, and pSu9(1–112)-DHFR with isolated yeast mitochondria were at 25°C for 5, 5, and 20 min, respectively. (**C** to **E**) Kinetic analysis of the distribution of different forms of Su9-DHFR fusion proteins between the mitochondrial pellet and the supernatant after centrifugation of import reactions. pSu9-DHFR fusion proteins were synthesized in reticulocyte lysate and then incubated in 100 μl of standard import buffer with mitochondria (250 μg/ml) that had been freshly isolated from *S. cerevisiae* strain D27310-B (*13*). Precursor- (p), intermediate- (i), and mature- (m) sized bands present in the mitochondrial pellet (P) and acetone precipitates of supernatants from reaction mixtures (S) were analyzed by SDS–polyacrylamide gel electrophoresis and fluorography (*13*).

In view of the known role of matrix adenosine triphosphate (ATP) in the import of proteins into mitochondria (*13*, *14*), we asked whether ATP is required to stabilize Mtx-arrested translocation intermediates with mitochondria. Precursors were incubated with mitochondria or mitoplasts in the presence of Mtx to allow accumulation of import intermediates (Fig. 2), then reaction mixtures were divided into two equal portions. One portion was treated with apyrase and oligomycin to reduce matrix ATP levels, but not alter $\Delta\Psi$ (*13*, *14*). The other portion served as a control. After further incubation, the translocation intermediates that remained associated with the import apparatus were determined. For Su9(1–86)-DHFR, depletion of matrix ATP caused release of the accumulated p form (Fig. 2). Similarly, after stalling Su9(1–94)-DHFR import in mitoplasts, the i form was released into the supernatant after matrix ATP depletion (Fig. 2). iSu9(1–112)-DHFR that accumulated in mitochondria in the presence of Mtx was also released at low matrix ATP, though not as efficiently as the two previous constructs (*8*). Prior to ATP depletion, the Mtx-arrested import intermediates were largely resistant to digestion by added protease because of the tight apposition of the DHFR domain to the mitochondrial surface (*15*). After ATP depletion, import intermediates that remained were completely sensitive to added protease, suggesting that the DHFR domain moved away from the membrane surface.

Thus, upon depletion of matrix ATP, membrane-spanning intermediates slid in a retrograde manner completely or partly out of the translocation machinery. Matrix ATP appeared to be required for tight association with components of the import apparatus that stabilize polypeptides in the import channel during transport into the matrix.

Previous studies suggested a role of mtHsp70 in driving translocation (*13*, *14*, *16*). To determine a possible interaction of mtHsp70 with precursor proteins as they emerge on the matrix side of the inner membrane, we asked whether this mitochondrial

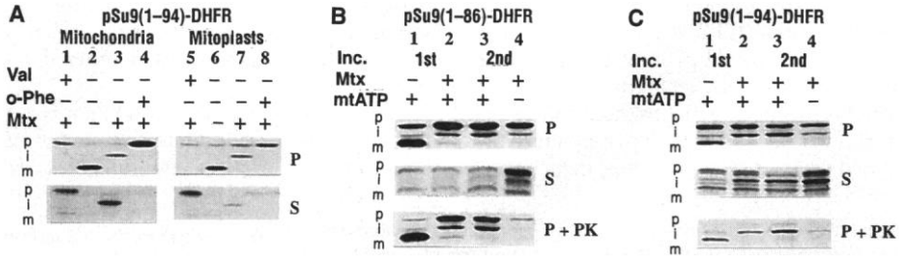

Fig. 2. Length and matrix ATP requirements for stabilization of pSu9-DHFR translocation intermediates in mitochondrial membranes. (**A**) Import of pSu9(1–94)-DHFR into mitochondria and mitoplasts. o-Phe denotes the addition of 0.4 mM *o*-phenanthroline and 4 mM EDTA to inhibit MPP (*10*). Mitoplasts were generated by swelling mitochondria in low–osmotic strength buffer (*11*). Analysis of samples was as in Fig. 1. (**B** and **C**) Depletion of matrix ATP promotes retrograde sliding out of mitochondria of Su9-DHFR import intermediates arrested by Mtx. Two successive incubations (Inc.) were performed for the respective precursor proteins. In the first reaction, pSu9-DHFR was incubated with mitochondria or mitoplasts for 10 min at 25°C to promote the accumulation of import intermediates. Samples were then removed for analysis. Reactions containing Mtx were further incubated in a second reaction for 10 min at 25°C in the presence (−mtATP) or absence (+mtATP) of apyrase (40 U/ml) and oligomycin (20 μM), which were added to deplete the matrix of ATP (*13*, *14*). Analysis of samples was as in Fig. 1. (B) Import of pSu9(1–86)-DHFR into mitochondria. (C) Import of pSu9(1–94)-DHFR into mitoplasts. Proteinase K treatments (PK; 75 μg/ml) were carried out on ice from 30 min prior to analysis of samples (*13*).

chaperone could be recovered in a complex with Mtx-arrested preproteins. pSu9(1–94)-DHFR was incubated with mitochondria or mitoplasts in the presence or absence of Mtx, and the relative amounts of the various forms of Su9(1–94)-DHFR that were associated with mitochondria under different import conditions were assessed (Fig. 3). The reactions were divided into two equal portions; in one portion, matrix ATP was depleted, whereas in the other, matrix ATP was kept high. Mitochondria were lysed with detergent, and coimmunoprecipitation with antibodies to mtHsp70 was performed. No coimmunoprecipitation was observed between mtHsp70 and pSu9(1–94)-DHFR that was bound to the surface of valinomycin-treated mitochondria. Thus, mtHsp70 did not bind to import intermediates after lysis of mitochondria (Fig. 3). When pSu9(1–94)-DHFR was completely imported into mitochondria and matrix ATP was kept high, little mSu9(1–94)-DHFR was coprecipitated with mtHsp70. In contrast, when matrix ATP was depleted, mSu9(1–94)-DHFR was detected in a complex with mtHsp70 (Fig. 3). A similar observation was made when Su9(1–94)-DHFR was imported into mitoplasts (Fig. 3) (*17*). DHFR domains of such fusion proteins fold after import into the matrix, so mtHsp70 must have remained bound to the 28–amino acid stretch of the Su9 portion.

When the import of Su9(1–94)-DHFR into mitochondria was arrested with Mtx, the resulting i form was not found in contact with mtHsp70, suggesting that the few residues protruding into the matrix were not bound by mtHsp70 (Fig. 3). By contrast, in mitoplasts, Mtx-arrested iSu9(1–94)-DHFR was firmly bound to mtHsp70 at high matrix ATP. At low matrix ATP, this same complex was not observed (Fig. 3).

Dissociation of complexes between mtHsp70 and a protein translocation intermediate upon ATP depletion was unexpected, because Hsp70-polypeptide complexes are generally stabilized at low ATP and high adenosine diphosphate concentrations (*18*), as was seen for completely imported proteins (Fig. 3). Thus, depletion of matrix ATP had differential effects on the binding of mtHsp70 to protein translocation intermediates and to fully imported forms. An ATP-dependent complex forms between mtHsp70 and MIM44 (*19*), a peripheral inner membrane protein also termed Mpi1p or Isp45 (*20*). MIM44 is essential for protein import and is located at the inner face of the inner membrane, where it has at least two activities: It interacts with precursor proteins as they enter the matrix and concentrates mtHsp70 at the outlet of the import channel (*19*). Depletion of matrix ATP reduces the level of complex between mtHsp70 and MIM44 and thus prevents efficient interactions between mtHsp70 and Su9-DHFR translocation intermediates. Thus, at low ATP concentrations, retrograde movement of import intermediates occurs at a higher rate than binding to mtHsp70, and polypeptides diffuse out of the import apparatus.

We next investigated whether mtHsp70 can bind the first part of the presequence as it emerges in the matrix. The p form of Su9(1–48)-DHFR was allowed to accumulate with mitoplasts in the presence of Mtx. In this situation, the NH_2-terminal 48 amino acid residues of this precursor spanned the inner membrane, but the cleavage site at position 35 was not accessible to MPP in the matrix (Fig. 4). A complex between mtHsp70 and import-arrested pSu9(1–48)-DHFR was observed by coimmunoprecipitation (Fig. 4). When matrix ATP was depleted, this complex was absent, whereas tight interaction of fully imported and processed preprotein with mtHsp70 was seen. Cross-linking experiments confirmed the ATP-dependent interaction of this preprotein with mtHsp70 (*8*). In addition, when import of pSu9(1–94)-DHFR was arrested and processing was simultaneously inhibited by addition of o-Phe, strong cross-links with both mtHsp70 and MIM44 were observed (*8*). Thus, the targeting sequence was bound by mtHsp70 as it entered the matrix.

This study sheds light on a number of aspects of the mechanisms and energy requirements of protein translocation. (i) Translocation of proteins into mitochondria is a fully reversible process up to a late stage, when segments of as many as 50 to 60 amino acid residues have been transported across the inner membrane. Unprocessed as well as processed preproteins can undergo retrograde movement. Reversible translocation has also been reported for bacterial secretion and translocation across the endoplasmic reticulum membrane (*21*). (ii) Stable translocation across the inner membrane requires not only $\Delta\Psi$ (*22*), but also the ATP-dependent action of mtHsp70. This explains the defects in presequence processing observed when mtHsp70 function is compromised (*13*). (iii) mtHsp70 can bind to both the targeting sequence and to mature segments of precursor proteins. (iv) There are two types of interactions of a preprotein with mtHsp70 in the matrix, one of which disappears when matrix ATP is depleted and another of which is stabilized by the same treatment. The first type holds for interactions with incoming segments of a preprotein, the second type for interaction of mtHsp70 with deeply or fully imported proteins. (v) Only ~50 amino acid residues of a preprotein are needed to span both the outer and inner membranes. MPP has access to the presequence of such intermediates, implying that the actual spanning segment is even shorter. The spanning segment is likely to be in an essentially extended conformation.

Reversibility of translocation could provide a mechanism for dual targeting of proteins to the matrix and cytosol. Such a dual targeting event would require a pause in import, such as might be caused by a folded domain ~50 to 60 residues beyond the MPP cleavage site. During this pause, the precursor would be processed and a fraction of the precursor would fall back into the

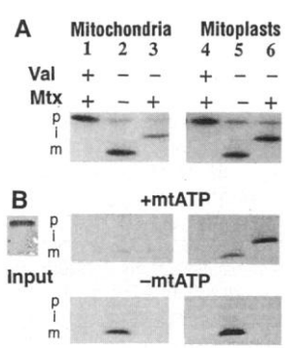

Fig. 3. Complex formation between Mtx-arrested import intermediates and mtHsp70. pSu9(1–94)-DHFR was imported into mitochondria or mitoplasts as described for Fig. 1. After import, the reaction was divided into three equal portions. One portion was treated for analysis of import (**A**). (**B**) Other portions of the import reaction were either incubated to maintain high levels of matrix ATP or treated with apyrase (40 U/ml) and oligomycin (20 μM) to deplete the matrix of ATP (*13*). Then, complex formation between mtHsp70 and the different forms of Su9(1–94)-DHFR was determined by coimmunoprecipitation with antibodies to mtHsp70 (*15*). Input represents 3% of the total material used for the coimmunoprecipitation reactions. Complex formation between mtHsp70 and translocation intermediates was not detected with preimmune antibodies. The levels of complex formation determined by this method were similar to those reported for other preproteins (*16*).

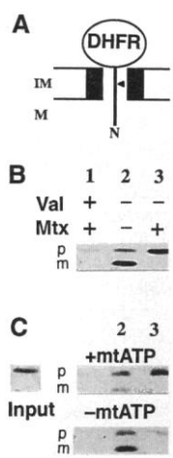

Fig. 4. Binding of the mitochondrial presequence by mtHsp70 during protein translocation across the inner membrane. Import of pSu9(1–48)-DHFR into mitoplasts was as described for Fig. 1. (**A**) Model of pSu9(1–48)-DHFR arrested with Mtx upon import across the membrane of mitoplasts. The arrowhead represents the MPP cleavage site at position 35. N, NH_2-terminus. (**B**) Analysis of precursor- (p) and mature- (m) sized Su9(1–48)-DHFR associated with mitoplasts. (**C**) Coimmunoprecipitation of p and m forms of Su9(1–48)-DHFR with mtHsp70 (see Fig. 3). Input represents 3% of the total sample used for the coimmunoprecipitation reaction.

cytosol, with the rest being completely imported. In fact, such a pathway might explain the dual targeting of fumarase in *Saccharomyces cerevisiae* (*23*).

Finally, our data suggest that the mitochondrial import machinery contains a passive import channel formed by components in both membranes. This import channel accommodates unfolded polypeptide chains, but components of the channel interact only weakly with mature segments of translocating chains as compared to mtHsp70.

REFERENCES AND NOTES

1. W. T. Wickner and H. F. Lodish, *Science* **230**, 400 (1985); T. A. Rapoport, *ibid.* **258**, 931 (1992); W. Neupert, F.-U. Hartl, E. A. Craig, N. Pfanner, *Cell* **63**, 447 (1990); K. P. Baker and G. Schatz, *Nature* **349**, 205 (1991); S. L. Sanders and R. Schekman, *J. Biol. Chem.* **267**, 9881 (1992).
2. W. T. Wickner, A. J. M. Driessen, F.-U. Hartl, *Annu. Rev. Biochem.* **60**, 101 (1991).
3. M. Kiebler, K. Becker, N. Pfanner, W. Neupert, *J. Membr. Biol.* **135**, 191 (1993); K. Hannavy, S. Rospert, G. Schatz, *Curr. Opin. Cell Biol.* **5**, 694 (1993).
4. S. M. Theg and S. M. Scott, *Trends Cell Biol.* **3**, 186 (1993).
5. A. Viebrock, A. Perz, W. Sebald, *EMBO J.* **5**, 565 (1982).
6. M. Eilers and G. Schatz, *Nature* **322**, 228 (1986).
7. J. Rassow, F.-U. Hartl, B. Guiard, N. Pfanner, W. Neupert, *FEBS Lett.* **275**, 190 (1990).
8. C. Ungermann, W. Neupert, D. M. Cyr, data not shown.
9. Fusion protein pSu9(1–112)-DHFR was prone to aggregation in translation lysates. This was reflected by its low import efficiency of approximately 20% of input. Import efficiencies of pSu9(1–86)-DHFR and pSu9(1–94)-DHFR were between 70 to 80% of input. Import efficiency of Su9(1–112)-DHFR increased by 50% in the presence of Mtx (Fig. 1). This was probably due to prevention of Su9(1–112)-DHFR aggregation through stabilization of the DHFR domain by Mtx (Fig. 1). However, the DHFR domain on all the pSu9(1–112)-DHFR molecules was not completely folded. This prevented a complete block in processing to the m form from occurring in the presence of Mtx (Fig. 1). A band corresponding in size to the m form of Su9-DHFR was detected in the supernatants of reaction mixtures (Fig. 1). This band was not generated because of import into the matrix. It was present in translation lysates (*8*), did not increase during incubations, and is likely to have resulted from translation initiation at an internal methionine.
10. F.-U. Hartl, N. Pfanner, D. W. Nicholson, W. Neupert, *Biochim. Biophys. Acta* **988**, 1 (1989).
11. G. Daum, P. C. Boehni, G. Schatz, *J. Biol. Chem.* **257**, 13028 (1982).
12. When experiments were conducted with mitoplasts (Fig. 2), some iSu9(1–94)-DHFR accumulated in the supernatants of import reactions. This was caused by protein import into mitochondria, with intact outer membranes contaminating mitoplast preparations (*8*).
13. D. M. Cyr, R. A. Stuart, W. Neupert, *J. Biol. Chem.* **268**, 23751 (1993).
14. R. A. Stuart *et al.*, *Eur. J. Biochem.* **220**, 9 (1994); C. Wachter, G. Schatz, B. S. Glick, *Mol. Cell. Biol.* **5**, 465 (1994).
15. E. Schwarz, T. Seytter, B. Guiard, W. Neupert, *EMBO J.* **12**, 2295 (1993).
16. R. A. Stuart, D. M. Cyr, E. A. Craig, W. Neupert, *Trends Biochem. Sci.* **19**, 87 (1994); P. J. Kang *et al.*, *Nature* **348**, 137 (1990); P. E. Scherer, U. C. Krieg, S. T. Hwang, D. Vestweber, G. Schatz, *EMBO J.* **9**, 4315 (1990); B. D. Gambill *et al.*, *J. Cell Biol.* **123**, 109 (1993).
17. The efficiency of pSu9(1–94)-DHFR import into mitoplasts was ~30% lower than that of import into mitochondria. In addition, a large portion of the m form remained sensitive to digestion by protease (Fig. 3). Thus, a portion of the m form was in a conformation in which it spanned both membranes. In mitoplasts, at both high and low matrix ATP, a slightly higher level of complex formation between Su9(1–94)-DHFR and mtHsp70 was detected as compared to that in mitochondria (Fig. 3). This difference apparently results from interaction of mtHsp70 with the m form that was not completely imported into the matrix.
18. M.-J. Gething and J. Sambrook, *Nature* **355**, 33 (1992).
19. H. C. Schneider, J. Berthold, K. Dietmeier, M. Brunner, W. Neupert, *Nature*, in press.
20. A. C. Maarse, J. Blom, L. A. Grivell, M. Meijer, *EMBO J.* **11**, 3619 (1992); M. Horst *et al.*, *ibid.* **12**, 3035 (1993).
21. E. Schiebel, A. J. M. Driessen, F.-U. Hartl, W. Wickner, *Cell* **64**, 927 (1991); C. E. Ooi and J. Weiss, *ibid.* **71**, 87 (1992).
22. M. Schleyer and W. Neupert, *ibid.* **43**, 339 (1985).
23. I. Stein, Y. Y. Peleg, S. Even-Ram, O. Pines, *Mol. Cell Biol.* **14**, 4770 (1994).

24. Supported by grants from the Sonderforschungsbereich 184, the Fonds der Chemischen Industrie, and the Human Frontier Science Program Organization (HFSPO) to W.N. D.M.C. was supported by a long-term fellowship from the HFSPO. C.U. is the recipient of a Boehringer Ingelheim predoctoral fellowship. We thank M. Brunner for antibodies to MIM44 and for discussions concerning MIM44 interactions with mtHsp70, and M. Scully for assistance with preparation of the manuscript.

28 July 1994; accepted 19 September 1994

Modulation of Epithelial Cell Growth by Intraepithelial γδ T Cells

Richard Boismenu and Wendy L. Havran*

The role played in immune surveillance by γδ T cells residing in various epithelia has not been clear. It is shown here that activated γδ T cells obtained from skin and intestine express the epithelial cell mitogen keratinocyte growth factor (KGF). In contrast, intraepithelial αβ T cells, as well as all lymphoid αβ and γδ T cell populations tested, did not produce KGF or promote the growth of cultured epithelial cells. These results suggest that intraepithelial γδ T cells function in surveillance and in repair of damaged epithelial tissues.

Thy-1$^+$ dendritic epidermal T cells (DETCs) (*1–3*) expressing the invariant $V_\gamma3$ $V_\delta1$ T cell receptor (TCR) (*4*, *5*) are found exclusively in the epidermis of the adult mouse (*6*), where they exist in intimate contact with keratinocytes. The expression of a monoclonal antigen recognition structure by DETCs contrasts with the large TCR repertoire of nonepithelial αβ and γδ T cells. This lack of diversity suggests that DETCs have a limited ligand recognition capacity and may have a different role in immunological surveillance from that of αβ T cells (*7*). We have shown that DETCs recognize self-antigen presented by cultured or stressed keratinocytes in an apparently non–major histocompatibility complex (MHC)–restricted fashion (*8*). The existence of a functional link between two cell types normally in close physical contact led us to investigate the possibility that this connection extends to the expression by DETCs of growth factors acting on keratinocytes.

Balb/MK keratinocytes are not viable when grown under defined serum-free conditions, except when supplemented with either exogenous KGF, acidic fibroblast growth factor (FGF), basic FGF, epidermal growth factor (EGF), or insulin growth factor–1 (Fig. 1A) (*9*, *10*). Other factors alone or in combination could conceivably support the proliferation of Balb/MK cells (*10*). We used the growth factor dependence of the Balb/MK line to devise an experimental system with membrane filters that allowed diffusible factors secreted by the 7-17 DETC line (*11*) to reach Balb/MK cells while preventing direct cell contact.

In transfilter experiments, we assessed the ability of 7-17 cells to produce a cytokine that would promote the proliferation of Balb/MK cells. The results of these experiments demonstrated that 7-17 cells, as well as three other DETC lines (*12*), release a soluble factor that can sustain the clonal growth of Balb/MK cells (Fig. 1, B and C). Stimulation of 7-17 cells beforehand with mitogen concanavalin A (Con A) potentiated this activity (Fig. 1D). In contrast, T cell lines bearing αβ receptors or γδ receptors other than $V_\gamma3$ $V_\delta1$ did not prevent Balb/MK cell death under identical conditions (Fig. 1D).

The ability of DETCs to inducibly secrete a mitogenic factor that targets keratinocytes represents a possible function for a T cell population expressing a monomorphic TCR that exclusively recognizes stressed or otherwise damaged keratinocytes. Consideration of cytokines with the potential to mediate this effect led us to investigate KGF as the most likely candidate because of its specificity toward keratinocytes (*13*, *14*).

Hybridization of a human KGF comple-

Department of Immunology, The Scripps Research Institute, La Jolla, CA 92037, USA.

*To whom correspondence should be addressed.