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6. Fluorescent labels were obtained from Molecular Probes (Eugene, OR) unless otherwise noted. Membrane labeling with head group–conjugated fluorescein-phosphatidylethanolamine (FL-PE) (unsaturated, Avanti Polar Lipids, Alabaster, AL; saturated, Molecular Probes) was slightly modified from (*11*). Briefly, freshly washed cells (10 μl) were suspended in phosphate-buffered saline (90 μl) and FL-PE (dissolved in methanol) was added (1 μl to ~20 μM). The mixture was incubated for 30 min at 25°C. The lipid probe $diIC_{18}$ (*5*) was similarly intercalated.
7. Band 3 on intact red cells was labeled with eosin 5-maleimide (*13*) or fluorescein 5-maleimide and observed through either a fluorescein or longer wavelength filter set. The same type of response for band 3 was observed with cells in which both band 3 and actin were labeled with nonoverlapping fluorophores. Similar results were also obtained by labeling band 3 with a fluorescein isothiocyanate (FITC)–monoclonal Fab designated BRAC-18. CD59 on intact cells was labeled with a rhodamine-tagged monoclonal Fab. The purity of all Fabs was verified, and antibodies were centrifuged before use. For studies of glycophorin C, Fabs were generated from a monoclonal immunoglobulin G designated BRIC-10.
8. Red cells can be reversibly permeabilized by cold, hypotonic lysis so that fluorescent probes specific to internal structures can diffuse into the cell "ghost" and bind (*22*) [J. F. Hoffman, in *The Use of Resealed Erythrocytes as Carriers and Bioreactors*, M. Magnani and J. R. DeLoach, Eds. (Plenum, New York, 1992), pp. 1–15; M. D. Scott *et al.*, *J. Lab. Clin. Med.* **115**, 470 (1990)]. Labeling of cytoskeletal actin with fluorescein- or rhodamine-phalloidin was accomplished by first air-drying 2 to 5 μl of phalloidin at a concentration of 1 mg/ml in methanol and then redissolving it in 20 to 50 μl of cold lysis buffer [7.5 mM sodium phosphate (pH 7.4 ± 0.1)]. Cold, packed red cells (5 μl) were added and, after 5 min, the suspension was adjusted to 100 mM KCl, 1 mM $MgCl_2$, and 0.1 mM dithiothreitol and incubated at 37°C for 20 to 60 min. Mechanical properties of the resealed membrane were not altered by the labeling procedure, as evaluated by both micropipette aspiration and ektacytometry. A concentration-dependent edge brightness indicated an apparent in situ dissociation constant of $\sim 3 \times 10^{-7}$ M, slightly less than in vitro assays. None of the protein solutions fluorescently labeled unlysed cells. Protein 4.1 was purified and labeled by previously described methods [J. M. Tyler, W. R. Hargreaves, D. Branton, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5192 (1979); A. Podgorski and D. Elbaum, *Biochemistry* **24**, 7871 (1985)] and stored at 0.25 mg/ml in 50 mM KCl, 10 mM sodium phosphate (pH 7.4), 0.2 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride. Protein 4.1–deficient cells were labeled by a process similar to that for phalloidin. Iodoacetimido-5-fluorescein was conjugated to a recombinant glutathione-S-transferase fusion protein containing the spectrin-actin–binding domain of protein 4.1 (*23*). Incorporation of protein 4.1 or the recombinant spectrin-actin–binding domain has been shown to normalize the mechanical strength of protein 4.1–deficient membranes (*22*, *23*). Polyclonal immunoglobulin G antibodies to the α-II(46) domain of α-spectrin were subjected to proteolysis to yield Fab fragments, which were then labeled with FITC. Incorporation provided a view of the gradient similar to that of phalloidin-labeled actin and labeling of protein 4.1. Ektacytometry has shown that anti-spectrin Fabs do not alter membrane deformability [K. Nakashima and E. Beutler, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3823 (1978)].
9. The micropipette system is based on previous descriptions [E. A. Evans, *Methods Enzymol.* **173**, 3 (1989)]. In the present studies [D. E. Discher, thesis, University of California at Berkeley and San Francisco (1993)], a conventional Nikon Diaphot was fitted with a standard video camera and also with a liquid N_2–cooled charge-coupled device camera (CH-260; Photometrics, Tucson, AZ) for accurate quantitation of very low level fluorescence [Y. Hiraoka, J. W. Sedat, D. A. Agard, *Science* **238**, 36 (1987)]. To collect light from the full depth of the fluorescing membrane cylinder, we used an objective lens with a depth of focus similar to the pipette diameter (Zeiss Neofluar; 40×, 0.75 numerical aperture). The interval of aspiration to the deformed state was varied from <1 s up to 5 min, and image collection ranged from 100 ms to 10 s; neither variable had a discernible effect. Similar images were obtained with both higher and lower power objectives. For fluorescein, excitation was at 480 ± 15 nm, dichroic at 510 nm, and emission at 535 ± 20 nm. For longer wavelengths, excitation was at 560 ± 20 nm, dichroic at 595 nm, and emission at 655 ± 20 nm. Although the aspiration process was fully reversible under brightfield illumination, prolonged excitation of fluorescence invariably resulted in photofixation, which could be demonstrated by the cell partially retaining its aspirated form and density gradient after expulsion from the micropipette.
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12. From (*3*), the distance between nodes is ~76 nm and the spectrin filament contour length is ~200 nm. The minimum relative density of an intact network is given by the squared ratio of these lengths, $\bar{\rho}_c \approx 0.14$.
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15. On the basis of excluded area interactions, a simplistic quantitative treatment of the complementarity shown in Fig. 4 leads to an estimate of the areal fraction occupied by coupled integral proteins such as band 3 on the nascent membrane of between 25 and 55%.
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20. The reference shape for the calculations was a discocyte (*2*); results changed little with both the variations expected in cell geometry and the range of pipette sizes. The pipette radius, R_p, was 1 μm, and relative to the cell-sphered radius R_o, $R_o/R_p = 3$.
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24. We thank A. Leung for technical assistance. D. Anstee (International Blood Group Reference Laboratory, Bristol, UK) provided BRAC-18 and BRIC-10. S. Test (Children's Hospital of Oakland Research Institute, Oakland, CA) provided rhodamine-tagged monoclonal Fab. S. Marchesi (Yale University) provided antispectrin. Supported in part by Department of Energy equipment funds and by NIH grants P01 DK32094-09 and R01 HL31579-13. N.M. has also been supported by NIH grant R01 DK26263-15.

31 May 1994; accepted 13 September 1994

Identification of Two GTP-Binding Proteins in the Chloroplast Protein Import Machinery

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Two of four proteins that associated with translocation intermediates during protein import across the outer chloroplast envelope membrane were identified as guanosine triphosphate (GTP)–binding proteins. Both proteins are integral membrane proteins of the outer chloroplast membrane, and both are partially exposed on the chloroplast surface where they were accessible to thermolysin digestion. Engagement of the outer membrane's import machinery by an import substrate was inhibited by slowly hydrolyzable or nonhydrolyzable GTP analogs. Thus, these GTP-binding proteins may function in protein import into chloroplasts.

At least six distinct chloroplast envelope proteins are specifically engaged by a protein import substrate during its translocation across the two membranes of the envelope in an in vitro chloroplast import system (*1*). These proteins, referred to as IAPs (import intermediate–associated proteins), coisolated specifically and in apparently stoichiometric amounts with tagged import intermediates after detergent solubilization of an envelope subfraction of chloroplasts. A subset of four of these IAPs, referred to as early IAPs, are specifically engaged by early import intermediates and, therefore, are proposed to represent components of the outer membrane (OM) import machinery (*1*).

We have characterized two of these early IAPs, IAP34 and IAP86. These two proteins are indeed integral proteins of the outer chloroplast membrane, and both pro-

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teins are GTP-binding proteins. Consistent with a role of these two GTP-binding proteins in protein import, we found that nonhydrolyzable GTP analogs inhibited engagement of the outer membrane's import machinery by the import substrate.

We obtained partial amino acid sequence data from IAP34 and used oligonucleotide probes to obtain an IAP34 complementary DNA (cDNA) clone (*2*). The deduced amino acid sequence (Fig. 1) revealed a protein with a calculated molecular size of 34,064 daltons. Analysis of the primary structure showed the presence of consensus motifs for a GTP-binding site (Fig. 1) (*3*) in an otherwise unique sequence when compared to the available protein sequences in the data banks.

IAP34 is indeed a GTP-binding protein. When it was expressed in *Escherichia coli* (*4*), resolved by SDS–polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose blots, and incubated with [α-^{32}P]GTP, there was specific labeling of IAP34 that was not competed by incubation with adenosine triphosphate (ATP) but was competed by unlabeled GTP (Fig. 2).

In vitro synthesis of IAP34 mRNA in a wheat germ cell–free translation system yielded a single band that comigrated with mature IAP34 (*5*) indicating that IAP34 is not synthesized as a larger precursor. To investigate the mechanism of integration of IAP34 into the outer chloroplast membrane, the translation mixture was depleted of ATP and then incubated with chloroplasts either in the presence of ATP or in the absence of ATP and in the presence of apyrase. Chloroplasts were then reisolated and a fraction containing all of the envelope proteins was analyzed by SDS-PAGE and autoradiography. Integration of IAP34 into the outer chloroplast membrane required neither ATP nor thermolysin-sensitive components of the import machinery (Fig. 3A). There was as much uptake of IAP34 into chloroplasts in the absence of ATP as there was in the presence of ATP (Fig. 3A); and incubation of chloroplasts with thermolysin did not affect uptake of IAP34 (Fig. 3A). IAP34 was apparently associated with the outer chloroplast membrane because it was largely recovered in the OM fraction of subfractionated chloroplasts (Fig. 3C). Moreover, IAP34 was integrated into the outer membrane in a manner resistant to alkali extraction independently of whether ATP was present in the import reaction; or whether chloroplasts were incubated with thermolysin before import (Fig. 3B). These results are consistent with the import characteristics of other outer membrane proteins (*6*, *7*). IAP34 integrated into the chloroplast membrane was degraded by incubation of chloroplasts with thermolysin (Fig. 3A) indicating that a majority of the protein is accessible on the chloroplast surface. The deduced amino acid sequence of IAP34 (Fig. 1) contains a single hydrophobic region (residues 267 to 283) that conforms to a predicted alpha helical transmembrane segment (*8*). If this sequence does represent the membrane

```
M A S Q Q Q T V R E W S G I N T F A P A T Q T K L  25
L E L L G N L K Q E D V N S L T I L V M G K G G V  50
G K S S T V N F I I G E R V V S I S P F Q S E G P  75
R P V M V S R S R A G F T L N I I D T P G L I E G 100
G Y I N D M A L N I I K S F L L D K T I D V L L Y 125
V D R L D A Y R V D N L D K L V A K A I T D S F G 150
K G I W N K A I V A L T H A Q F S P P D G L P Y D 175
E F F S K R S E A L L Q V V R S G A S L K K D A Q 200
A S D I P V V L I E N S G R C N K N D S D E K V L 225
P N G I R W I P H L V Q T I T E V A L N K S E S I 250
F V D K N L I D G P N P N Q R G K L W I P L I F A 275
L Q Y L F L A K P I E A L I R R D I A T E T K P A 300
W E T R D V G D R K                              310
```

Fig. 1. Deduced amino acid sequence of IAP34 cDNA (*2*). Residues corresponding to the putative transmembrane domain (*8*) are indicated by a broken underline. Residues corresponding to peptide sequence obtained from IAP34 are indicated by the solid underlines. The residues corresponding to conserved motifs of known GTP-binding proteins (*3*) are indicated in bold type. The GenBank accession number for the IAP34 cDNA is L36856.

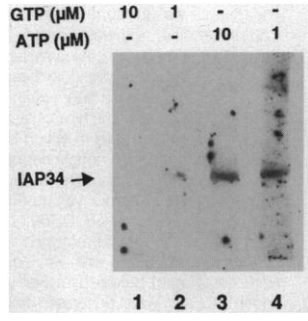

Fig. 2. GTP binding by IAP34. A DNA fragment encompassing the entire IAP34 coding region of pBS-IAP34 (*2*) was inserted into pET21d (Novagen, Inc.) under the control of the inducible Ø10 promoter. The resulting plasmid, pET21d-IAP34, was introduced into *E. coli* BL21 (DE3). Expression of IAP34 was induced by isopropylthio-β-galactoside (IPTG), and IAP34 was recovered in an insoluble fraction containing inclusion bodies (*14*). Proteins in this fraction (containing approximately 1 μg of recombinant IAP34) were resolved by SDS-PAGE and transferred to nitrocellulose filters. GTP binding was assayed by a modification of the method of Bhullar and Haslam (*15*). Briefly, the filters containing *E. coli*–expressed IAP34 were incubated in 50 mM tris-HCl (pH 7.5), 50 μM $MgCl_2$, 0.3% Tween-20, 5 nM [α-^{32}P]GTP (3000 Ci/mmol) for 2 hours at 4°C in the absence or presence of ATP or GTP as indicated. After the incubation, the filters were washed five times in 50 mM tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.3% Tween-20 at 4°C and exposed to Kodak XAR-5 film with intensifying screens for 18 hours. No [α-^{32}P]GTP binding was observed in extracts from *E. coli* containing the pET21d vector alone (*5*).

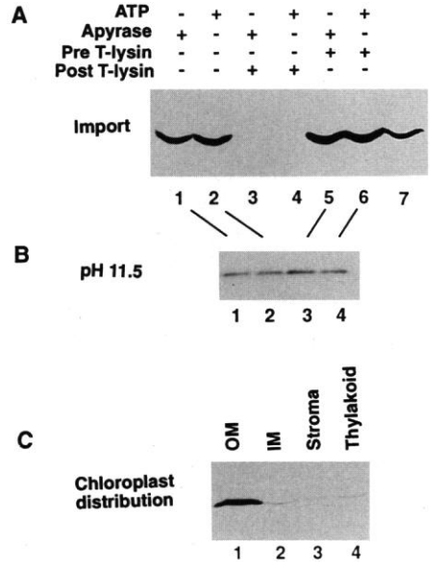

Fig. 3. Import of IAP34 into the outer envelope membrane of isolated chloroplasts. (**A**) [^{35}S]IAP34 was synthesized in a wheat germ extract (*16*). Import of [^{35}S]IAP34 was assayed in a standard import reaction (*17*) containing ATP-depleted chloroplasts (*16*). The import reactions contained 2 mM MgATP (+ATP) or 15 U of the ATP-consuming enzyme, apyrase (+Apyrase). Where indicated, chloroplasts were treated with thermolysin before (Pre T-lysin) or after (Post T-lysin) the import reaction (*18*). After import and protease treatment, the chloroplasts were reisolated and separated into membrane and stromal fractions (*19*). The membrane fractions were analyzed by SDS-PAGE and fluorography. Lane 7 contains a portion of the [^{35}S]IAP34 equivalent to 10% of that added to each import reaction. (**B**) The chloroplast membrane fractions corresponding to lanes 1, 2, 5, and 6 of (A) were treated with 0.1 M Na_2CO_3 (pH 11.5) as described (*1*). The membranes were recovered by centrifugation and analyzed by SDS-PAGE and fluorography. (**C**) Chloroplasts corresponding to lane 2 of (A) were reisolated after import, diluted with unlabeled chloroplasts (equivalent to 1 mg of chlorophyll) and fractionated into envelope, thylakoid and stromal components by lysis and flotation into linear sucrose gradients (20 to 38%) as described (*1*). Proteins from outer envelope membranes (OM), inner envelope membranes (IM), stromal extract (Stroma), and thylakoid membranes (Thylakoid) were analyzed by SDS-PAGE and fluorography to determine the distribution of imported [^{35}S]IAP34. Each lane contains 5 μg of protein.

anchor for IAP34, IAP34 is likely to be oriented with the short COOH-terminal segment in the intermembrane space and the large NH_2-terminal domain (including the GTP-binding site) extending into the cytosol.

We obtained amino acid sequence data from IAP86 and corresponding cDNA clones (*2*). The deduced amino acid sequence showed a protein of 96,685 daltons (Fig. 4A). This size is considerably larger than that estimated from the mobility of IAP86 on SDS-PAGE (86 kD). The amino acid sequence obtained from one peptide derived from IAP86 includes the COOH-terminal amino acid of the deduced IAP86 sequence (Fig. 4A). Therefore, the primary translation product of IAP86 might be proteolytically processed at its NH_2-terminus to yield the mature protein. Like IAP34, IAP86 contains consensus motifs for a GTP-binding site (*3*). Except for the GTP-binding site motif, IAP86 lacks similarity to other protein sequences in the data banks. However, it does share sequence similarity with IAP34 outside of the GTP-binding site motif (Fig. 4B).

To confirm the location of IAP86 in the outer chloroplast membrane, we obtained antibodies to IAP86 (anti-IAP86) (*9*). When chloroplasts were subfractionated, anti-IAP86 reacted specifically with an 86-kD polypeptide that was present in the light outer membrane fraction and to a much lesser extent in the inner membrane fraction (probably because of outer membranes attached through contact sites) but was not detectable in the thylakoid or stroma fractions (Fig. 5).

To determine whether IAP86 is a GTP-binding protein, an isolated OM fraction was incubated with [α-^{32}P]GTP and subjected to crosslinking with ultraviolet (UV) light in the absence or presence of ATP or GTP. An 86-kD polypeptide was labeled specifically; labeling was diminished in the presence of unlabeled GTP but not in the

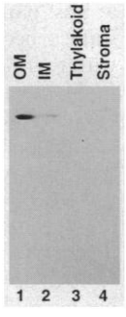

Fig. 5. Distribution of IAP86 in the chloroplast envelope membrane. Isolated chloroplasts (equivalent to 10 mg of chlorophyll) were fractionated into envelope, thylakoid, and stromal components by lysis and flotation into linear sucrose gradients (20 to 38%) as described (*1*). Proteins of gradient fractions containing outer envelope membranes (OM), inner envelope membranes (IM), stromal extract (Stroma), and thylakoid membranes (Thylakoid) were resolved by SDS-PAGE transferred to nitrocellulose and immunoblotted with anti-IAP86 (*9*). Each lane contains 10 μg of protein.

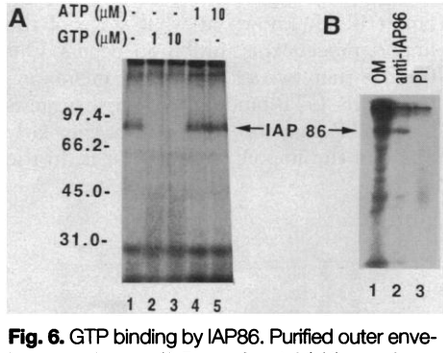

Fig. 6. GTP binding by IAP86. Purified outer envelope membranes (0.5 mg of protein) (*1*) were incubated for 5 min on ice in a total volume of 1 ml containing 50 mM tris-HCl (pH 7.5), 100 μM $MgCl_2$, 50 nM [α-^{32}P]GTP (3000 Ci/mmol) in the absence or presence of ATP or GTP as indicated. After incubation, the samples were irradiated at 366 nm with a hand-held UV lamp (UVP model UVL 56) at a distance of 5 cm for 10 min. The irradiated membranes were diluted with a large volume of buffer excluding [α-^{32}P]GTP and recovered by centrifugation at 200,000*g* for 1 hour at 4°C. (**A**) [α-^{32}P]GTP–crosslinked outer envelope membrane proteins were analyzed by SDS-PAGE and autoradiography. Each lane contains 10 μg of protein. The position of IAP86 is indicated by the arrows. (**B**) [α-^{32}P]GTP–crosslinked outer envelope membranes (10 μg of protein) corresponding to lane 5 of (A) were solubilized in SDS and incubated with affinity-purified anti-IAP86 or immunoglobulin G derived from preimmune serum (PI) in a standard immunoprecipitation reaction (*20*). The immunoprecipitates were analyzed by SDS-PAGE and autoradiography. Lane 1 contains [α-^{32}P]GTP–crosslinked outer envelope membranes equivalent to 50% of that used in each immunoprecipitation reaction. The molecular sizes of standard proteins in kilodaltons are indicated at the left of (A).

A

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M  D  D  G  S  H  V  E  A  A  V  D  H  H  I  D  R  E  I  D  D  L  L  S  D   25
S  K  D  E  S  M  I  F  G  G  S  D  S  A  N  K  Y  L  E  E  L  E  K  Q  I   50
R  D  S  E  S  S  Q  G  D  R  I  D  G  Q  I  V  T  D  S  D  E  E  D  V  S   75
D  E  E  G  G  S  K  E  L  F  D  T  A  T  L  A  A  L  L  K  A  A  S  G  A  100
G  G  E  D  G  G  G  I  T  L  T  A  Q  D  G  S  R  L  F  S  V  E  R  P  A  125
G  L  G  P  S  L  Q  T  G  K  P  A  V  R  S  I  R  P  N  L  F  A  P  S  M  150
S  R  A  G  T  V  V  S  D  T  D  L  S  E  E  D  K  K  K  L  E  K  L  Q  E  175
I  R  I  K  Y  L  R  V  I  Q  R  L  G  F  T  T  E  E  S  I  A  A  Q  V  L  200
Y  R  L  T  L  V  A  G  R  Q  I  G  E  M  F  S  L  D  A  A  K  E  S  A  S  225
R  L  E  A  E  G  R  D  D  F  A  F  S  L  N  I  L  V  L  G  K  T  G  V  G  250
K  S  A  T  I  N  S  I  F  G  E  T  K  T  S  F  S  A  Y  G  P  A  T  T  S  275
V  T  E  I  V  G  M  V  D  G  V  E  I  R  V  F  D  T  P  G  L  K  S  S  A  300
F  E  Q  S  Y  N  R  K  V  L  S  T  V  K  K  L  T  K  K  S  P  P  D  I  V  325
L  Y  V  D  R  L  D  L  Q  T  R  D  M  N  D  L  P  M  L  R  S  V  T  S  A  350
L  G  P  T  I  W  R  N  V  I  V  T  L  T  H  A  A  S  A  P  P  D  E  Q  Q  375
G  S  P  L  S  Y  D  V  F  V  A  Q  R  S  H  I  V  Q  Q  A  I  G  Q  A  V  400
G  D  L  R  L  M  N  P  N  L  M  N  P  V  S  L  V  E  N  H  P  S  C  R  K  425
N  R  D  G  Q  K  V  L  P  N  G  Q  S  W  K  P  L  L  L  L  L  C  Y  S  M  450
K  I  L  S  E  A  T  N  I  S  K  T  Q  E  A  A  D  N  R  R  L  F  G  F  R  475
S  R  A  P  P  L  P  Y  L  L  S  W  L  L  Q  S  R  A  H  P  K  L  P  D  Q  500
A  G  I  D  N  G  D  S  D  I  E  M  A  D  L  S  D  S  D  G  E  E  G  E  D  525
E  Y  D  Q  L  P  P  F  K  P  L  K  K  S  Q  I  A  K  L  N  G  E  Q  R  K  550
A  Y  L  E  E  Y  D  Y  R  V  K  L  L  Q  K  K  Q  W  R  E  E  L  K  R  M  575
R  D  M  K  K  R  G  K  N  G  E  N  D  Y  M  E  E  D  E  E  N  G  S  P  A  600
A  V  P  V  P  L  P  D  M  V  L  P  Q  S  F  D  S  D  N  P  A  Y  R  Y  R  625
F  L  E  P  N  S  Q  L  L  T  R  P  V  L  D  T  H  S  W  D  H  D  C  G  Y  650
D  G  V  N  I  E  N  S  M  A  I  I  N  K  F  P  A  A  V  T  V  Q  V  T  K  675
D  K  Q  D  F  S  I  H  L  D  S  S  V  A  A  K  H  G  E  N  G  S  T  M. A  700
G  F  D  I  Q  N  I  G  K  Q  L  A  Y  I  V  R  G  E  T  K  F  K  N  F  K  725
R  N  K  T  A  A  G  V  S  V  T  F  L  G  E  N  V  S  T  G  V  K  L  E  D  750
Q  I  A  L  G  K  R  L  V  L  V  G  S  T  G  T  V  R  S  Q  N  D  S  A  Y  775
G  A  N  V  E  V  R  L  R  E  A  D  F  P  V  G  Q  D  Q  S  S  L  S  L  S  800
L  V  Q  W  R  G  D  L  A  L  G  A  N  F  Q  S  Q  I  S  L  G  R  S  Y  K  825
M  A  V  R  A  G  L  N  N  K  L  S  G  Q  I  N  V  R  T  S  S  S  D  Q  L  850
Q  I  A  L  I  A  I  L  P  V  A  K  A  I  Y  K  N  F  W  P  G  V  T  E  N  875
Y  S  I  Y                                                                  879
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B

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IAP86  238 SLNILVLGKTGVGKSATINSIFGETKTSFSAYGPATTSVTEIVGMVDGVEIRVFDTPGLKSSAFEQSYNRKVLSTV
           || |||*|| |||||*|*|||*||   |*|** *       *     | * **|||||  ***    |  |* *
IAP34   39 SLTILVMGKGGVGKSSTVNSIIGERVVSISPFQSEGPRPVNVSRSRAGFTLNIIDTPGLIEGGY---INDMALNII

IAP86  314 KKLTKKSPPDIVLYVDRLDLQTRDMNDLPMLRSVTSALGPTIWRNVIVTLTHAASAPPDEQQGSPLSYDVFVAQR
           | *      |**|||||||| *  *** | * ***| **|  ||** ||*|||| *|||   |  |*|| | **|
IAP34  112 KSFLLDKTIDVLLYVDRLDYRVDNLDKL-VAKAITDSFGKGIWNKAIVALTHAQFSPPD---G--LPYDEFFSKR

IAP86  389 SHIVQQAIGQAVGDLRLMNPNLMNPVSLVENHPSCRKNRDGQKVLPNGQSWKPLLLLLCYSMKILSEATNISKTQ
           |  * | * ** * |*          || |*||   | ||   *||||||  | | |*     *  |* * *|  *
IAP34  182 SEALLQVV-RSGASLKKDAQASSIPVVLIENSGRCNKNDSDEKVLPNGIRWIPHLVQTITEV-ALNKSESIFVDK

IAP86  464 EAADNRRLFGFRSRAPPLPYLLSWLLQSRAHPKLPDQAGIDNGDSDIEMADLSD
           *  |       |**  || * |  |* ***   |  *    *  *  |  | *|
IAP34  255 NLIDGPNP-NQRGKLIPLIFALQYLFLAKPIEALIRRDIATETKPAWETRDVGD
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Fig. 4. Deduced amino acid sequence of IAP86 cDNA (*2*) and its comparison to IAP34. (**A**) Deduced amino acid sequence of a cDNA corresponding to IAP86. Residues corresponding to peptide sequence obtained from IAP86 are indicated by the solid underlines. The GenBank accession number for the IAP86 cDNA is L36857. (**B**) Alignment of the deduced amino acid sequences of IAP34 and IAP86. Identical amino acids are indicated by bars. Conserved amino acids are indicated by asterisks. The residues corresponding to conserved motifs of known GTP-binding proteins (*3*) are indicated as bold type.

presence of unlabeled ATP (Fig. 6A). The [α-^{32}P]GTP-labeled 86-kD polypeptide was indeed IAP86 as it was immunoprecipitated with anti-IAP86 (Fig. 6B).

IAP86 appears to be an integral membrane protein of the outer membrane, because it was not extracted from chloroplasts by high concentrations of salt or at pH 11.5 (Fig. 7A). Moreover, IAP86 appears to be at least partially exposed on the chloroplast surface because incubation of chloroplasts with thermolysin caused its degradation to a 50-kD fragment that was still reactive with anti-IAP86 (Fig. 7B). The nature of the membrane anchor of IAP86 is not apparent; standard hydrophilicity analysis of the partial IAP86 sequence did not identify a segment of sufficient length and hydrophobicity to span the membrane as an alpha helix (*8*). An 86-kD outer membrane protein also has been identified by crosslinking of chloroplast-bound pS (*10*) and in a detergent-extracted outer membrane fraction that contains bound pS (*11*). It remains to be seen whether either of these 86-kD proteins is identical to IAP86.

Nonhydrolyzable GTP analogs interfere with protein import into chloroplasts (*12*), but it is not known at what stage of the import process this inhibition occurs. Our finding that two of four outer membrane IAPs are GTP-binding proteins suggests that GTP may be required in the early phase of the import process, that is, in the engagement of the precursor with the import machinery in the outer membrane. To detect a GTP requirement in the early phase of import, we incubated urea-denatured import substrate with chloroplasts in the absence or presence of GTP or GTP analogs. The chloroplasts were then reisolated and bound precursors were detected by SDS-PAGE and autoradiography. There was a small amount of binding in the absence of nucleotides, and there was considerable binding in the presence of 0.1 mM ATP alone, whereas in the presence of only GTP there was little binding (Fig. 8). Moreover, in the presence of both ATP and GTP, binding was only slightly higher than in the presence of ATP alone. These data confirmed the requirement of binding for ATP (*12*, *13*), but they were equivocal with regard to a requirement for GTP. However, a requirement for GTP in the binding process became clearly evident when either guanosine-5′-[thio]-triphosphate (GTP-γ-S) or guanosine-5′-[β,γ-imido]triphosphate (GMP-PNP) (*5*) was added together with 0.1 mM ATP (Fig. 8). Both GTP analogs inhibited binding of precursors to the chloroplast outer membrane. The inhibition by GMP-PNP suggests that the binding reaction requires GTP hydrolysis. The binding reaction may still occur in the absence of added GTP because sufficient amounts of GTP might be contributed to the reaction either by the chloroplasts themselves (IAP34 and IAP86 may contain bound GTP) or with the added ATP (which might be contaminated by small amounts of GTP).

IAP34 and IAP86 may function in regulating presentation of the transit sequence of chloroplast precursors to the outer membrane protein conducting channel through a GTP-binding and hydrolysis cycle (*1*). In this scenario, IAP34 or IAP86 could function by directly interacting with the transit sequence (as import receptors) or by regulating the interactions of other IAPs that bind directly to the precursor. The isolation and characterization of these IAPs provides the opportunity to investigate their functions in chloroplast import.

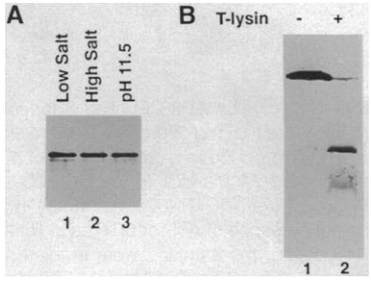

Fig. 7. Characteristics of the membrane association of IAP86. (**A**) Isolated envelope membranes (10 μg of protein) were resuspended in 2 ml of 50 mM Tricine-KOH (pH 7.5), 2 mM EDTA (TE buffer) (Low Salt), TE buffer containing 1 M NaCl (High Salt), or 0.1 M Na_2CO_3, (pH 11.5). Each sample was homogenized by sonication in a water bath for 30 s. The extracted membranes were collected by centrifugation at 200,000*g* for 30 min. The supernatant fraction was discarded and the sedimented membranes were analyzed by SDS-PAGE and immunoblotting with affinity-purified anti-IAP86 (*9*). (**B**) Isolated chloroplasts (equivalent to 100 μg of chlorophyll) were incubated with thermolysin (200 μg/ml) (+ T-lysin) for 30 min on ice (*18*). After protease treatment, the chloroplasts were reisolated, lysed, and separated into membrane and stromal fractions by differential centrifugation (*19*). The membrane fractions were analyzed by SDS-PAGE and immunoblotting with affinity-purified anti-IAP86 (*9*).

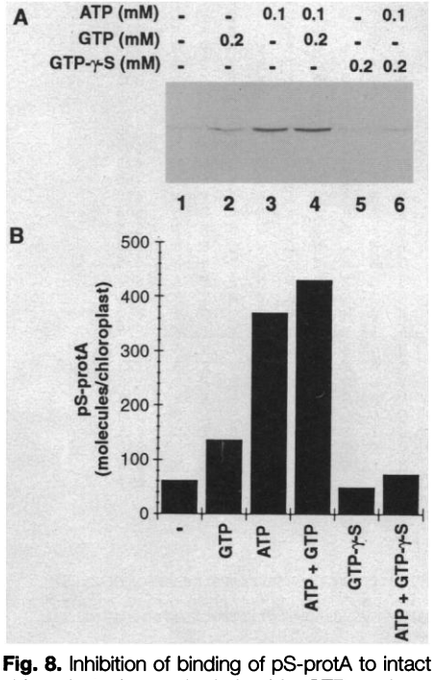

Fig. 8. Inhibition of binding of pS-protA to intact chloroplasts by nonhydrolyzable GTP analogs. ATP-depleted chloroplasts were incubated with urea-denatured [^{35}S]pS-protA in a standard precursor binding assay as described (*21*). The binding reactions contained ATP, GTP, or GTP-γ-S at the concentrations indicated. After the binding reaction, the chloroplasts were reisolated over 40% percoll silica gel and fractionated to yield crude envelope membranes (*19*). (**A**) The envelope membranes were analyzed by SDS-PAGE and fluorography. (**B**) Quantitation of the data presented in (A). Quantitation was performed by direct radioanalytic processing of SDS-PAGE gels on a PhosphorImager (Molecular Dynamics, Inc.).

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2. IAPs from the 2.5-min time point in pS-protA import (*1*) were resolved by SDS-PAGE, transferred to polyvinyledine difluoride membrane, and visualized with 0.1% amido black in 0.1% acetic acid. The protein bands corresponding to 34 kD and 86 kD were excised and cleaved with endopeptidase Lys-C as described (*22*). Several internal peptides for each protein were subjected to NH_2-terminal sequence determination. For IAP34, a 51-bp probe corresponding to the sequence of one of these peptides (residues 159 to 178 of the deduced IAP34 sequence in Fig. 1) was synthesized by the polymerase chain reaction (PCR) (*23*) with oligo(dT)-primed cDNA as a template. A degenerate sense primer [GT(A, T, C, or G)GCI(T or C)TIAC(A, T, C, or G)CA(T or C)GC] corresponding to residues 159 to 164 and a degenerate antisense primer [AA(G or A)AA(T or C)TC(G or A)TC(G or A)TA(A, T, C, or G)GG] corresponding to residues 173 to 178 of the deduced IAP34 sequence (Fig. 1) were used for PCR. The 51-bp probe was used to screen an unamplified lambda gt11 cDNA library from pea (*1*) in a standard hybridization reaction containing 30% (v/v) formamide (*24*). A 1.3-kb IAP34 cDNA was isolated and subcloned into the Not 1 site of pBluescript II (Stratagene, Inc). The construct is referred to as pBS-IAP34. For IAP86, a degenerate oligonucleotide [(T or C)TTIGTIAC(T or C)TGIACIGTIACIGCIGCIGG] corresponding to the antisense strand encoding one of these peptides (residues 599 to 610, Fig. 4) was synthesized and used to screen an unamplified lambda gt11 cDNA library from pea (*1*) by standard aqueous hybridization (*24*). A 2.3-kb cDNA representing a partial IAP86 cDNA was isolated. This cDNA was used to screen a second lambda gt11 cDNA library from pea (Clontech Laboratories). A 1.5-kb cDNA containing the remaining 5′ sequences of the IAP86 cDNA was isolated. The two IAP86 cDNA clones were used to construct a 2.9-kb cDNA that contained the entire coding region of the IAP86 mRNA. The resulting plasmid is referred to as pBS-IAP86. The nucleotide sequences of both strands of the IAP34 and IAP86 cDNAs were determined using Sequenase Version 2.0 (United States Biochemical Co.)
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4. The IAP34 coding region of pBS-IAP34 (nucleotides

98 to 1027) was synthesized by PCR (*23*) with primers that incorporated in-frame Nco 1 and Bam H1 sites at the 5′ and 3′ ends of the DNA, respectively. The DNA was inserted into the Nco 1 and Bam H1 sites of pET21d (Novagen, Inc.). The pET21d-IAP34 plasmid was transformed into *E. coli* BL21 (DE3) and expression was induced with IPTG as recommended by the suppliers. The inclusion body fraction containing IAP34 was purified from *E. coli* by the lysis and TX-100–washing method (*14*). IAP34 constituted approximately 50% of the total protein in the inclusion body fraction as estimated by Coomassie blue staining. The IAP34 was not further purified for the [α-^{32}P]GTP overlay assay.

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9. Purified outer envelope membranes were resolved by SDS-PAGE and the region corresponding to IAP86 was excised and used to raise antibodies in rabbits. Strips of nitrocellulose containing bound IAP86 were prepared from SDS-PAGE resolved IAPs (*1*). Antibodies specific for IAP86 were selected by incubating aliquots of anti-IAP86 with the nitrocellulose strips, and bound antibodies were eluted by treatment with low pH buffer (*25*). Affinity-purified antibodies to IAP86 were used for all experiments. SDS-PAGE and immunoblotting were done with standard methods as described (*26*). Immunoprecipitation was done as described (*20*).
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16. [^{35}S]IAP34 was synthesized in a coupled transcription-translation system containing a wheat germ extract (Promega Corp.) with pBS-IAP34 as the template. The translation mixture was centrifuged for 22 min at 150,000*g* at 4°C to yield a postribosomal supernatant (PRS), and the PRS was gel filtrated on Sephadex G-25 to remove free nucleotides. Import of IAP34 was assayed in a standard import reaction (*17*) containing ATP-depleted chloroplasts (*13*) (equivalent to 50 μg chlorophyll) and 2 μl of gel-filtered PRS for 20 min at 26°C in the dark. The thermolysin treatments were done as described (*18*). For alkaline extractions, chloroplast membrane fractions from the import reaction were resuspended in 2 ml of 0.1 M Na_2CO_3 (pH 11.5) by homogenization in a waterbath sonicator for 30 s. The extracted membranes were collected by centrifugation at 200,000*g* for 30 min. Quantitative analysis indicated that 75 to 80% of the [^{35}S]IAP34 was recovered with the sedimented membrane of each sample after alkaline extraction.
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28 July 1994; accepted 13 October 1994

Prevention of T Cell Anergy by Signaling Through the γ_c Chain of the IL-2 Receptor

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When stimulated through their antigen receptor without requisite costimulation, T cells enter a state of antigen-specific unresponsiveness, termed anergy. In this study, signaling through the common γ chain of the interleukin-2 (IL-2), IL-4, and IL-7 receptors in the presence of antigen was found to be sufficient to prevent the induction of anergy. After culture with IL-2, IL-4, or IL-7, Jak3 kinase was tyrosine-phosphorylated, which correlated with the prevention of anergy. Therefore, a signal through the common γ chain may regulate the decision of T cells to either clonally expand or enter a state of anergy.

Although little is known about the molecular mechanisms responsible for the induction and maintenance of anergy (*1*), the critical costimulatory signal necessary to prevent the induction of anergy is probably mediated through the CD28 molecule on the T cell surface (*2–4*). After T cell receptor (TCR) signaling, ligation of CD28 by either of its natural ligands, B7-1 or B7-2, induces secretion of a number of cytokines, most notably IL-2, which results in T cell clonal proliferation and effector function (*5*). In the absence of CD28-mediated costimulation, addition of exogenous IL-2 during TCR activation can also prevent the induction of anergy (*6–8*). Therefore, the critical signal necessary to prevent anergy might not be mediated directly through CD28, but might be delivered by signaling through the IL-2 receptor (IL-2R). However, T cells from IL-2– and CD28-deficient mice are neither anergic nor severely defective in function (*9*), which suggests that additional signals, potentially delivered through one or more cytokine receptors, might also be capable of preventing the induction of anergy.

To address this issue, human T cell clones specific for the human leukocyte antigen class II molecule HLA-DR7 were stimulated with cell lines that expressed different costimulatory molecules (Fig. 1). All experiments were done with at least two T cell clones. T cells were stimulated with the Epstein-Barr virus (EBV)–transformed B lymphoblastoid cell line LBL-DR7, which is homozygous for HLA-DR7 and expresses B7-1, B7-2, LFA-1, LFA-3, and ICAM-1, with NIH 3T3 fibroblasts transfected with HLA-DR7 alone (t-DR7), or with NIH 3T3 fibroblasts transfected with both HLA-DR7 and the B7-1 costimulatory molecule (t-DR7.B7-1). HLA-DR7–specific alloreactive T cell clones stimulated with either LBL-DR7 or t-DR7.B7-1 cells proliferated in response to secondary rechallenge with LBL-DR7 cells. In contrast, when T cell clones were first cultured with either LBL-DR7 cells in the presence of α fusion protein consisting of the extracellular domain of CTLA4 and an immunoglobulin G (IgG) chain (CTLA4-Ig) (*3*) (to block B7 family–mediated costimulation) or with t-DR7 cells, they were anergized and did not respond when rechallenged with LBL-DR7 cells. Addition of IL-2, IL-4, or IL-7 to the primary culture of T cell clones with either LBL-DR7 cells plus CTLA4-Ig or with t-DR7 cells prevented the induction of anergy (Fig. 1, A and B). In contrast, addition of various concentrations of interferon γ (IFN-γ), tumor necrosis factor α (TNF-α), IL-6, IL-10, or IL-12 to the primary culture of T cell clones with either LBL-DR7 cells plus CTLA4-Ig or with t-DR7 cells alone did not prevent the induction of anergy. However, IFN-γ, IL-6, IL-10, and IL-12 each alone induced proliferation of the T cell clones (*10*), which shows that proliferation

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