Sec-Independent Protein Translocation by the Maize Hcf106 Protein

A. Mark Settles, Ann Yonetani,* Aimee Baron, Daniel R. Bush,† Kenneth Cline, Rob Martienssen‡

The bacterial Sec and signal recognition particle (*ffh*-dependent) protein translocation mechanisms are conserved between prokaryotes and higher plant chloroplasts. A third translocation mechanism in chloroplasts [the proton concentration difference (ΔpH) pathway] was previously thought to be unique. The *hcf106* mutation of maize disrupts the localization of proteins transported through this ΔpH pathway in isolated chloroplasts. The *Hcf106* gene encodes a receptor-like thylakoid membrane protein, which shows homology to open reading frames from all completely sequenced bacterial genomes, which suggests that the ΔpH pathway has been conserved since the endosymbiotic origin of chloroplasts. Thus, the third protein translocation pathway, of which HCF106 is a component, is found in both bacteria and plants.

Protein translocation across lipid bilayers is accomplished by a small number of highly conserved pathways in both eukaryotes and prokaryotes (*1*). Two classes of preprotein recognition complexes have been identified in prokaryotes, and both utilize nucleotide triphosphates as an energy source mediating translocation. SecA-dependent translocation requires adenosine triphosphate (ATP) and is inhibited by NaN_3 (*2*), whereas signal recognition particle (SRP) substrates require guanosine triphosphate (GTP) (*3*). The proton motive force supports each of these pathways in bacteria (*4*). In higher plant chloroplasts, many of the protein components of the photosynthetic electron transport complexes are encoded by nuclear genes. These proteins are synthesized in the cytoplasm and transported into the plastid and, when appropriate, across the thylakoid membrane (*5*). Preproteins are targeted to the thylakoid by translocation machinery that is homologous to the SecA and SRP secretory machinery in *Escherichia coli* (*6*). A third targeting mechanism depends on the proton concentration difference (ΔpH) across the thylakoid membrane and is independent of soluble factors (*7*). This third pathway was previously thought to be unique to higher plant chloroplasts because substrates for this pathway are not found in cyanobacteria, which are the presumptive progenitors of endosymbiotic plant chloroplasts (*8*). Substrate competition studies show that several molecular components regulate the translocation of thylakoid proteins in each pathway (*9*).

In maize, high-chlorophyll fluorescent (*hcf*) mutants are seedling-lethal nonphotosynthetic mutants that possess near normal pigment levels but lack one or more elements of electron transport activity (*10*). *hcf106* mutants are deficient in several thylakoid membrane complexes (photosystem I and II and the cytochrome f/b6 complex) but retain others [light-harvesting complex (LHCP) and the coupling factor ATPase] (*11*). In addition, the *hcf106* mutation results in unusual thylakoid membrane morphology (*12*). On the basis of these observations, it was previously postulated that *hcf106* might encode a protein required for thylakoid protein uptake and assembly (*11*). Mutant *hcf106* seedlings indeed accumulate precursors to proteins normally translocated by the ΔpH pathway, which indicates that they had not been translocated (*13*). Thylakoid proteins transported by the other two pathways are unaffected. The maize mutant *tha1* (thylakoid assembly) has similar pleiotropic effects on thylakoid membrane complexes, but *tha1* accumulates preproteins taken up by the chloroplast SecA (cpSecA) pathway, which suggests that *tha1* may be a component of that pathway (*13*). The specific and complementary nature of the *tha1* and *hcf106* mutations supports the notion that the corresponding genes encode components of each of these two translocation pathways and *tha1* encodes a protein homologous to pea cpSecA (*14*). Here we show that *hcf106* mutant chloroplasts are defective in thylakoid protein uptake and that *Hcf106* encodes a receptor-like chloroplast membrane protein that is closely related to bacterial genes of previously unknown function. These results imply that this third protein translocation pathway evolved first in prokaryotes.

hcf106 mutants accumulate low levels of ΔpH-transported proteins (*13*). The impact of the *hcf106* mutation on the ΔpH pathway suggests that either the HCF106 protein is a component of the targeting machinery or the loss of HCF106 dissipates the requisite ΔpH, thereby inhibiting this pathway. To differentiate between these possibilities, we measured the ΔpH gradient maintained by thylakoid membrane vesicles from *hcf106*, *tha1* (cpSecA), and wild-type seedlings (*15*). Methylamine was used as a reporter of the transmembrane proton concentration difference. Energized wild-type membrane vesicles accumulate 16.6 times more methylamine than uncoupled vesicles that were de-energized with nigericin and valinomycin ($n = 10$). This accumulation ratio is consistent with a ΔpH of at least one pH unit. Vesicles made from *hcf106* thylakoid membranes accumulated 4.3 times more methylamine ($n = 6$) and *tha1* vesicles accumulated 4.7 times more methylamine than did uncoupled controls ($n = 4$). These data show that both mutations diminish the ability of these nonphotosynthetic thylakoids to generate and maintain a transmembrane pH difference. Nevertheless, the *tha1* mutants accumulate wild-type levels of ΔpH-targeted proteins (*13*), thus demonstrating that this level of energization is sufficient to drive ΔpH-dependent translocation. We conclude from these data that the lower ΔpH observed in *hcf106* mutants is not the cause of the translocation deficiency.

To confirm that the *hcf106* mutant chloroplasts are deficient in protein translocation, purified chloroplasts from wild-type seedlings and seedlings homozygous for the null *hcf106* allele (*16*) were used for in vitro targeting and processing assays. We used the 17-kD and 33-kD polypeptides of the oxygen-evolving complex (OE17 and OE33) as substrates for the ΔpH- and SecA-dependent pathways, respectively. In mutant and wild-type chloroplasts, radiolabeled precursors for the ΔpH (pOE17) and SecA (pOE33) pathways were successfully imported into the stroma and processed to intermediate forms [iOE17 and iOE33, respectively (Fig. 1)]. Translocation into the lumen of the thylakoid results in a second processing step (*6*, *7*) that removes an NH_2-terminal lumen targeting domain and produces mature forms of the proteins (OE17 and OE33, respectively). Wild-type maize chloroplasts target and process pOE17 and pOE33 in the same way as pea chloroplasts,

A. M. Settles, A. Yonetani, D. R. Bush, R. Martienssen, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724, USA.
A. Baron, Department of Psychiatry and Behavioral Sciences, Emory University School of Medicine, Atlanta, GA 30322, USA.
K. Cline, Department of Horticultural Sciences, University of Florida, Gainesville, FL 32611, USA.
*Present address: Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143, USA.
†Permanent address: Photosynthesis Research Unit, U.S. Department of Agriculture—Agricultural Research Service, and Department of Plant Biology, University of Illinois, Urbana, IL 61801, USA.
‡To whom correspondence should be addressed.

with pOE17 being sensitive to ionophores and pOE33 sensitive to azide (Fig. 1, lanes 2 through 4). Dissipation of the ΔpH with nigericin and valinomycin results in the specific accumulation of iOE17 (lane 4), whereas inhibition of the SecA pathway with azide results in accumulation of iOE33 (lane 3). The *hcf106* mutant chloroplasts transport iOE33 correctly but fail to transport iOE17. Mutant chloroplasts accumulate iOE17 but only process small quantities of mature protein (lane 5). The limited accumulation of mature OE17 can be inhibited by ionophores (lane 7).

If HCF106 is a component of the targeting machinery, biochemical studies predict that it should be a thylakoid membrane protein (*17*). The *hcf106-mum1* allele was cloned by exploitation of a *Mu1* transposable element inserted in the promoter of the *Hcf106* gene (*18, 19*). Full-length cDNA clones were obtained (*20*) and found to encode a peptide containing 243 amino acids (Fig. 2), with a predicted membrane-spanning domain from amino acid 68 to 89. The sequence NH_2-terminal to this domain has a net positive charge, whereas the COOH-terminal domain has a net negative charge and a predicted isoelectric point (pI) of 4.2.

Antibodies directed against recombinant fusion protein detect a 30-kD protein in wild-type chloroplasts that is absent from mutant chloroplasts [Fig. 3A, lanes 1 and 2 (*21*)]. HCF106 fractionates with thylakoid membranes after hypotonic lysis of intact chloroplasts (Fig. 3A, lanes 3 and 4). Membrane-bound HCF106 is partially extractable with 4 M and 6 M urea but not with alkali or bromide extractions (Fig. 3B). In contrast, the peripheral membrane protein OE33 is extractable with all of these chaotropes. HCF106 is sensitive to thermolysin digestion (Fig. 3C). In contrast, the lumenal OE33 protein was protected in this assay. Thus, the bulk of the HCF106 protein is exposed to the stromal compartment. These results are consistent with HCF106 having a direct role in the ΔpH pathway, because soluble factors are not required and substrate uptake is also sensitive to mild protease treatment (*7*). The Ffh receptor FtsY and the membrane-bound form of SecA in bacteria are also oriented in this way (*22*).

Database searches with the HCF106 sequence revealed similarity to expressed sequence tags (ESTs) from *Arabidopsis thaliana* and rice, as well as to a homologous class of hypothetical bacterial proteins identified by genome sequence analysis of the bacteria *Escherichia coli*, *Mycobacterium leprae*, *Mycobacterium tuberculosis*, *Azotobacter chroococcum*, *Synechocystis* PCC6803, *Neisseria gonorrhoeae*, *Bacillus subtilis*, and *Haemophilus influenzae* (*23*). In each case, the homology was restricted to the membrane-spanning domain and approximately 40 residues of the adjacent COOH-terminal domain, which is predicted to form an amphipathic alpha helix (Fig. 4). The remainder of the protein sequences are unrelated at the amino acid level, although six out of the eight bacterial open reading frames (ORFs) have similar negative charge in the COOH-terminal domain (mean pI 4.9). The conserved membrane-spanning domains of the bacterial proteins are at the NH_2-terminus, but HCF106 and the *Arabidopsis* EST have an additional NH_2-terminal extension. Both maize and *Arabidopsis* NH_2-terminal sequences are unrelated but

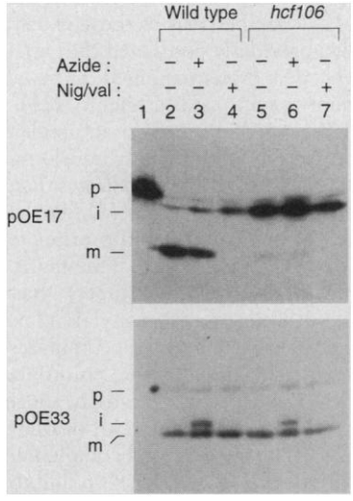

Fig. 1. In vitro import assays comparing *hcf106-mum3* mutant and wild-type chloroplasts, using ΔpH- and cpSecA-dependent precursors (*31*). Radiolabeled precursors (p) of OE17 and OE33 (lane 1) were assayed for import and processing in *hcf106-mum3* mutant and wild-type chloroplasts. Mutant chloroplasts process OE17 into a mature (m) form but to a much lower extent than wild-type chloroplasts (compare lanes 2 and 5). In addition, mutants accumulate a large amount of iOE17 (i), which indicates that the precursor is targeted to chloroplasts but is not translocated across the thylakoid efficiently. Residual translocation is still dependent on a pH gradient, because ionophores completely block processing of mature OE17 (lanes 4 and 7). The mutants show wild-type accumulation of mature OE33 (compare lanes 2 and 5), which indicates that translocation via the Sec pathway is normal. This translocation is sensitive to azide (lanes 3 and 6), and the degree of inhibition is equivalent to that seen in pea chloroplasts (*5*). Nig/val, nigericin/valinomycin.

Fig. 2. Hcf106 predicted amino acid sequence (*32*). Hcf106 was cloned as described (*20*). The Hcf106 cDNA sequence is available through GenBank (accession number AF027808). The predicted membrane domain is underlined.

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MTPTANLLLP APPFVPISDV RRLQLPPRVR HQPRPCWKGV EWGSIQTRMV      50
SSFVAVGSRT RRRNVICASL FGVGAPEALV IGVVALLVFG PKGLAEVARN     100
LGKTLRAFQP TIRELQDVSR EFRSTLEREI GIDEVSQSTN YRPTTMNNNQ     150
QPAADPNVKP EPAPYTSEEL MKVTEEQIAA SAAAAWNPQQ PATSQQQEEA     200
PTTPRSEDAP TSGGSDGPAA PARAVSDSDP NQVNKSQKAE GER            243
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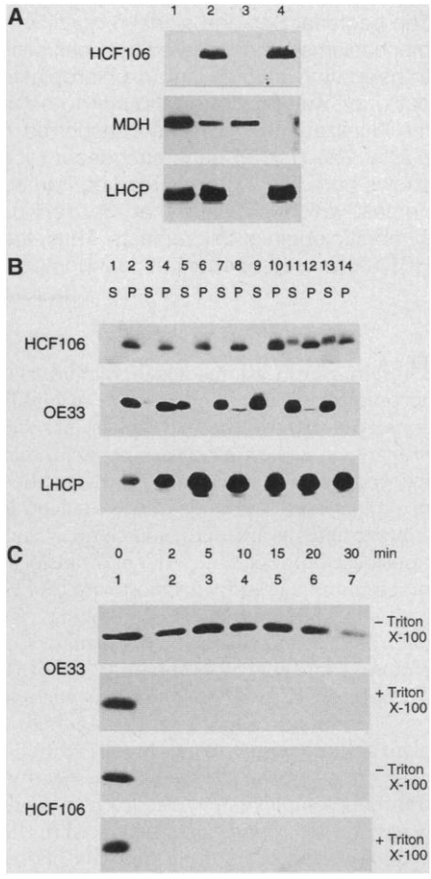

Fig. 3. HCF106 is a thylakoid membrane protein (*33*). (**A**) Purified whole chloroplasts from *hcf106* mutants and wild-type siblings (lanes 1 and 2) and the supernatant and pellet of hypotonically lysed chloroplasts (lanes 3 and 4) are shown. The proteins were separated by SDS-PAGE, blotted, and probed with antibodies against HCF106, the stromal protein malate dehydrogenase (MDH), and a membrane protein that contains multiple transmembrane domains (LHCP). (**B**) Purified thylakoid membranes were extracted with sonication and a variety of chaotropic agents (*34*). Protein gel blots were probed with antibodies against HCF106, a peripheral membrane protein (OE33), and LHCP. Lanes 1, 3, 5, 7, 9, 11, and 13, extracted supernatants (S); lanes 2, 4, 6, 8, 10, 12, and 14, thylakoid membrane–associated proteins (P). The membranes were untreated (lanes 1 and 2); sonicated (lanes 3 and 4); or extracted with 0.2 M Na_2CO_3 (lanes 5 and 6), 0.1 M NaOH (lanes 7 and 8), 2 M NaBr (lanes 9 and 10), 4 M urea (lanes 11 and 12), or 6 M urea (lanes 13 and 14). (**C**) HCF106 faces the stroma. Thylakoids were treated with thermolysin as described (*35*). Samples were removed before digestion (lane 1) or after 2 min (lane 2), 5 min (lane 3), 10 min (lane 4), 15 min (lane 5), 20 min (lane 6), or 30 min (lane 7).

resemble chloroplast-targeting domains. This would account for the difference in migration of the in vitro translated and mature HCF106 protein on SDS gels and is consistent with the localization of HCF106 in chloroplasts (*21*).

The *E. coli* homolog of HCF106 is predicted to be within an operon encoding several other hypothetical proteins. Two of these ORFs were also found in *A. chroococcum*, *N. gonorrhoeae*, *B. subtilis*, *H. influenzae*, *M. leprae*, and *M. tuberculosis* by BLAST searches (*23*). The gene linkage and order are the same in each organism. Although the functions of these genes are not known in most of these bacteria, the *Azotobacter* operon is required for H_2-dependent respiration (*24*). H_2-dependent respiration uses secreted hydrogenases to recycle H_2 produced by nitrogen fixation and fermentation (*25*). The β subunits of these bacterial hydrogenases have signal sequences that contain an essential twin arginine motif (*26*). The same motif is also essential for efficient translocation of ΔpH-targeted proteins across the thylakoid membrane in chloroplasts (*17, 27*). Berks (*28*) has proposed that this motif may define a common secretory pathway for proteins binding complex redox factors in bacteria and for a subset of chloroplast thylakoid preproteins. In contrast, Robinson and Klösgen (*8*) have proposed that the ΔpH pathway in chloroplasts could be a relatively late evolutionary development because none of the known photosynthetic substrates are found in cyanobacteria. Membrane-bound hydrogenase activity in *A. chroococcum* is mislocalized to soluble fractions when the HCF106 homolog is disrupted (*24*). The discovery that homologous genes regulate each pathway leads us to propose that the chloroplast ΔpH pathway and the bacterial redox protein secretory pathway are closely related. In plastids, this same pathway has apparently been recruited to deliver photosystem proteins that evolved after the divergence of chloroplasts and cyanobacteria.

Why do bacteria and chloroplasts have multiple secretion pathways? Possibly these pathways are needed to avoid catastrophic feedback when demands on protein secretion are high. If there were only one set of targeting machinery, then the machinery itself would need to be incorporated into the membrane in competition with its own substrates, leading to fewer export sites. A similar argument can be made for substrates having a choice between alternate pathways, for which there is some evidence in *Chlamydomonas* and pea chloroplasts and in *E. coli* (*29*). Alternatively, multiple pathways may simply reflect the diversity of membrane and secreted proteins that are translocated in prokaryotes. Complex redox factors have special folding requirements for protein translocation, and the dedication of the *Hcf106* pathway in bacteria to membrane assembly of these proteins may be related to these requirements (*28*).

Note added in proof: There is a second *E. coli* open reading frame, ybeC, that should be grouped with this homologous class of genes. ybeC shows better similarity to the gene family after the recent corrections to the *E. coli* genome sequence (*30*).

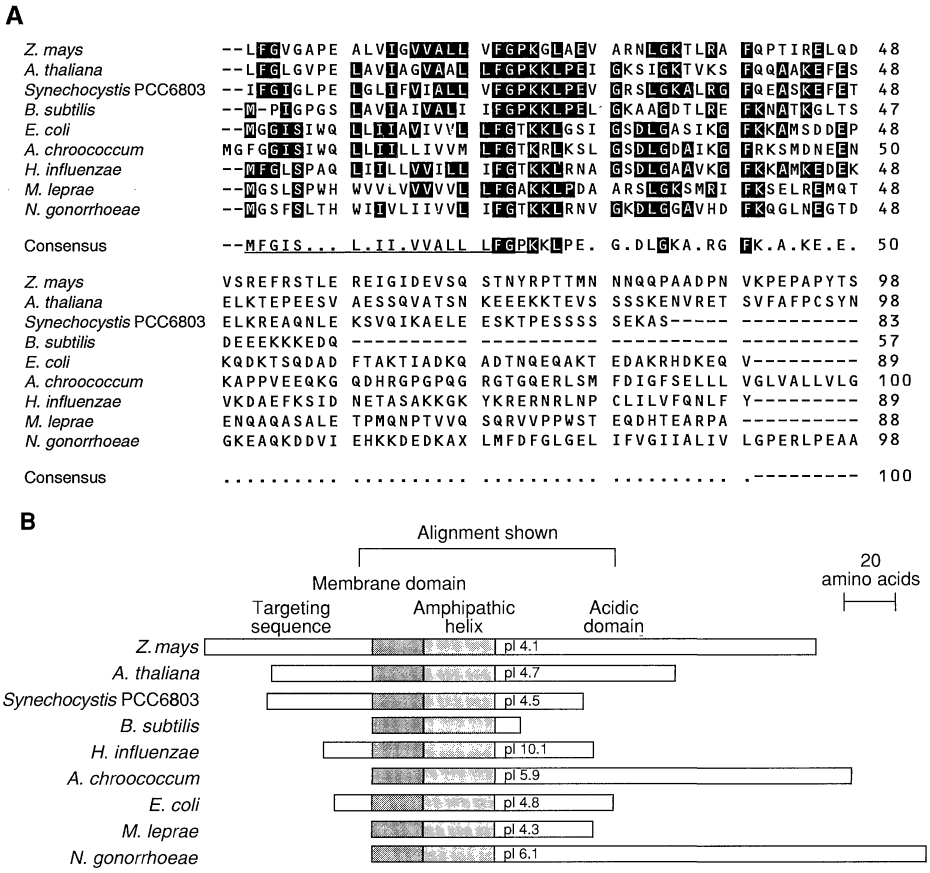

Fig. 4. HCF106 has homology to several ORFs from bacterial genomes (*31*). (**A**) Alignment of HCF106 with predicted peptides from a variety of prokaryotic species as well as an *A. thaliana* EST. Highlighted residues show identity to the consensus sequence. Residues that are identical in all of the peptides are highlighted in the consensus, and the predicted membrane domain is underlined. The predicted amphipathic helices are conserved. (**B**) Schematic of the alignment. The COOH-terminal domains of all of these proteins have acidic pIs, with the exception of the *H. influenzae* protein. The *B. subtilis* sequence appears to be truncated. The maize and *A. thaliana* sequences have predicted chloroplast-targeting domains. The *Synechocystis* sp. and *H. influenzae* NH_2-terminal domains do not have predicted functions.

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15. Thylakoids were purified from seedlings 10 to 14 days old. Approximately seven seedlings were pooled for each methylamine assay. Seedling leaf tissue was cut and then homogenized in grinding buffer as in (9). The homogenate was filtered through Miracloth, and chloroplasts were pelleted. The pellet was resuspended in lysis buffer [10 mM Hepes-KOH (pH 8.0) and 5 mM $MgCl_2$] and repelleted. Thylakoids were resuspended to chlorophyll (0.8 mg/ml) for the wild type and to an equivalent volume based on fresh mass for mutants. Mutant thylakoids were confirmed for phenotype by protein gel blots with antibodies against HCF106 (for *hcf106* mutants) or OE23 and PC (for *tha1* mutants). Methylamine assays were conducted in lysis buffer with 3 mM Mg-ATP, 5 mM dithiothreitol, $^{14}CH_3NH_2$ (1.67 μCi/ml), and thylakoids (chlorophyll, 0.2 mg/ml). Assays were conducted at 25°C in the presence of light. Thylakoids were collected on a 0.45-μm Millipore filter at 0, 2, and 5 min (~60 μg of chlorophyll per time point) and washed with lysis buffer. Methylamine accumulation was measured by scintillation spectroscopy. Unenergized thylakoids, generated by the addition of nigericin (0.75 μM final concentration) and valinomycin (1.5 μM final concentration), were used as a measure of background accumulation [H. Rottenberg, *Methods Enzymol.* **55**, 547 (1979)]. Average accumulations at 2 min in counts per minute ± SEM were as follows: wild-type, 9559 ± 1398; wild type with ionophores, 623 ± 106; *hcf106*, 2900 ± 961; *hcf106* with ionophores, 697 ± 260; *tha1*, 2528 ± 259; and *tha1* with ionophores, 616 ± 177.
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20. 3′ rapid amplification of PCR ends (RACE) polymerase chain reaction products were generated from seedling RNA with the primer 5′-ATCTCCAACCTGTGAACGGTGAAC-3′. The products were cloned and sequenced and found to correspond to a single 1.1-kb transcript. A genomic clone spanning the transcription unit was obtained by screening of a library prepared from the inbred maize line B73 (the same line was used to make the cDNA). The transcription unit spans 7 kb, comprising five exons and four introns. Northern (RNA) blotting revealed a mature transcript of 1.1 kb (*18*) and a larger transcript of about 7 kb that was reduced in relative abundance in polyadenylated rather than total RNA. The larger transcript likely corresponds to unprocessed precursor RNA. Both transcripts are increased in abundance in light-grown, rather than etiolated, seedlings and are present in much greater amounts in leaves than in roots [R. Martienssen, unpublished data; (*18*)].
21. The HCF106 polyclonal antibodies were raised against a full-length TrpE::HCF106 fusion protein by standard methods. The antibodies were affinity-purified with the use of a MalE::HCF106 protein. Both unpurified and purified sera immunoprecipitate a 35-kD protein in wheat germ extract translations programmed with in vitro–transcribed Hcf106 cDNA. Protein immunoblot analysis detects a 30-kD HCF106 protein from maize leaf extracts. The 30-kD protein copurifies with chloroplasts and is protected by intact chloroplasts from thermolysin digestion. We have further confirmed that HCF106 is a chloroplast protein through import of in vitro–translated HCF106 into purified pea and maize chloroplasts (K. Cline and A. M. Settles, unpublished data). Both precursor and mature proteins migrate on SDS gels much more slowly than predicted by the deduced full-length protein sequence (27 kD).
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25. At least three gene clusters have been identified that are important for hydrogenase activity in *E. coli*. These loci encode subunits of the hydrogenases, proteins required for Ni and Fe metabolism (hydrogenases are metalloproteins), and regulatory genes [J. H. Lee, P. Patel, P. Sankar, K. T. Shanmugam, *J. Bacteriol.* **162**, 344 (1985); S. Lutz *et al.*, *Mol. Microbiol.* **5**, 123 (1991)].
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31. Import assays were conducted as in (*9*). Chloroplasts were purified from maize seedlings 10 to 12 days old, grown during 16-hour days at 26°C and 8-hour nights at 12°C. *hcf106* mutants were selected by their pale green phenotype, the aerial portions of the seedlings were harvested, and chloroplasts were purified with continuous Percoll gradients. Mutant chloroplasts were confirmed by protein immunoblot. Equal numbers of whole wild-type and *hcf106-mum3* mutant chloroplasts were incubated with radiolabeled maize precursors for OE33 (*13*) and OE17 [W. F. Ettinger and S. M. Theg, *Plant Physiol.* **99**, 791 (1992)] for 10 min at 25°C with light and Mg-ATP (5 mM). Chloroplasts were incubated with or without nigericin (0.75 μM), valinomycin (1.5 μM), and NaN_3 (10 mM) on ice 10 min before the addition of diluted precursor. The chloroplasts were post-treated with thermolysin (0.1 mg/ml) on ice for 40 min and repurified on a 35% Percoll cushion with 5 mM EDTA. The radiolabeled proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and visualized with fluorography.
32. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
33. Chloroplasts were prepared as described (*30*). Pelleted chloroplasts were hypotonically lysed by resuspension in 10 mM Hepes-KOH (pH 8.0) and 5 mM $MgCl_2$ and incubation on ice for 10 min. Thylakoids were purified by slow-speed centrifugation and washed once in lysis buffer. Supernatant proteins were concentrated by trichloracetic acid (TCA) precipitation. Membrane and supernatant pellets were resuspended in equal volumes of loading buffer, separated by SDS-PAGE, and blotted to nitrocellulose. The blot was incubated and stripped, and reprobed with several antibodies with the use of enhanced chemiluminescence.
34. Thylakoid membranes were prepared as described (*15*). Thylakoids were pelleted and resuspended (in 50 μg of chlorophyll per milliliter) in each chaotropic solution with 1 mM phenylmethylsulfonyl fluoride. The membranes were then incubated on ice for 30 min. Sonicated membranes were treated in a bath sonicator four times for 10 s each time. The extracted thylakoid membranes were separated by differential centrifugation. Supernatants were concentrated by TCA precipitation. The samples were separated by SDS-PAGE, and protein gels were blotted with antibodies against HCF106, OE33 (*13*), and LHCP.
35. Purified chloroplasts were lysed, and thylakoids were separated by low-speed centrifugation. The thylakoids were then resuspended in lysis buffer or 1% Triton X-100 (0.3 mg of chlorophyll per milliliter) and treated with thermolysin (0.1 mg/ml final concentration) for 30 min on ice. Protease digestion was stopped by addition of EGTA (50 mM final concentration), the samples were separated by SDS-PAGE, and protein gels were blotted with antibodies against HCF106 and OE33 (*13*).
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CD4-Independent Binding of SIV gp120 to Rhesus CCR5

Kathleen A. Martin, Richard Wyatt, Michael Farzan, Hyeryun Choe, Luisa Marcon, Elizabeth Desjardins, James Robinson, Joseph Sodroski,* Craig Gerard,* Norma P. Gerard*

CCR5 and CD4 are coreceptors for immunodeficiency virus entry into target cells. The gp120 envelope glycoprotein from human immunodeficiency virus strain HIV-1(YU2) bound human CCR5 ($CCR5_{hu}$) or rhesus macaque CCR5 ($CCR5_{rh}$) only in the presence of CD4. The gp120 from simian immunodeficiency virus strain SIVmac239 bound $CCR5_{rh}$ without CD4, but $CCR5_{hu}$ remained CD4-dependent. The CD4-independent binding of SIVmac239 gp120 depended on a single amino acid, Asp^{13}, in the $CCR5_{rh}$ amino-terminus. Thus, CCR5-binding moieties on the immunodeficiency virus envelope glycoprotein can be generated by interaction with CD4 or by direct interaction with the CCR5 amino-terminus. These results may have implications for the evolution of receptor use among lentiviruses as well as utility in the development of effective intervention.

HIV-1 entry is mediated by the viral envelope glycoprotein complex consisting of the exterior glycoprotein, gp120, and the transmembrane glycoprotein, gp41 (*1*). CD4 acts as the primary target cell receptor, binding to the gp120 glycoprotein (*2*). The gp120-CD4 complex requires an additional target cell coreceptor of the seven-transmembrane-spanning G protein–coupled receptor family (3–5). Formation of this complex is believed to induce a conformational change exposing the gp41 ectodomain, which mediates fusion of the viral and target cell membranes (6).