lanes 1 and 2) and with two antibody positive healthy homosexual males (Fig. 3, lanes 7 and 8; these are the same sera as in Fig. 2B, lanes 5 and 6). No proteins of related sizes were detected in sera from antibody-negative healthy homosexual males (Fig. 3, lanes 9 and 10) or with sera from laboratory workers (Fig. 3, lanes 11 and 12).

None of the human serum samples that we tested contained antibodies to p24 or other *gag*-related HTLV-III antigens without also containing readily detectable antibodies to gp120 and gp160. Conversely, when examined in the same RIP/SDS-PAGE preparation, less than half of the AIDS patients that had antibodies to gp120 also revealed detectable antibodies to p24 (Table 1). Although this ratio was higher for ARC patients and antibody-positive healthy homosexual males, only about three-fourths of the individuals in these categories that had readily detectable antibodies to gp120 and gp160 also had readily detectable antibodies to p24. Other, more sensitive assay systems could conceivably reveal a higher proportion of individuals with antibodies to p24. However, for assays based on equimolar amounts of undenatured antigen, the gp120/160 complex, which has been mapped to the *env* gene of HTLV-III, would presumably be the antigen of choice. This might be particularly important for patients involved in differential diagnoses for AIDS, since such individuals had lower amounts of antibody to p24.

We recently reported that asymptomatic hemophiliacs that tested positive by MIF also regularly had antibodies to gp120/160 (*11*). At that time we had not established that these proteins were encoded by HTLV-III. However, as we now find with AIDS patients, ARC patients, and healthy homosexual males, a significant fraction of the asymptomatic hemophiliacs had antibodies to gp120/gp160 in the absence of antibodies to p24 that could be detected with the same procedure. We also recently reported that four healthy individuals from "high risk" backgrounds carried infectious HTLV-III but had no antibodies detectable by the MIF, ELISA (enzyme-linked immunosorbent assay), or Western blotting procedures (*12*). Three of these individuals were also tested by RIP/SDS-PAGE and were also found to be negative by this procedure.

These results suggest that RIP/SDS-PAGE should be considered as a possible confirmatory test for establishing reactivity of selected serum samples that give uninterpretable results by procedures such as ELISA and Western blotting, especially potential "false positives." Although the ELISA and Western blotting procedures are clearly more adaptable for use in broad-scale screening, to our knowledge the RIP/SDS-PAGE described here is the only assay currently available that regularly reveals a reactivity with a spectrum of the major HTLV-encoded proteins of both the *gag* and *env* genes, virtually eliminating the possibility that false positives would be a problem with this procedure.

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In Vivo Function and Membrane Binding Properties Are Correlated for *Escherichia coli* LamB Signal Peptides

Abstract. *Wild-type and pseudorevertant signal peptides of the* lamB *gene product of* Escherichia coli *interact with lipid systems whereas a nonfunctional deletion mutant signal peptide does not. This conclusion is based on (i) interaction of synthetic signal peptides with a lipid monolayer-water surface, (ii) conformational changes induced by presence of lipid vesicles in an aqueous solution of signal peptide, and (iii) capacities of the peptides to promote vesicle aggregation. Analysis of the signal sequences and previous conformational studies suggest that these lipid interaction properties may be attributable to the tendency of the functional signal peptides to adopt α-helical conformations. Although the possibility of direct interaction between the signal peptide and membrane lipids during protein secretion is controversial, the results suggest that conformationally related amphiphilicity and consequent membrane affinity of signal sequences are important for function in vivo.*

Secreted proteins are generally synthesized as precursors with an amino-terminal extension termed a signal sequence (*1–4*). The signal sequence is required for protein export, but the mechanism of its action is unclear. Biochemical and genetic evidence suggests that the signal sequence plays a number of roles, interacting at various times in the secretion process with one or more species including the ribosome, soluble and membrane-bound proteins, lipid head groups, and hydrocarbon chains. Although signal sequences exhibit little sequence homology, they may be interchanged with retention of function, even among widely different proteins and organisms (*5–9*). Several investigators (*10–12*) have proposed a "conformational homology" as the basis for this interchangeability; that is, signal sequences all take on the same general conformation (or conformations) during the secretion process. Inspection of known signal sequences (*13*) supports this idea. Signal sequences are similar in length, generally having 15 to 30 amino acid residues. One to three charged residues, usually basic, are located near the NH_2-terminus, and are adjacent to a stretch of hydrophobic residues. This hydrophobic core region is usually about 9 to 18 residues long, and has been predicted (*14*) and observed in synthetic signal peptides (*15, 16*) to adopt an α-helical conformation, a structural feature often found in membrane-binding proteins. Biochemical and biophysical studies have, in fact, shown

that signal sequences can interact with membranes (*17, 18*). However, there has been little direct evidence to show that such interactions are significant in protein translocation.

Emr and Silhavy's genetic analysis (*19*) of the signal sequence of the lambda receptor (an outer membrane protein in *E. coli* coded for by the *lamB* gene) provides an excellent system for investigating correlations of signal sequence properties with biological function. A mutant deficient in translocation of the lambda receptor across the bacterial inner membrane was found to have four amino acids deleted from the central portion of the signal sequence. This deletion brings proline and glycine, both of which destabilize the propagation of α-helical structure (*20*), into proximity (Fig. 1). Pseudorevertants (double mutants with restored export competence) were subsequently isolated in which the proline or glycine was replaced by leucine or cysteine, respectively (designated Pro>Leu and Gly>Cys). These changes were predicted (*19, 21*) to restore α-helix–forming tendency, suggesting a conformational requirement for activity. Previous work in one of our laboratories (*15*) tested these conformational hypotheses. Peptides that included the hydrophobic portions of these LamB signal sequences (Fig. 1) were synthesized, and their conformations were analyzed by circular dichroism (CD). In helix-promoting environments (trifluoroethanol or sodium dodecyl sulfate micelles), wild-type and pseudorevertant peptides had CD spectra characteristic of a partially helical conformation, whereas the deletion mutant did not, an indication that the intrinsic properties of the isolated signal peptide in the absence of the mature protein are correlated to their activity in vivo.

We now extend our previous studies of the conformational properties of genetically defined signal peptides to include conformation-dependent interactions with phospholipid-water interfaces. Indeed, we find that the ability of these peptides to interact with phospholipids correlates with their in vivo function.

The interaction of the previously studied (*15*) synthetic LamB signal peptide fragments with a planar lipid surface was studied by injecting the peptides beneath a phospholipid monolayer (to simulate one side of a bilayer) and measuring the consequent change in surface pressure while holding surface area constant. To minimize the possibility of incomplete mixing in the monolayer, that is, the formation of patches of pure peptide, we spread the lipids to a surface pressure

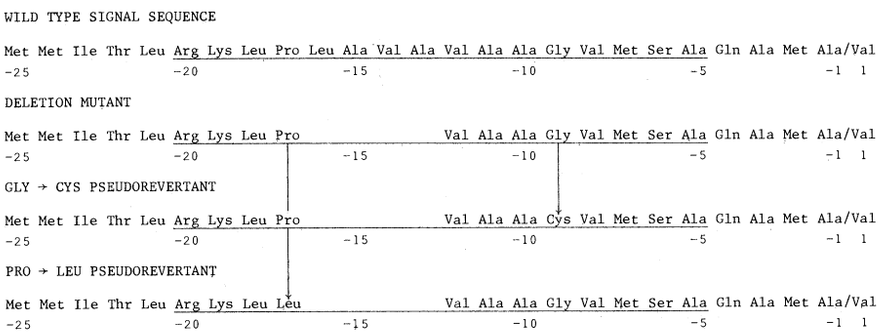

Fig. 1. The amino acid sequences of LamB wild-type and mutant signal peptides. A slash indicates the site of cleavage by signal peptidase. The portions of the signal peptides that we have synthesized are underlined. Details of synthesis, purification, and characterization are reported in (*15*).

greater than the peptide's equilibrium spreading pressure at an air-water interface (*22*). Thus an increase in surface pressure indicated that the peptide was interacting with the lipid components of the monolayer. In each experiment, a portion of peptide was added to the subphase (5 m*M* tris-HCl, *p*H 7.3) of a monolayer consisting of egg phosphatidylethanolamine (PE) and egg phosphatidylglycerol (PG) (65 to 35, mole ratio) (*23*) at an initial pressure of 20 mN/meter and constant area, and the increase in surface pressure (which stabilizes to a plateau value after 30 to 90 minutes) was observed. The increase in surface pressure was dependent on peptide concentration (Fig. 2). The maximum increases in surface pressure for the wild-type and Pro>Leu pseudorevertant peptides (*24*) were approximately 10 and 8 mN/meter, respectively. The peptide concentrations at half-maximal surface pressure increase, approximating the dissociation constant for monolayer binding (*25*), for the wild-type and Pro>Leu pseudorevertant peptides were 0.3 and 0.1 μ*M*, respectively. In contrast, the maximal surface pressure increase for the deletion mutant peptide was only 1.5 mN/meter, and its concentration at half-maximal surface pressure was 0.8 μ*M*.

Similar experiments in the presence of 0.15*M* NaCl showed smaller surface pressure increases, an indication of interaction between the positively charged NH_2-terminus and side chains of the signal peptides and the negatively charged head groups of phosphatidylglycerol. The maximal increases in surface pressure at saturating peptide concentrations were 5 and 3 mN/meter for wild-type and Pro>Leu pseudorevertant, respectively, although the midpoints were not greatly affected. The deletion mutant did not increase the surface pressure of the lipid monolayer, even when its concentration was 3 μ*M*.

The ability of one surface-active molecule to "penetrate" a monolayer of another is dependent on the initial monolayer surface pressure (*26*). The lipid monolayer pressure above which the penetrating molecule no longer inserts (thus causing a pressure change) is called the critical pressure of insertion, and is obtained by measuring the dependence of the surface pressure increase on initial monolayer surface pressure and extrapolating to a pressure increase of zero. Critical pressures of insertion of the signal peptides (Fig. 3) were determined at saturating peptide concentrations (1 μ*M* for the wild-type and Pro>Leu pseudorevertant peptides, and 2 μ*M* for the deletion mutant peptide, in 0.15*M* NaCl, 5 m*M* tris, *p*H 7.3). The critical pressures of insertion of the wild-type and Pro>Leu pseudorevertant peptides are 28.1 and 26.3 mN/meter, respectively, which are similar to those of membrane-associated proteins such as rat apolipoprotein AI (*27*) and colicin A (*28*). In contrast, the critical pressure of insertion for the deletion mutant is 16 mN/meter, which is similar to that of bovine serum albumin (*27*), a soluble protein. These values yield a rough measurement of the point at which the forces favoring transfer of the peptide from the subphase to the monolayer are balanced by the compressional forces opposing the addition of material to the surface. The critical pressures, when multiplied by the cross-sectional area per peptide molecule (*29*), provide estimates of the energies of insertion of the peptides into the monolayer. Insertion energies for the functional signal peptides are nearly double those for the nonfunctional signal peptide. The increased insertion energies of the functional versus nonfunctional signal peptides (three to nine times thermal energy) suggest that signal peptide-lipid interactions contribute significantly to lowering energy barriers to protein translocation.

Circular dichroism spectra of the peptides (data not shown) were obtained in the presence and absence of PE-PG (65:35) vesicles (preformed at 3 m*M* total lipids in 0.15*M* NaCl, 10 m*M* tris, *p*H 8.0; peptide to lipid ratios of 1:100). Under these conditions, there is little aggregation of the vesicles so that light-scattering artifacts are minimal (*30*). The CD spectrum of the deletion mutant is identical to its spectrum in aqueous solution in the absence of vesicles. In contrast, both the wild-type and Pro>Leu pseudorevertant peptides gave spectra distinctly different from those in aqueous solutions and similar to those previously observed in detergent micelles (*15*), an indication that they form complexes of phospholipid and peptide with altered conformational properties. At ratios of peptide to lipid greater than 1:50, both the wild type and Pro>Leu pseudorevertant caused vesicle aggregation, as judged by increases in light scattering and changes in elution volume on size exclusion chromatography. The deletion mutant did not cause vesicle aggregation, even at ratios of peptide to lipid of up to 1:10.

Our results show that the ability of these signal peptides to interact with phospholipid-water interfaces correlates with their in vivo activity. We believe that this correlation arises because of the different tendencies of these peptides to adopt secondary structures that minimize amide group surface exposure and thus enhance the hydrophobicity of the uncharged core region and the amphiphilicity of the signal region overall. The distinctions among the peptides are most clearly illustrated by comparing the pseudorevertant and deletion mutant peptides. Although the hydrophobicities of the side chains are nearly equal, their lengths are the same, and their charged residues are identical, the pseudorevertant has a greater propensity to form secondary structure and interacts more strongly with phospholipid surfaces than does the deletion mutant.

Although these experiments were performed on a small set of peptides from only one organism, the results may bear on more general questions of protein secretion. One major point of controversy is how the signal sequence and the secreted protein interact with the membrane components. In the signal hypothesis (*4*), a proteinaceous pore which permits the secreted protein to cross the membrane is postulated; thus the signal sequence and nascent protein never come in contact with the membrane lipids. At the other extreme, Wickner's membrane trigger hypothesis holds that no proteinaceous export machinery is necessary (with the exception of signal peptidase), and that both the signal sequence and the secreted protein interact extensively with lipid (*3*). Genetic evidence suggests that translocation of the *lamB* gene product to the outer membrane is a multistep process involving the cooperation of many proteins (*1*).

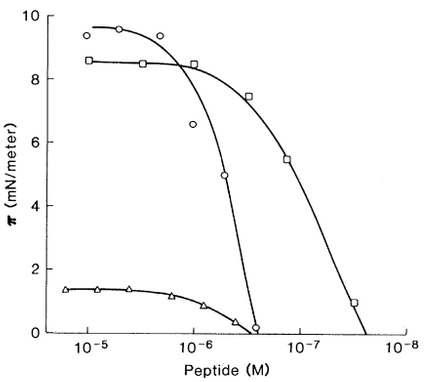

Fig. 2. The increase in surface pressure of phospholipid monolayers as a function of signal peptide concentration (semi-logarithmic plot). A monolayer of egg phosphatidylethanolamine and egg phosphatidylglycerol (65:35) (Avanti, Birmingham, Alabama) was spread from a benzene solution onto 5 m*M* tris HCl, *p*H 7.3, yielding a final surface pressure of 20 mN/m after evaporation of the benzene. The peptide was added by injecting a concentrated solution below the lipid-water interface. The surface pressure was measured by the du Noüy ring method (*31*) with a Fisher Autotensiomat equipped with a platinum-iridium ring. The plateau values are plotted as a function of the peptide concentration for the wild-type (○), pseudorevertant (□), and deletion mutant (△) peptides.

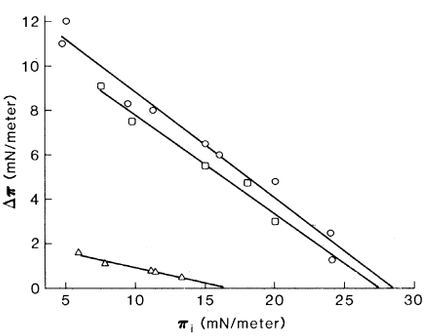

Fig. 3. Dependence of final surface pressure increase on initial surface pressure. A monolayer of egg PE and egg PG was spread from benzene (see text) to yield the desired initial surface pressure. Peptide was injected below the lipid surface to a final concentration of 1 μ*M* for the wild-type and Pro>Leu revertant peptides, and 2 μ*M* for the deletion mutant peptide. Surface pressure plateau values are plotted versus the initial surface pressure for wild type (○), pseudorevertant (□), and deletion mutant (△) peptides.

Nonetheless, direct interactions with the phospholipid constituents of membranes may also be involved in the process. Our data indicate that the ability of these signal peptides to interact with ordered phospholipid assemblies correlates with their biological activity. While the data in no way diminish the probability that various proteins are necessary for protein transport, and may indeed interact with the signal sequence, the implication that signal sequences may also have some contact with the membrane lipids during secretion is clear.

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22. A concentrated solution of peptide was injected into 5 m*M* tris, *p*H 7.3, and the change in surface tension at the air-water interface was observed. The maximal increase in surface pressure was 19

mN/meter at concentrations greater than 0.25 μM for wild type; 13 mN/meter at greater than 2 μM for the Pro>Leu revertant; and 4 mN/meter at greater than 5 μM for the deletion mutant signal peptides.
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Reversibility of Progression of the Transformed Phenotype in Ad5-Transformed Rat Embryo Cells

Abstract. *The carcinogenic process is extremely complex and is affected by diverse environmental and host factors. The mechanism for the gradual development of the transformed phenotype (a process termed "progression") was studied in type 5 adenovirus (Ad5)-transformed rat embryo cells. Progression was not correlated with major changes in the pattern of integration of viral DNA sequences. Instead, it was associated with an increased methylation of integrated viral sequences other than those corresponding to the E1 transforming genes of Ad5. A single exposure of progressed cells to the demethylating agent 5-azacytidine (Aza) resulted in a stable reversion to the unprogressed state of the original parental clone. A further selection of cells after growth in agar allowed the isolation of Aza-treated clones that had regained the progressed phenotype. These observations indicate that progression is a reversible process and suggest that progression may be associated with changes in the state of methylation of one or more specific genes.*

On the basis of in vivo and in vitro studies, the neoplastic process has been divided into three (sometimes overlapping) phases termed initiation, promotion, and progression (*1, 2*). The process of progression involves the development of unique phenotypes or the further elaboration of transformation-associated phenotypes during the evolution of a transformed cell (*1*). Specific cellular phenotypes that may be used to monitor progression include stable changes in anchorage independence (the ability of a cell to grow in agar, agarose, or methylcellulose) and stable alterations in a cell's tumorigenic potential (*1*). Progression can occur naturally after treatment with a chemical carcinogen or after viral transformation and repeated subculturing, and can be accelerated by exposing cells to the potent tumor-promoting agent 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) (*3, 4*). TPA induces an irreversible acquisition of anchorage independence and increases the ability of carcinogen-treated mouse epidermal cells (*4*) and Ad5-transformed rat embryo cells (*3*) to grow in agar. In addition, TPA can enhance the tumorigenic potential of cells transformed by herpes simplex virus type 2 (*5*). We now present evidence that progression of the transformed phenotype in Ad5-transformed rat embryo cells can be reversed by exposing cells to the demethylating agent 5-azacytidine (Aza) and can be accelerated by a second isolation of clones after growth in agar suspension.

The E11 cell line is a cloned culture isolated from secondary Sprague-Dawley rat embryo cells that had been infected with a temperature-sensitive mutant of Ad5 (H5ts125) (*6*). The biological and biochemical properties of E11 cells are similar to those of rat embryo cells transformed by wild-type Ad5 (*3*). Liquid and filter hybridization analysis has shown that E11 cells contain one complete copy of the Ad5 genome (*3*). The E11-NMT subclones are clones of Ad5-transformed cells reisolated from tumors induced in nude mice by the parental E11 cell line. Passage of E11 cells through agar (in the presence or absence of TPA) or in a nude mouse results in stable subclones of E11 cells displaying further progression of the transformed phenotype, as indicated by their higher cloning efficiency in agar (*7*). Here we have used the stable acquisition of enhanced anchorage independence and enhanced tumorigenic potential in nude mice as markers for progression.

Analysis of the pattern of viral DNA integration by DNA filter hybridization (*8*) in parental E11 cells and E11 subclones isolated from agar and nude mouse tumors indicated that progression was not associated with detectable alterations in the state of integration of the Ad5 genome in cellular DNA (Fig. 1). In addition, hybridization of Xba I–cleaved cellular DNA with the Xho I-C fragment of Ad5 (0 to 15.5 map units), which contains the primary region of Ad5 DNA involved in transformation (*9*), indicated that this region of viral DNA was conserved during progression (*10*). Chromosomal DNA from agar- and tumor-derived E11 subclones was then cleaved with the restriction endonuclease Hpa II, which recognizes the tetranucleotide sequence C-C-G-G (C, cytosine; G, guanine) but does not cleave if the internal C is methylated (*11*). The migra-

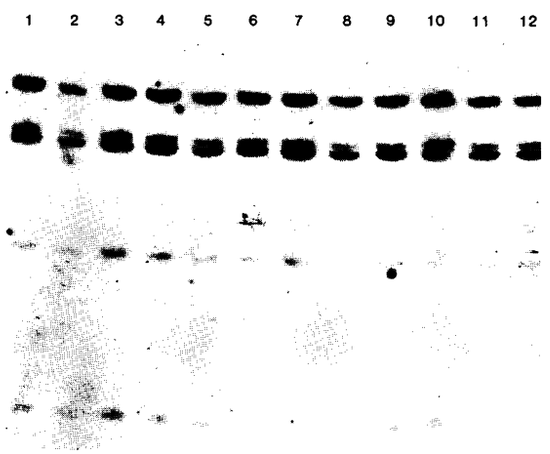

Fig. 1. Analysis of Ad5 DNA sequences persisting in clone E11-L and its agar-, tumor-, and Aza-derived subclones. E11-L (lane 1); E11-L nude mouse tumor (lane 2); E11-AT-1a (lane 3); E11-AT-1a nude mouse tumor (lane 4); E11-NMT subclones Cl 1, 16, 18, and 12 isolated from monolayer culture (lanes 5 to 8, respectively); E11-NMT-Aza subclones 10-1, -6, -9, and -11, isolated from E11-NMT cells treated for 24 hours with 10 μM Aza (lanes 9 to 12, respectively). DNA (10 μg) was cleaved with the restriction endonuclease Xba I, separated by electrophoresis in 0.7 percent agarose, transferred to nitrocellulose sheets by blotting, and hybridized to Ad5 DNA that had been labeled with ^{32}P by nick-translation.