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Membrane Association of the Tnp and Inh Proteins of IS*50*$_R$

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Using a radioimmunoassay for the IS*50*$_R$ proteins Tnp and Inh, we found that both proteins were present primarily in the cytoplasm, but 3 to 11% of Tnp and 3 to 5% of Inh were found in association with the inner membrane. The fractions of total Tnp and Inh that became membrane bound were unaffected by the amount of Tnp and Inh synthesized in whole cells, provided that the ratio of total Tnp to total Inh was not changed. In addition, Inh was not found in the membrane fraction in Tnp$^-$ IS*50*$_R$ mutants, indicating that Tnp is required for Inh localization.

IS*50*$_R$ is the insertion sequence (IS) element that is responsible for transposition of transposon Tn*5*. The IS*50*$_R$ *tnp* gene codes for a transposase (Tnp), and the *inh* gene codes for a diffusible inhibitor of transposition (Inh) (3, 6, 13). The *inh* gene is nested within *tnp*, and the *tnp* transcripts are translated in the same reading frame, so that the amino acid sequences of the Inh and Tnp proteins differ only at their amino termini; Tnp is 55 amino acids longer than Inh (1, 7, 9). Whereas other IS elements code for regulatory gene products that control synthesis of their respective transposase proteins, the IS*50*$_R$ Inh protein acts posttranslationally to block transposition (3, 6). The mechanism of Inh action is unknown, though a model for inhibition in which Inh forms a complex with Tnp, inhibiting Tnp action through direct protein-protein contact, is consistent with the observed data.

In an initial biochemical characterization, it was found that Tnp could be detected in purified membrane fractions both in wild-type cells overproducing the transposition proteins and in maxicells (4). It has been noted for several other proteins that either overproduction or synthesis in maxicells (heavily UV-irradiated cells) can result in artifactual association of the protein in question with membrane material. Therefore, in the present work, we determined the localization of Tnp and Inh synthesized from a wild-type IS*50*$_R$ in growing cells.

The distribution of IS*50*$_R$ protein products in cytoplasmic and membrane subcellular fractions from wild-type cells is shown in Fig. 1. The proteins were assayed by standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), electroblotting, and radioimmunological detection procedures (10). The procedure for preparing cytoplasmic and membrane fractions is a slight modification of that of Yamato et al. (11). The antibody used in these experiments was raised against Tnp/Inh-β-galactosidase fusion proteins and was detected on the nitrocellulose paper by labeling with ^{125}I-labeled *Staphylococcus aureus* A protein.

Figure 1 (lane 2) shows the proteins present in a whole-cell extract of cells carrying IS*50*$_R$. Two prominent bands corresponding to the 58- and 54-kilodalton (kDa) Tnp and Inh proteins were evident. Densitometric analysis of this autoradiogram revealed that the Tnp protein was approximately fivefold less abundant than the Inh protein (Table 1). This value corresponds with the previously reported Tnp/Inh ratios (3, 6, 7). Both IS*50*$_R$ proteins fractionated largely with the cytoplasmic components (lane 4), while a smaller portion (about 10% of the total Tnp and approximately 3% of the total Inh) was found in the membrane fraction (Fig. 1, lane 6, and Table 1). In this experiment, the ratio of Tnp to Inh was approximately 1:5 in the whole-cell extract as well as in the cytoplasmic fraction; this ratio increased to about 1:1.5 in the membrane fraction. Similar results were obtained with pBR322::IS*50*$_R$ in independent fractionation experiments (data not shown).

To control for cytoplasmic contamination of the membrane fraction, we stained the proteins with Coomassie blue for both the cytoplasm and membranes. The major cytoplasmic bands were not visible in the membrane fraction. To obtain a more quantitative picture of cytoplasmic contamination, the activity of glucose-6-phosphate isomerase, a cytoplasmic enzyme, was assayed in each purified fraction. These assays detected 1% of this cytoplasmic marker enzyme in the membrane material (data not shown).

The membrane fraction in Fig. 1 contains both inner and outer membrane material. Separation of inner and outer membrane was routinely omitted to minimize proteolytic degradation of Tnp and Inh, although, in agreement with published results (4), further fractionation experiments demonstrated that only inner membrane fractions contained IS*50*$_R$ products (data not shown). The smaller proteins that can be seen in Fig. 1 are probably proteolytic degradation products of Inh and Tnp that arose after cell lysis. These smaller proteins are probably not generated in vivo, since they were not evident in fresh extracts of whole cells; rather, they accumulated in extracts that were frozen and thawed and those that were stored at 0°C. Because the membrane fractions required 2 days of preparation time, the small proteins were relatively abundant in membrane fractions.

Isberg and Syvanen (4) reported that some Tnp was membrane associated, while Inh was confined entirely to the cytoplasmic compartment. The results presented here show that a small amount of Inh consistently fractionated with membrane material. Two factors probably account for this difference. First, in most of the earlier experiments, proteins produced in maxicells were analyzed; maxicell systems employ heavily UV-irradiated cells that can yield unreliable

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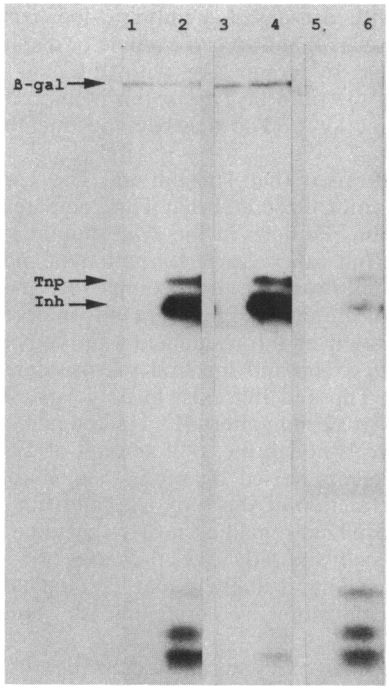

FIG. 1. Localization of IS*50*$_R$ products in wild-type cells. IS*50*$_R$ proteins were revealed by autoradiography of SDS-polyacrylamide gels that had been blotted onto nitrocellulose paper and reacted with anti-β-galactosidase-Tnp-Inh antibody and then with ^{125}I-labeled protein A. Lanes 1 and 2, Whole-cell extracts; lanes 3 and 4, cytoplasmic fractions; lanes 5 and 6, purified membrane fractions. Lanes 1, 3, and 5, SY900(pUC19) (no IS*50*$_R$); lanes 2, 4, and 6, SY900(pADL70) (pUC19::IS*50*$_R$). β-gal, β-Galactosidase. Molecular weights were determined by comparing the migration of the bands labeled with ^{125}I-protein A to the migration of prestained protein molecular weight standards.

fractionation data. Second, in the experiments comparable to those presented here, Isberg and Syvanen (4) analyzed labeled Tnp and Inh in fractions containing a labeled *Escherichia coli* inner membrane protein that comigrated with Inh; this protein probably obscured the presence of Inh in the membrane fraction. The immunoassay used in the current work is specific for Tnp and Inh.

The fact that membrane fractions are enriched for Tnp

TABLE 1. Membrane localization of Tnp and Inh[a]

Source of Tnp and Inh	Fig. no.	% of total Tnp and Inh in purified membrane fractions	
		Tnp	Inh
pUC19::IS*50*$_R$	1	11	3.5
pBR322::IS*50*$_R$ (wild-type strain)[b]	3	5–10	5–10
pBR322::IS*50*$_R$ (*dam* strain)	3	24	6

[a] The relative levels of Tnp and Inh were determined directly from the autoradiograms shown in Fig. 1 and 3 by using a densitometer. The ratios of Tnp to Inh (in whole cells) were 0.2 to 0.3.

[b] The copy number of pBR322 is at least 10-fold lower than that of pUC19; the levels of Tnp and Inh in extracts of SY900(pBR322::IS*50*$_R$) (shown in Fig. 3) were correspondingly lower than in extracts of SY900(pUC19::IS*50*$_R$) (shown in Fig. 1). This lower abundance introduces greater uncertainty in Tnp and Inh amounts.

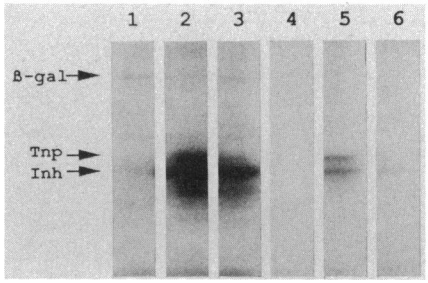

FIG. 2. Inh is not membrane associated in cells producing Inh alone. ^{35}S-labeled subcellular fractions were immunoprecipitated with anti-Tnp-Inh antibody. Immunoprecipitated complexes were boiled in SDS and electrophoretically separated on a 12% polyacrylamide gel. The gel was treated with salicylate fluor, dried, and exposed to X-ray film. Lanes 1 through 3, Cytoplasmic fractions; lanes 4 through 6, inner membrane fractions. Lanes 1 and 4, SY900(pBR322); lanes 2 and 5, SY900(pRI162) (Tnp$^+$ Inh$^+$); lanes 3 and 5, SY900(pRI173) (Tnp$^-$ Inh$^+$). β-gal, β-Galactosidase.

suggests that Tnp may have greater affinity for membrane material than does Inh. To further investigate this question, localization was examined in cells overproducing either Inh alone or both Tnp and Inh. A *tnp*$^+$ *inh*$^+$ plasmid (pRI162) and a *tnp inh*$^+$ plasmid (pRI173) overproduce IS*50*$_R$ products under the control of an inducible promoter. Both plasmids were made by inserting a DNA fragment containing the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible *tac* promoter (2, 4) into IS*50*$_R$. In pRI173, the *tac* promoter is inserted at about position 165 and drives transcription of *inh* only. The IS*50*$_R$ proteins in ^{35}S-labeled extracts of cells carrying either pRI162 or pRI173 were assayed by immunoprecipitation after IPTG induction of the promoter.

As expected, in cells carrying pRI162, both Tnp and Inh were found in the membrane fraction (Fig. 2, lane 5). In contrast, the amount of Inh in the membrane fraction of Inh$^+$ Tnp$^-$ cells was greatly reduced (lane 6). Cells carrying pRI162 and pRI173 produced similar amounts of Inh (compare the Inh bands in lanes 2 and 3), and the membranes of cells carrying pRI162 contained an easily detectable amount of Inh (lane 5). In the absence of Tnp, however, the Inh produced by pRI173 appeared to be excluded from the pool of membrane-associated proteins (lane 6). Indeed, the barely detectable level of Inh (<1% of the total) seen in lane 6 can be accounted for by cytoplasmic contamination of the membrane fraction.

Two points can be drawn from the results in Fig. 2. First, neither the Tnp/Inh ratio in the inner membrane nor the ratio of these proteins between cytoplasmic and membrane compartments was changed by a coordinate increase in the levels of Tnp and Inh. Second, Tnp was required for membrane association of Inh. This latter result also provides a control for the significance of Inh that we did see in the membrane fraction. For example, it could be argued that this was due to cytoplasmic contamination of the membranes.

We also examined the pattern of membrane localization of Inh and Tnp when the ratio of Tnp to Inh was altered. This was accomplished by analyzing the localization pattern in an *E. coli dam* mutant strain (8). In *dam* mutants, Tnp is produced in greater amounts than Inh (13). Therefore, analyzing the distribution of the IS*50*$_R$ proteins in extracts of Dam$^+$ and Dam$^-$ cells carrying IS*50*$_R$ allowed evaluation of the localization of the transposition proteins at two different Tnp:Inh ratios. The results of this experiment are displayed in Fig. 3.

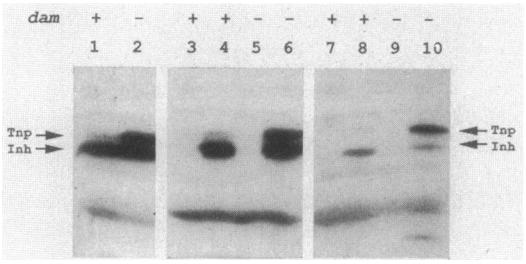

FIG. 3. *dam*::Tn*9* allele increases the proportion of Tnp that is membrane associated. IS*50*$_R$ proteins were analyzed as described in the legend to Fig. 1. Lanes 1 and 2, Whole-cell extracts; lanes 3 through 6, cytoplasmic fractions; lanes 7 through 10, membrane fractions. Lanes 1, 4, and 8, Wild type with IS*50*$_R$ [SY900(pRI43)]; lanes 2, 6, and 10, *dam* mutant with IS*50*$_R$ [SY1089(pRI43)]; lanes 3 and 6, SY900(pBR322); lanes 5 and 9, SY1089(pBR322).

Two aspects of the localization pattern in the *dam* mutant were different from that of the wild-type strain. First, there was a much greater accumulation of Tnp in extracts made from the *dam* strain than in those made from the wild-type strain (compare lanes 2 and 1, Fig. 3). This increased accumulation altered the Tnp/Inh ratio, which was 1:1.5 in whole-cell extracts made from the *dam* mutant strain and 1:5 in extracts from the wild-type strain (Table 1). Second, we recovered more Tnp in the membrane fraction of *dam* cells (lane 10) than in the membrane fractions of wild-type cells (Fig. 3, lane 8; Table 1). Thus, in the *dam* strain there appeared to be more Tnp in membrane fractions, although it was slightly less abundant than Inh in whole-cell extracts.

The possibility that the *dam* mutations in these strains affected Tnp and Inh membrane association directly (as opposed to affecting the amount of Tnp and Inh synthesized) was tested by examining the effects of the *dam* mutation on localization of Inh alone. In strain SY1092(pRI173) (Dam$^-$), *inh* is under control of the *tac* promoter, and Inh is inducibly overproduced. The *dam* mutation in this strain does not alter the amount of Inh produced when the *tac* promoter is induced with IPTG. As in the Dam$^+$ parent strain [SY900(pRI173)], only background levels of Inh were found in membrane fractions from the SY1092(pRI173) (Dam$^-$) strain (data not shown). These results indicate that an increase in the ratio of Tnp to Inh results in an increased membrane localization of Tnp.

Tnp contains the determinant of membrane association, and Inh lacks this determinant. At the primary sequence level, the sole difference between the Inh and Tnp proteins is the presence of the amino-terminal 55 amino acids of Tnp. Some function or characteristic of these residues must account for the differential localization of the two proteins, although examination of the amino acid sequence of Tnp does not reveal any signals that would be predicted to direct membrane association.

These results provide support for the hypothesis that Inh and Tnp interact with each other directly. We have shown that Tnp is required for the membrane localization of Inh; the simplest explanation for this is that Inh becomes associated with the inner membrane only by forming a complex with Tnp. The alternative explanation, that Tnp might act to prepare a membrane-binding site for Inh, is not supported by the decreased membrane localization of Inh in the *dam* mutant strain. If Tnp acted to prepare a site for Inh binding, then a higher fraction of Inh should be membrane bound when membrane localization of Tnp is increased. However, we show that increased membrane association of Tnp is correlated with decreased membrane localization of Inh. This result argues that Tnp can exclude or displace Inh from the membrane by competing with Inh and furthermore suggests that the ratio of Tnp to Inh is important in determining the fraction of Tnp that becomes membrane associated.

Our hypothesis is that Tnp-Inh and Tnp-Tnp complexes are membrane localized and that Tnp competes with Inh in oligomerization. Perhaps in the *dam* mutant strain the increased Tnp/Inh ratio allows formation of more Tnp-Tnp complexes (and fewer Tnp-Inh complexes) and the altered distribution of protein complexes results in decreased membrane association of Inh. Sedimentation velocity measurements of native Tnp and Inh in a sucrose density gradient indicate that Tnp and Inh exist in polydisperse complexes containing 6 to 12 monomers (E. Hanley and M. Syvanen, unpublished). Although the total amount of Tnp and Inh in these experiments varied by more than 30-fold (data not shown), the fraction of the total Tnp and Inh that became membrane localized remained nearly the same. Thus, Tnp-Inh binding is not saturated over the range of concentrations tested here, making it unlikely that Tnp excludes Inh from membrane localization by competing for some membrane component.

The significance of membrane localization to the transposition mechanism itself is difficult to assess. A mutation that increases Tn*5* transposition frequency (*dam*) increases the amount of Tnp that is found in cytoplasmic membrane fractions. In addition, coordinate overexpression of Tnp and Inh does not stimulate transposition (5), nor does it alter the pattern of Tnp and Inh localization. These results point to a correlation between Tnp activity and its membrane localization.

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