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Membrane insertion of the bacterial signal transduction protein ToxR and requirements of transcription activation studied by modular replacement of different protein substructures

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The *Vibrio cholerae* protein ToxR is an integral membrane protein that acts as a transcription activator in response to environmental signals; it controls expression of toxin genes *ctxA* and *ctxB*, along with a variety of other genes related to pathogenicity. Here it is shown that: (i) ToxR has a modular architecture and that activation of transcription starting at the *ctx* promoter depends strictly on dimerization of the periplasmic ToxR domain; (ii) the transmembrane (TM) region of ToxR is sufficient as a topogenic signal but not for stable membrane anchoring of the protein; (iii) the TM region has no special function in signal transduction and (iv) a proline residue located within the TM region minimizes background transcription activation, most plausibly by reducing TM–TM interaction. Possible applications of ToxR as a technical tool for analysing protein–protein interactions between pairs of arbitrary TM domains are discussed.

Keywords: gene regulation/membrane protein/protein dimerization/signal transduction/*Vibrio cholerae*

Introduction

Upon invasion of its host, the human pathogen *Vibrio cholerae* synthesizes a number of virulence factors which enable the organism to colonize the intestine and cause diarrhoeal disease. Expression of virulence genes is coordinately regulated in response to environmental signals by gene products of the *toxRS* operon (DiRita, 1992).

ToxR is an integral membrane protein of M_r 32 500, probably anchored in the *V.cholerae* cytoplasmic membrane by a single membrane-spanning region (Figure 1). The protein is oriented in the membrane such that the N-terminal domain faces the cytoplasm; this domain shares sequence homology with several prokaryotic transcription activators (Miller *et al.*, 1987). ToxR stimulates transcription from the cholera toxin gene promoter *ctx*, most likely by direct binding to DNA element TTTTGAT present in different isolates of *V.cholerae* in three to eight tandemly repeated copies upstream of the *ctx*AB structural genes (Miller *et al.*, 1987).

Transcription activation is thought to be initiated by environmental stimuli which cause the periplasmic ToxR domain to form a homodimer. This, in turn, tethers together the two cytoplasmic ToxR domains, which can now bind to the control region of the *ctx* promoter (Miller *et al.*, 1987; DiRita and Mekalanos, 1991). A second membrane-associated protein, ToxS (M_r 19 000), is required for maximal activation of the *ctx* promoter; most plausibly it stabilizes the ToxR dimer by direct contact (Miller *et al.*, 1989; DiRita and Mekalanos, 1991; compare Figure 1).

Indirect experimental support for the dimerization model was derived mainly from two findings. First, replacement of the periplasmic ToxR domain by alkaline phosphatase, by itself a dimeric, soluble periplasmic enzyme (Schlesinger, 1967), leads to constitutive high transcription from the *ctx* promoter in a ToxS-independent fashion (Miller *et al.*, 1987). This suggests either that the C-terminal part of ToxR is dispensable or that alkaline phosphatase substitutes for part of its functions. Second, it was shown recently that ToxR can drive the dimerization of the DNA binding domain of phage λ cI repressor fused to the ToxR N-terminus, either with the assistance of ToxS or without auxiliary protein in case of PhoA forming the periplasmic moiety of the protein chimera. (Dziejman and Mekalanos, 1994).

ToxR can be regarded as a one-component signal transduction system, the periplasmic domain being the sensor and the cytoplasmic domain the regulator. In contrast to bacterial two-component systems (Parkinson, 1993), transmitter and receiver modules are missing, communication between sensor and regulator domain being mediated by the transmembrane (TM) module.

We investigated the role of the putative TM region in cellular localization of ToxR and signal transduction. Since the *toxR* gene does not provide coding sequence for a leader peptide, it must be assumed that the major or only topogenic signal of ToxR is its TM segment itself. A candidate membrane-spanning region is a stretch of 16 hydrophobic amino acids extending from residues 183 to 198 which is flanked by an arginine on its N-terminal and by several polar uncharged residues on its C-terminal side (Miller *et al.*, 1987). This motif is found in many integral membrane proteins (Kyte and Doolittle, 1982; Engelman *et al.*, 1986) which adds credence to the above assignment. Beyond this question, it was not clear until now whether the putative TM segment plays a role of its own in ToxR dimer formation or contributes to signal transduction in some other specific fashion.

Here we address these questions using modular replacement of various substructures of the ToxR molecule as the main tool of investigation. For that purpose, we have constructed various ToxR derivatives in which the periplasmic domain and/or the TM region are replaced by other structural modules of known behaviour and tested the resulting chimeric proteins for their ability to activate transcription from the *ctx* promoter.

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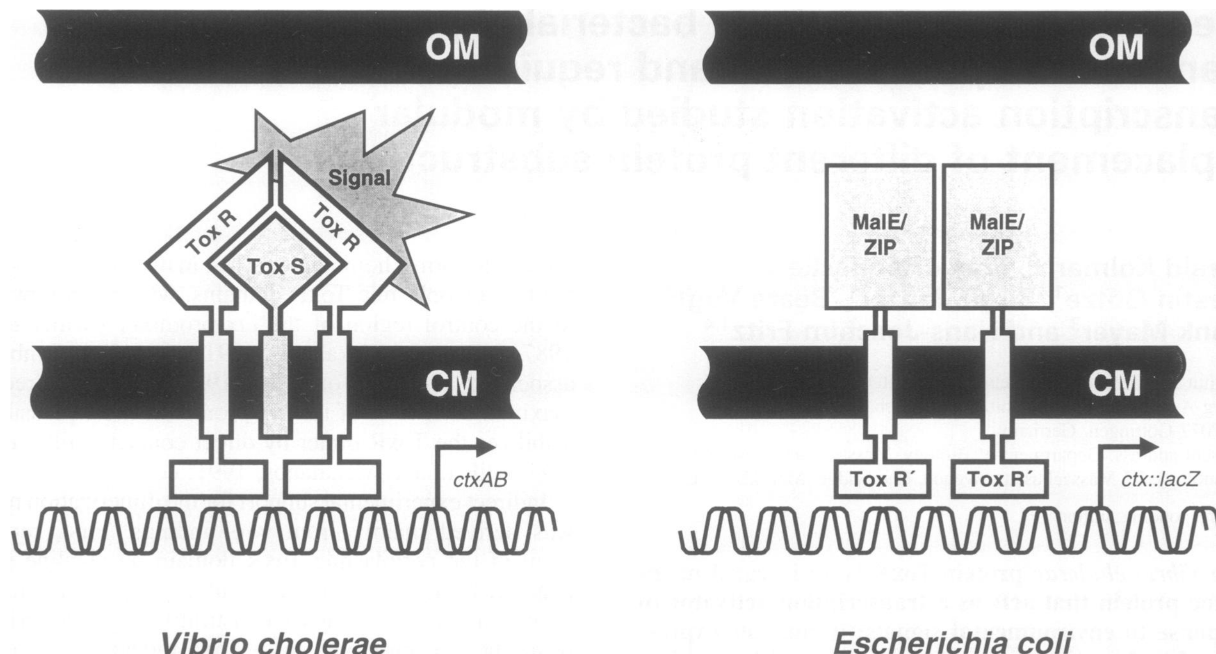

Fig. 1. Activation of transcription from the *V.cholerae ctx* promoter (Miller *et al.*, 1987). Left: *ctx*AB gene activation in *V.cholerae*. Signal-mediated interaction between periplasmic domains of membrane proteins ToxR and ToxS leads to passive dimerization of cytoplasmic ToxR domains with the consequence of sequence-specific binding to *ctx* promoter DNA and, hence, activation of transcription. Right: Positive regulation of *ctx* promoter by ToxR, modelled in *E.coli.* A C-terminal leucine zipper (ZIP) brings about dimerization of a ToxR/MalE fusion protein and leads to *ctx* activation. The arrows indicate direction of transcription. OM, outer membrane; CM, cytoplasmic membrane.

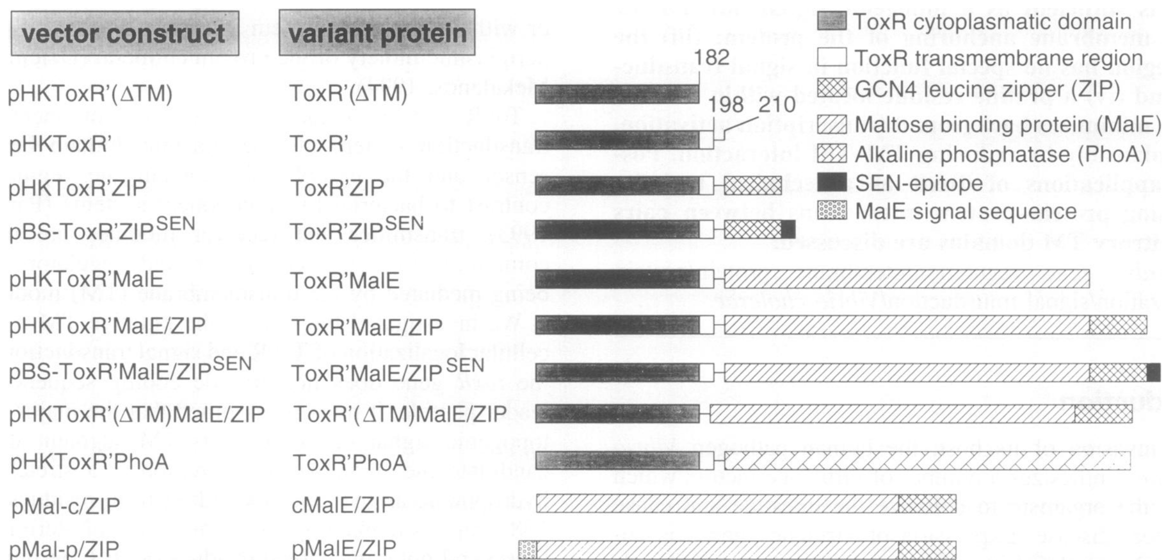

Fig. 2. Modular organization of chimeric proteins employed in this study. In the vector series pHKToxR′ (Kolmar *et al.*, 1994), chimeric *toxR′* genes are under transcriptional control of *V.cholerae toxR* promoter. For overexpression, the respective constructs were cloned into vector pBS [pBluescript II SK(−)™]. Here, the fusion genes are under additional control of the *lac* promoter. Soluble MalE/ZIP fusion proteins were accumulated alternatively in the cytoplasmic or the periplasmic compartment of *E.coli* by expression of the respective genes residing in vectors pMal–c/ZIP and pMal–p/ZIP; in the latter case, an N-terminal leader sequence is provided. For details of construction of the respective fusion genes, see Materials and methods.

Results

The starting point of the present study was a series of genes encoding chimeric proteins, the structural organization of which is summarized in Figure 2. The reference module is a ToxR fragment, termed ToxR′, which extends from amino acid residues 1–210 and encompasses the cytoplasmic domain (residues 1–182) and the putative TM region (residues 183–198), the entire C-terminus of ToxR (residues 211–294) being deleted. In two cases, ToxR′(ΔTM) and ToxR′(ΔTM)MalE/ZIP, the TM region was also deleted. Another experimental parameter was the dimerization state of the chimeric proteins. This was controlled by fusing to the ToxR′ C-terminus various proteins or protein domains with known dimerization tendency, among them ZIP, the leucine zipper domain of

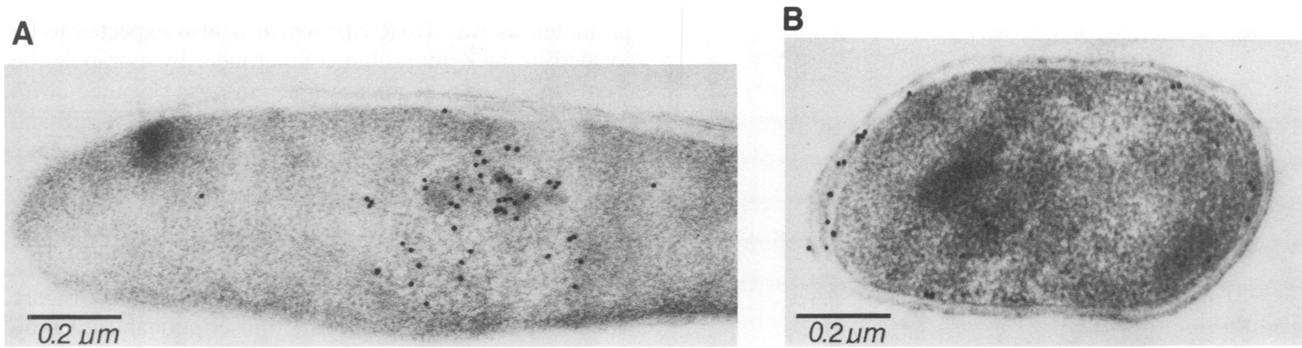

Fig. 3. Immunogold labelling of chimeric ToxR proteins in ultrathin sections through *E.coli*. Ultrathin cell sections of *E.coli* strain BMH71-18, transformed with pBS-ToxR′ZIP^{SEN} (**A**) or pBS-ToxR′MalE/ZIP^{SEN} (**B**), respectively, were stained with anti-Sendai antibody and goat anti-mouse coupled to ultra-small gold particles.

Saccharomyces cerevisiae transcriptional activator GCN4 (Hope and Struhl, 1987; O'Shea *et al.*, 1989), MalE, the *Escherichia coli* maltose binding protein (Spurlino *et al.*, 1991) and PhoA, alkaline phosphatase of *E.coli* (Sowadski *et al.*, 1985). For convenient immunochemical analysis, a linear epitope (SEN) consisting of the 13 C-terminal amino acid residues of Sendai virus L-protein (Einberger *et al.*, 1990) was included in some of the chimeric proteins. cMalE/ZIP and pMalE/ZIP are derived from MalE by a C-terminal ZIP extension and are located in the cytoplasmic or the periplasmic cell compartment, respectively (the location being controlled by absence/presence in the corresponding genes of DNA coding for a secretion leader sequence).

The hydrophobic region 183–198 of ToxR is necessary but not sufficient for membrane localization

The ToxR region extending from residue 183 to 198 exclusively contains non-polar amino acid residues and is thus a good candidate for a TM region (Miller *et al.*, 1987). To visualize directly the cellular localization of ToxR-derived proteins by *in situ* immunogold labelling of ultrathin sections of *E.coli*, genes *toxR′malE/zip^{sen}* and *toxR′zip^{sen}* were inserted into expression vector pBluescript SK (Stratagene) for increased cellular concentration of the epitope-tagged variants ToxR′MalE/ZIP^{SEN} and ToxR′ZIP^{SEN}; the resulting plasmids were pBS-ToxR′ZIP^{SEN} and pBS-ToxR′MalE/ZIP^{SEN}.

Upon immunogold labelling of ultrathin sections of *E.coli* cells transformed by the plasmids described above, ToxR′MalE/ZIP^{SEN} was found to be located exclusively in the cytoplasmic membrane (Figure 3B). Surprisingly, however, the derivative differing only in the absence of the MalE moiety was located in the cytoplasm, apparently associated with the nucleoid (Figure 3A). In a control experiment of antibody specificity, non-transformed cells of expression host BMH71-18 were found not to be labelled by the anti-Sendai antibody (data not shown). Thus, the TM region is by itself not sufficient for stable membrane integration. The necessary presence of the TM region as a topogenic signal to direct the various ToxR derivatives to the cytoplasmic membrane was confirmed separately by heterodimer formation with interaction partners of known cellular location and by the inability of ToxR′(ΔTM)MalE to sustain growth of a *malE* strain on maltose minimal medium (see below).

Correlation between dimer formation of ToxR derivatives and activation of transcription

According to the model illustrated in Figure 1, activation of transcription by ToxR rests on communicating the dimerization of its periplasmic domain to the cytoplasmic side of the membrane. This is achieved by keeping the cytoplasmic domains of the ToxR homodimer in close vicinity and could conceivably also involve changes of their conformation. Beyond anchoring the molecule in the membrane, this defines a second role for the ToxR TM region, namely that of a connector between the sending and the receiving domain and the question arises whether this role is sufficiently described as a passive physical link or whether the TM region contributes in a specific way to the stabilization of that particular ToxR structure which is able to activate transcription.

This question is addressed here by genetically manipulating the TM region in different ways and measuring the influence on transcription activation using expression of the *E.coli lacZ* gene under control of the *V.cholerae ctx* promoter as an *in vivo* indicator (Figure 1; Miller and Mekalanos, 1984; Kolmar *et al.*, 1994). Specific activities of β-galactosidase in cell lysates (determined as described under Materials and methods, final paragraph) are ranked on a signal scale, the end-points of which had to be determined first by measuring specific β-galactosidase activity of cells producing ToxR derivatives that were *bona fide* either monomeric or dimeric, a parameter controlled by choice of the C-terminal (i.e. periplasmic) fusion partner of ToxR′.

As a monomeric fusion partner we chose the maltose binding protein of *E.coli* (MalE), which is present in the *E.coli* periplasm (Duplay *et al.*, 1984). While ToxR′PhoA displays elevated transcription activation of the *ctx* promoter (Miller *et al.*, 1987; Figure 4A), transcription activation mediated by ToxR′MalE is low. This finding defines the background end of the scale and is consistent with the established monomeric nature of MalE (Richarme, 1982).

It has been shown earlier (Blondel and Bedouelle, 1991) that maltose binding protein can be converted from a monomer to a homodimer with a K_D below 0.1 μM by fusing to its C-terminus the 33 amino acids of the leucine zipper of *S.cerevisiae* protein GCN4 (ZIP; Hope and Struhl, 1987; O'Shea *et al.*, 1989). In accord with the working hypothesis that dimerization of the periplasmic protein moiety is the crucial parameter, ToxR′MalE/ZIP

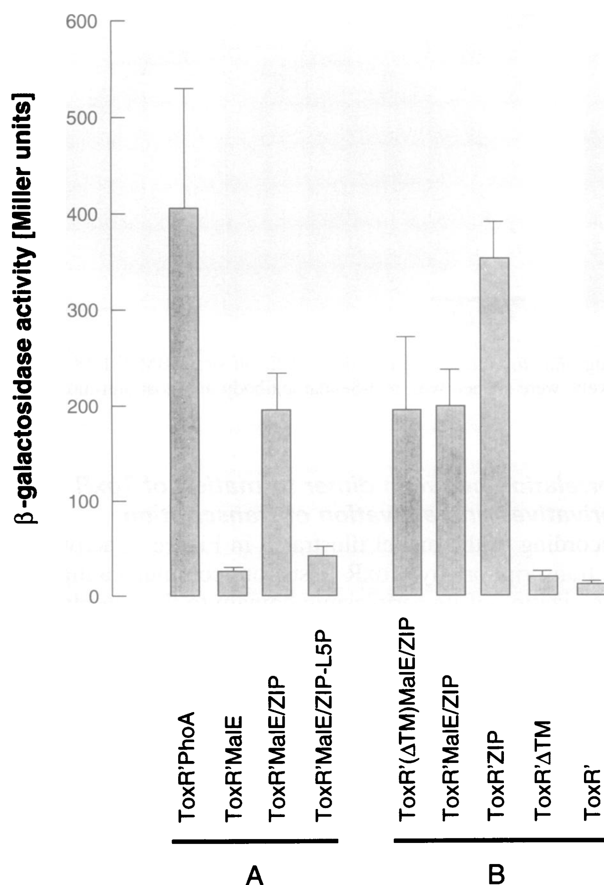

Fig. 4. (**A**) Transcription activation of *ctx* promoter mediated by ToxR′ fusion proteins that have their periplasmic domain substituted by various other proteins. (**B**) Transcription activation of *ctx* promoter mediated by various cytoplasmic or membrane-bound ToxR′ fusion proteins (see Figure 2). Each bar displays specific β-galactosidase activity in crude cell lysates obtained from overnight cultures of six individual colonies. Standard deviations are indicated by error bars.

exhibits a 7.5-fold higher transcription activation compared with ToxR′MalE.

The dimer interface of the leucine zipper is formed largely by contacts between hydrophobic side chains with an alternating spacing of three and four amino acid residues along the polypeptide chain (Hu *et al.*, 1990; Zhou *et al.*, 1992a,b). A ZIP mutant with Leu5 replaced by proline was shown to be non-functional in a genetic assay of dimer formation of a λ-repressor–ZIP fusion protein (Hu *et al.*, 1990). Accordingly, fusion protein ToxR′MalE/ZIP–L5P was found to be unable to mediate transcription activation. These results strongly support the dimerization model that was previously proposed by Mekalanos and co-workers mainly on the basis of high constitutive activation of the *ctx* promoter mediated by a ToxR′PhoA fusion protein (DiRita and Mekalanos, 1991).

Localization in the cytoplasmic membrane is not required for activation of transcription by dimeric ToxR derivatives

In a first round of investigation into the functions of the ToxR TM region, the polypeptide segment ranging from residues 183–198 of ToxR′MalE/ZIP was deleted. The resulting cytoplasmic protein ToxR′(ΔTM)MalE/ZIP was tested for its ability to activate transcription from the *ctx* promoter, as was ToxR′ZIP which is also expected to be located in the cytoplasmic compartment by extrapolation from the electron microscopic results obtained with ToxR′ZIP^{SEN}. To this end, phagemids pHKToxR′ZIP and pHKToxR′(ΔTM)MalE/ZIP which contain the respective genes under control of the *V.cholerae toxR* promoter, were introduced into indicator strain FHK12 by transformation. Specific β-galactosidase activities of crude cell lysates were measured and compared with those of FHK12 producing membrane-anchored ToxR′MalE/ZIP (Figure 4B). In the same experiment, transcriptional activation mediated by ToxR′, lacking a periplasmic domain, and ToxR′ΔTM was determined as a control. All variants endowed with a C-terminal dimerization segment were found to be able to mediate transcription activation irrespective of their presumed cellular location.

Activation of transcription by ToxR derivatives can be suppressed by heterodimer formation with non-ToxR partner proteins

The preceding paragraph shows passive dimerization of the cytoplasmic domain of ToxR to be the only prerequisite of its function as a transcriptional activator. As a corollary to that notion, it must be assumed that suppressing homodimerization of a fully competent ToxR derivative should abolish its transcription-activating effect. This can be achieved by co-production of a large excess of proteins capable of interaction with selected ToxR derivatives but themselves devoid of a ToxR moiety. This renders the ToxR transcription activation domain trapped in an unproductive heterodimer (see Figure 5). If this is done with competing dimerization partners of known cellular location, the experiment would confirm simultaneously the cellular location of the various ToxR derivatives under scrutiny. This scheme was followed for both a membrane-bound and a cytoplasmically located ToxR derivative using ToxR′MalE/ZIP and ToxR′(ΔTM)MalE/ZIP as transcription activators and soluble MalE/ZIP located in the periplasmic or, respectively, the cytoplasmic compartment as interaction partners for heterodimer formation.

For accumulation of MalE/ZIP within the *E.coli* periplasm, the ZIP coding sequence was fused in frame to the *mal*E gene residing in expression vector pMal-p (Maina *et al.*, 1988). Likewise, this segment was fused to the *mal*E gene of vector pMal-c lacking the codons for the MalE signal sequence (Maina *et al.*, 1988). Since both vectors carry an ampicillin resistance marker, which also resides in indicator strain FHK12, a tetracycline-resistance gene was instead introduced to form pMal–p/ZIP and pMal–c/ZIP, respectively. In these plasmid constructs, MalE/ZIP expression is under *tac* promoter control. Indicator strain FHK12 was transformed alternatively with pMal–p/ZIP and with pMal–c/ZIP, induced by IPTG and tested by cell fractionation for the location of the corresponding protein chimeras. As expected from earlier work (Bedouelle and Duplay, 1988), the presence/absence of a secretion leader sequence determined the accumulation of MalE/ZIP in the periplasmic or, respectively, in the cytoplasmic cell compartment (data not shown). FHK12 containing pMal–p/ZIP or pMal–c/ZIP was transformed with pHKToxR′MalE/ZIP or pHKToxR′(ΔTM)MalE/ZIP, respectively. Double transformants (phenotype: Tc^R, Cm^R)

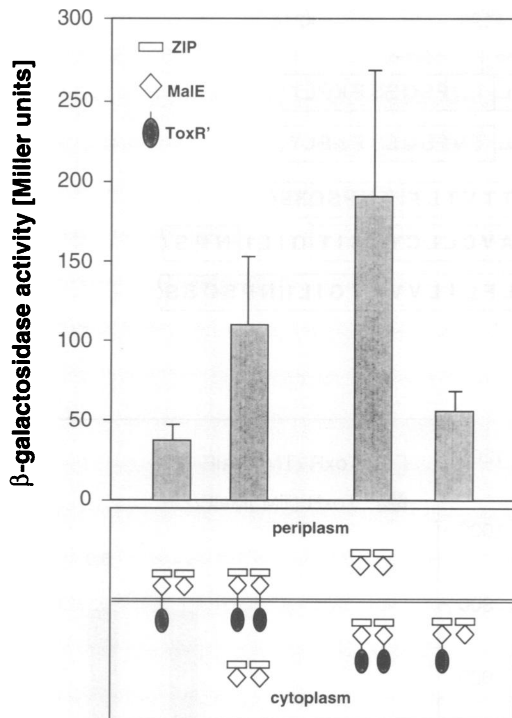

Fig. 5. Suppression of ToxR′-mediated *ctx* promoter activation by co-production of a soluble dimerization partner. The bar graph shows specific β-galactosidase activities resulting from various combinations of soluble and/or membrane-bound fusion proteins as indicated schematically below each bar. In all cases, the MalE/ZIP protein was produced in larger quantities than the chimera containing a cytoplasmic ToxR domain (gene expression under control of *lac* and, respectively, *toxR* promoter). Hence, heterodimer formation (situation shown on far left and far right) must be accomplished by formation of MalE/ZIP homodimers. These are omitted from the illustration for the sake of clarity. *E.coli* strain FHK12 was transformed with pMal–p/ZIP or pMal–c/ZIP, respectively. A single colony of each transformed FHK12 was used for preparation of competent cells and introduction of pHKToxR′MalE/ZIP or pHKToxR′(ΔTM)MalE/ZIP, respectively. Ten individual liquid cultures were started from 1/10 of the transformation mixture each and assayed for β-galactosidase activity after overnight growth at 37°C. Standard deviations within each set of ten individual measurements are indicated by error bars.

were induced by IPTG and β-galactosidase activities of crude cell lysates were measured (Figure 5).

In both constructs, transcription from the *ctx* promoter was found to be reduced if free MalE/ZIP accumulated in a cellular compartment that allows contact to the MalE/ZIP moiety of a given ToxR derivative tested (Figure 5). On the other hand, dimerization incompetent periplasmic MalE/ZIP–L5P did not interfere with transcription activation mediated by ToxR′MalE/ZIP, nor had free periplasmic or cytoplasmic MalE/ZIP an influence on transcription activation mediated by a ToxR′PhoA chimera (data not shown).

As argued above, these findings also confirm ToxR′-(ΔTM)MalE/ZIP to be present in the cytoplasmic compartment and, hence, the notion of the TM region being a necessary pre-requisite of guiding ToxR to the inner membrane.

A variety of different heterologous TM helices can functionally substitute for the putative TM segment of ToxR

The finding that removal of the hydrophobic region 183–198 of ToxR′MalE/ZIP results in cytoplasmic localization confirms the crucial role of this segment in directing the ToxR protein to and anchoring it in the cytoplasmic membrane. To determine whether this sequence can be modified, or even functionally replaced by TM regions of other membrane proteins, various monomeric ToxR′MalE and dimeric ToxR′MalE/ZIP chimera differing in the TM region were constructed (Figure 6A).

At position 192, the ToxR TM region contains a single proline, a residue known to kink polypeptide helices. Its presence within the TM region might therefore have special structural reasons linked to the function of signal transduction. To test this possibility, variant P192A was constructed (Figure 6A). Moreover, the ToxR TM region was completely replaced by several TM regions of eukaryotic origin, that of membrane-bound immunoglobulin M heavy chain (Rogers *et al.*, 1980), that of interleukin 4 receptor α chain (Idzerda *et al.*, 1990), and that of c-neu receptor (Bargmann *et al.*, 1986). All these proteins are membrane-anchored with a single TM region.

We first investigated if the modified or heterologous TM regions (Figure 6A) promote localization and proper orientation of the fusion proteins in the cytoplasmic membrane, i.e. with the DNA-binding domain located in the cytoplasm and the MalE/ZIP moiety facing the periplasm. To this end, the respective pHKToxR′-(TM^x)MalE/ZIP derivatives were introduced by transformation into *E.coli* strain PD28 (Duplay *et al.*, 1987), which lacks the *malE* gene. Due to the absence of functional maltose binding protein, this strain is unable to grow on M9 minimal medium containing maltose as the only carbon source. As expected, PD28 transformed with plasmid pHKToxR′, or with pHKToxR′(ΔTM)MalE/ZIP producing intracellularly located MalE/ZIP, was unable to grow on maltose minimal medium (Figure 6B). However, *toxR′MalE/ZIP* and also the variants with TM regions different from the natural one are able to complement the MalE deficiency. This indicates that in all these constructs, the MalE/ZIP moiety of the fusion proteins faces the periplasm, the cellular localization of the maltose binding protein where it exerts its function in energy-dependent translocation of maltodextrins through the cell envelope (Kellermann and Szmelcman, 1974; Hengge and Boos, 1983).

With respect to transcription activation, all chimeras tested display the same qualitative behaviour as the corresponding ToxR′MalE proteins with the natural TM region in that the derivatives with a dimerizing ZIP domain activate several times stronger than the corresponding derivatives without ZIP (Figure 6C). Interestingly, the natural ToxR TM region leads to the lowest gross activation but the highest factor of increase upon dimerization. Clearly, a wide variety of modified and heterologous TM regions can substitute for the natural ToxR membrane-spanning segment; the higher background levels in the absence of the ZIP domain may be caused by weak homodimerization tendencies of the respective chimeras due to side-by-side interaction of the TM regions themselves (Bargmann *et al.*, 1986; Lemmon *et al.*, 1992; Rutledge *et al.*, 1992; Lemmon and Engelman, 1994).

Discussion

The results presented above confirm the *V.cholerae* transcription activator ToxR to be an integral membrane

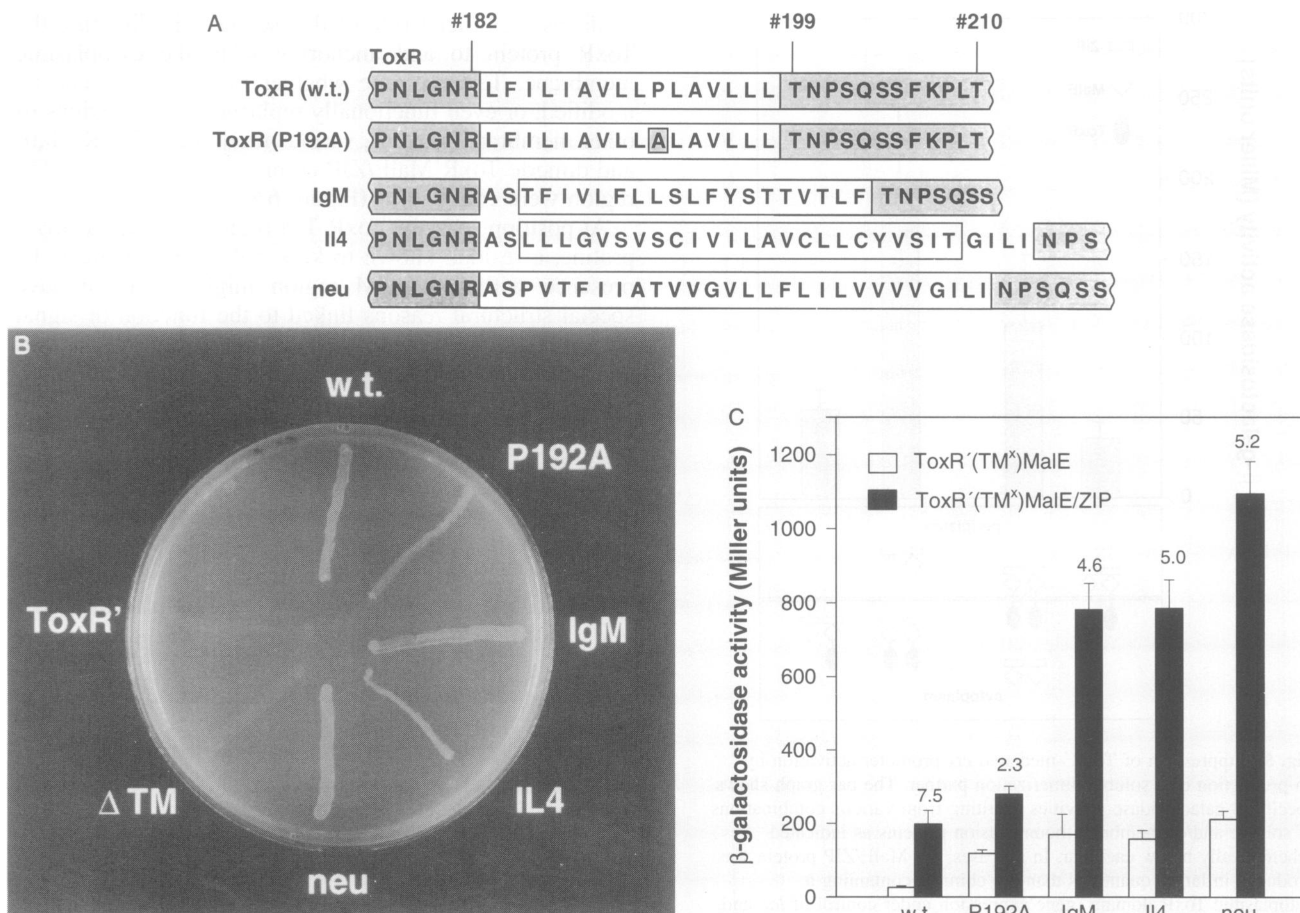

Fig. 6. **(A)** Amino acid sequence of the TM region (light boxes) employed in protein chimeras with the cytoplasmic ToxR domain and MalE or MalE/ZIP as the periplasmic moiety. Flanking residues derived from ToxR′ are indicated in shaded boxes; residues between boxes are a result of the respective gene construction, neither derived from the respective TM region, nor from ToxR′. w.t.: amino acid sequence of ToxR, numbers of amino acid residues in ToxR wild-type protein are given above the sequence. P192A: ToxR′ TM region with residue Pro192 replaced by Ala. IgM: TM region derived from residues 18–38 of membrane-anchored mouse IgM (Rogers *et al.*, 1980); Il4: TM region derived from residues 233–256 of the interleukin receptor α chain (Idzerda *et al.*, 1990); neu: TM region derived from residues 653 to 678 of the *neu* proto-oncogene derived receptor (Bargmann *et al.*, 1986). **(B)** Complementation of growth deficiency of *E.coli* strain PD28 (Duplay *et al.*, 1987) on M9-maltose by expression of various fusion genes derived from *toxR′* and/or *malE*. *Escherichia coli* strain PD28 was transformed with pHKToxR′MalE (w.t.), pHKToxR′(TM^{P192A})MalE (P192A), pHKToxR′(TM^{Il4})MalE (Il4), pHKToxR′(TM^{IgM})MalE (IgM), pHKToxR′(TM^{Neu})MalE (Neu), pHKToxR′(ΔTM)MalE (ΔTM) and pHKToxR′ (ToxR′), respectively. Cells were plated on rich medium containing chloramphenicol (25 μg/ml). After overnight growth at 37°C, individual colonies were picked and streaked onto M9-agar containing 2% maltose as the only carbon source. **(C)** Activation of transcription from the *ctx* promoter mediated by chimeric ToxR proteins that have their membrane-spanning segment substituted by various other TM regions. Each column displays β-galactosidase activity of crude cell lysates obtained from overnight cultures of six individual colonies. Standard deviations are indicated by error bars. The factor of *ctx* promoter activation observed upon addition of the leucine zipper to the ToxR′MalE constructs is given in each case above the respective bar.

protein with a modular architecture consisting of a periplasmic domain, a membrane-spanning (TM) region and a cytoplasmic transcription activator domain. At least the former two can be replaced individually by heterologous protein modules without loss of the core function of ToxR, i.e. activation of transcription from the *ctx* promoter. The periplasmic ToxR domain was replaced by a series of *bona fide* monomeric or, respectively, homodimeric proteins and a strict correlation was observed between dimerization of the individual proteins studied and activation of transcription, thought to be brought about by binding of the cytoplasmic ToxR domains to the *ctx* control region. Thus, our present systematic study supports conclusions on the key role of ToxR dimerization drawn earlier from sporadic experiments in which ToxR′ was fused to PhoA (DiRita and Mekalanos, 1991) or REI_V (Kolmar *et al.*, 1994), both homodimeric proteins by themselves. In the case of REI_V, the variable domain of a human immunoglobulin κ light chain, derived from a Bence-Jones protein, it had been possible to link the extent of transcription activation to the equilibrium constant of dimer formation (Kolmar *et al.*, 1994).

Location of ToxR in the cytoplasmic membrane was demonstrated by immunogold electron microscopy; an observed selective accumulation in the region of the cell poles may have functional implications. The TM region of ToxR is necessary and seems to be sufficient for guiding the protein to the cytoplasmic membrane; stable membrane anchoring, however, requires a sizeable globular domain on the periplasmic side, most simply viewed as a mechanical block to slippage. ZIP, (33 amino acids, α-helical fold) is not sufficient for stable membrane anchoring; whereas REI_V (108 amino acids, β-sandwich domain) is—as are all larger globular proteins tested to date. ToxR′ZIP^{SEN}, a derivative without a sizeable periplasmic domain, was found associated with the *E.coli*

nucleoid by immunogold electron microscopy. This finding confirms the DNA-binding properties of the cytoplasmic ToxR domain and at the same time it hints at possible functioning as a transcription activator even of soluble ToxR derivatives. Indeed, when the TM region of ToxR'MalE/ZIP was deleted, the resulting ToxR'(ΔTM)MalE/ZIP accumulated as soluble protein in the cytoplasmic cellular compartment, yet it was able to mediate activation of transcription.

The ToxR TM region can be replaced by a variety of TM regions derived from other membrane proteins without loss of function; thus, in first approximation, it acts as a passive connector of the periplasmic and the cytoplasmic domains. It is conspicuous, though, that all heterologous TM regions examined lead to relatively high transcription levels even when fused to a monomeric periplasmic domain, as if these TM regions by themselves could mediate to some extent the dimerization of the corresponding ToxR derivatives. Most revealing in that respect is mutation P192A in the natural ToxR TM region. In comparison with wild-type, this mutation leads to a ~1.5-fold increase of transcription level with a dimeric, but to a ~4.5-fold increase of background transcription with a monomeric periplasmic domain, so that the 'induction ratio' upon dimerization drops by a factor of about three. It seems plausible, therefore, that the proline residue at position 192 has the important function of suppressing background signalling caused by TM–TM dimerization; a proline-induced helix kink could well be at the core of this function. Thus, it would seem both possible and attractive to exploit the ToxR molecule as a genetic test-stand of intermolecular interactions between membrane-spanning protein domains. This could expand the technical utility of ToxR beyond acting as a genetic indicator for various properties of periplasmic protein domains, namely dimerization (Kolmar *et al.*, 1994) and folding stability (Kolmar *et al.*, 1995).

Since this manuscript was prepared, a paper has been published (Ottemann and Mekalanos, 1995) which challenges the notion of protein dimerization being the key step in transcription activation by ToxR. Its authors now favour ToxR membrane localization as the crucial pre-requisite, irrespective of the monomer/dimer status of the protein. Ottemann and Mekalanos rest their argument mainly on the following results: they find β-lactamase (Bla), a monomeric protein, to lead to high constitutive transcription when fused to ToxR' (similar value to that obtained with a corresponding ToxR'PhoA chimera). Second, they find a ToxR'ZIP fusion protein (our nomenclature) to be a transcription activator but not ToxR'-(ΔTM)ZIP, and it is implicit in their argument that ToxR'ZIP is anchored in the inner membrane and ToxR'(ΔTM)ZIP is not. It is this very last point with which we agree. For the rest, it is important to bear in mind that there is no experimental evidence for ToxR'ZIP being integrated into the inner membrane; on the contrary, our electron microscopy results obtained with ToxR'-ZIP^{SEN} argue against it. The logically consistent possibility, however, remains that the immunotag of ToxR'ZIP^{SEN} actively prohibits stable anchoring in the membrane. Even if that were so, the notion of membrane-anchoring being the main and possibly only pre-requisite of transcription activation by ToxR would still be at variance with our data; ToxR'(ΔTM)MalE/ZIP is clearly both a cytoplasmic protein and an activator of transcription from the *ctx* promoter. The failure reported by Ottemann and Mekalanos (1995) of ToxR'(ΔTM)ZIP to activate transcription may be due to any of a number of reasons, among them rapid proteolysis of this particular chimera in *E.coli* (the authors demonstrate its accumulation only in *V.cholerae* cells) or unfavourable steric arrangement of the DNA-binding domains in the assumed ToxR'(ΔTM)/ZIP homodimer. Finally, a ToxR'Bla chimera in our hands leads to only background activation of transcription (approximately the same value as with ToxR'MalE; data not included in this study). The reasons for this discrepancy of experimental data obtained with near-identical constructs are not understood.

Materials and methods

Enzymes and chemicals

RNase A, T4 polynucleotide kinase and T4 DNA ligase were purchased from Boehringer Mannheim; *Taq* DNA polymerase was from Boehringer Mannheim and Cetus. Sequenase™ was from United States Biochemicals. Restriction enzymes were from Boehringer Mannheim, New England Biolabs and Gibco/BRL. All other chemicals were of analytical grade and purchased from Merck or Sigma. The monoclonal antibody (VII-E-7) against a 13 residue C-terminal epitope of Sendai virus L-protein (DGSLGDIEPYDSS, Einberger *et al.*, 1990) was a gift from H.Einberger and H.P.Hofschneider (Max-Planck-Institut für Biochemie, Martinsried bei München).

Microbiological procedures

The medium used for growth and maintenance of *E.coli* strains was dYT (1% Bacto yeast extract, 1.6% Bacto tryptone, 0.5% NaCl) and M9 minimal medium (Miller, 1972). Isopropyl-thio-β-D-galactoside (IPTG, BTS Biotech) was added to agar plates or to liquid media at a final concentration of 0.4 mM. Ampicillin, chloramphenicol and tetracycline were used at a final concentration of 100, 25 and 12.5 μg/ml, respectively.

Escherichia coli strains

BMH71-18: F' *lacI*^{q} *lacZ*ΔM15, *pro*A$^+$B$^+$; Δ(*lac-pro*AB), *sup*E, *thi*. PD28 (Duplay *et al.*, 1987): F$^-$, *thi*A, *rel*A, *ara*D139, Δ*lac*U169, *rps*L, *malT*^{c}1, Δ*mal*E444, Δ(*srl*R–*rec*A)306::Tn*10*. *Escherichia coli* K12 strain FHK12 [F' *lacI*^{q} *lacZ*ΔM15, *pro*A$^+$B$^+$; *ara*, Δ(*lac-pro*AB), *rps*L, (ϕ80 d*lacZ*ΔM15), *att*B::(*ctx*::*lacZ*)] was constructed in two steps: FHK11 was constructed from strain JM83 {F$^-$, *ara*, Δ(*lac-pro*AB), *rps*L, [ϕ80 d*lac*Δ(*lacZ*)M15]} (Yanish-Perron *et al.*, 1985) by chromosomal integration of the *lac*Z gene under control of the *V.cholerae ctx*AB promoter from plasmid pLDR10–*ctx*::*lac*Z (see section Plasmid constructions) at the phage λ *attB* site (Diederich *et al.*, 1992). In FHK11 the *ctx*::*lacZ* fusion integrated at the *att*B site is tagged with the ampicillin resistance gene. FK12 was subsequently constructed from FHK11 by F-duction (Miller, 1972) with BMH71-18 (F' *lacI*^{q} *lacZ*ΔM15, *pro*A$^+$B$^+$; Δ[*lac-pro*AB], *sup*E, *thi*) (source, B.Müller-Hill). F-ductants were selected on M9 minimal medium containing ampicillin (100 μg/ml).

DNA procedures

Standard DNA procedures, such as plasmid isolation, ligation, restriction analysis of plasmids and isolation of DNA fragments were as described by Sambrook *et al.* (1989). Site-directed mutagenesis was performed as described by Kunkel *et al.* (1987). The identity of all mutations was confirmed by nucleotide sequence analysis using Sequenase™ as described by Ansorge *et al.* (1987). Unless stated otherwise, polymerase chain reaction (PCR) using *Taq* polymerase (Higuchi *et al.*, 1988) was as follows: 45 s denaturation at 94°C, 60 s annealing at 37°C and 2 min elongation at 72°C, 30 cycles.

Oligonucleotide synthesis

Synthetic 2'-deoxyoligonucleotides for directed mutagenesis and PCR were prepared using the Applied Biosystems 380B synthesizer as described (Beaucage and Caruthers, 1981) and purified via polyacrylamide gel electrophoresis (Wu *et al.*, 1984). CTXUP (53 mer):

5′-GTGTGTGATACGAAACGAAGCATTGGATCCTAGAAGTGAA-ACGGGGTTTACCG; IL4RTM1 (28 mer): 5′-TCGAGCTAGCCTCCT-GCTGGGGGTCAGC-3′; IL4RTM2 (29 mer): 5′-ATCAGGATCCCGG-TGATGCTGACATAGCA-3′; IMG097 (54 mer): 5′-GCCCTGAG-CAGCCCGGTTTTCCAGAACAGGCGTTAGGGGTTTAAAGCTGG-ATTG; IMG249 (34 mer): CATCTGGATATCCTACGTTAGGGG-TTTAAAGCTG; IMG329 (31 mer): 5′-CACGACGTTGTAGTACTA-CCTTTACCATATA; IMG382 (34 mer): 5′-GAAAGATATCTT-GCAAAGAATGAAACAACTTGAA; IMG383 (35 mer): 5′-GTAAA-TGAAAGCTTCAGCGTTCGCCAACTAATTTC; IMG385 (45 mer): 5′-TTGGCTTGGGTTGATCAGGATCCCAAGCTAGCTCGATTCCC-CAAG; IMG388 (50 mer): 5′-TCGAGCTAGCCCGGTTACCTTCAT-CATCGCTACCGTTGAAGGAGTACTGC; IMG390 (59 mer): 5′-ATC-AGGATCCCAACCACGACAACCAGGATCAGGAACAGCAGTACT-CCAACAACGGTAGC; IMG413 (39 mer): 5′-GAAAGATATCTTGC-AAAGAATGAAACAACCTGAAGACAA; IMG436 (23 mer): 5′-GAC-AACCAGGTCATCGATTCCCC; IMG437 (87 mer): 5′-TGCTCTAG-ATTAAGAGGAGTCATATGGTTCGATGTCACCCAAGGATCCGTC-GCCGCGAGTGATTTGAAGCTTGCGTTCGCCAACTAA; IMG454 (89 mer): 5′-GGCTTGGGTTAGTGAACAACGTGACCGTGGTGCT-ATAGAACAGGCTCAGCAGGAACAGCACGATGAAGGTGCTGGC-TCGATTCCCCAAG; IMG470 (32 mer): 5′-GTAATACTGCGAGG-GCTAGCAAGACCGCTATC; IMG659 (24 mer): 5′-AAGAGATAT-CAAAATCGAAGAAGG; RSPU (18 mer): 5′-GTGAATTTCGACC-TCTAG.

Phasmid–plasmid constructions

For construction of pLDR10-ctx::lacZ, cells of a pathogenic *V.cholerae* strain (gift from H.Hesemann, University of Würzburg) were lysed in water by boiling at 100°C for 10 min. From this crude cell lysate the *ctx*AB promoter/operator region (Mekalanos *et al.*, 1983) was amplified via PCR and the primers CTXUP and IMG329 (45 s denaturation at 94°C, 60 s annealing at 37°C and 2 min elongation at 72°C, 30 cycles). The resulting amplified DNA, which contains the *ctx* promoter/operator region plus codons 1–4 of *ctx*A (ranging from nucleotide pairs 357–527 of GenEMBL accession No. X00171) was fused to the coding sequence of the *lac*Zα gene fragment (starting at nucleotide pair 34 according to GenEMBL accession No. V00296) from pMa5–b9 (Kolmar *et al.*, 1990) via PCR using the primer pair CTXUP and RSPU. The resulting *ctx::lac*Zα fragment which is bordered by a *Bam*HI and an *Xba*I site was cloned into *Bam*HI–*Xba*I-cleaved pMc5Δbla, a derivative of pMc5–8 (Stanssens *et al.*, 1989) with the *bla* gene deleted. Comparison of the nucleotide sequence of the PCR-amplified *ctx*A promoter/operator region with the published *ctx*A gene sequence (Mekalanos *et al.*, 1983; GenEMBL accession No. X00171) indicated a perfect identity with the exception that the upstream regulatory element TTTTGAT (Miller *et al.*, 1987) is repeated 7-fold instead of 8-fold. In a second round, the complete *lac*Z coding sequence was introduced by gapped duplex DNA formation (Kramer and Fritz, 1987) of *Bgl*I–*Xba*I cleaved pMc5Δbla–*ctx::lac*Zα with single-stranded DNA of pMaLINKLAC (a gift from R.Frank, GBF Braunschweig), which is a derivative of pMa5–8 (Stanssens *et al.*, 1989) that contains the *lac*Z coding sequence bordered by *Bam*HI restriction sites. After transformation of the DNA mismatch repair-deficient strain HKF1 (Kolmar *et al.*, 1990) and selection for chloramphenicol resistance, cells were identified that carried pMc5Δbla::ctxlacZ. Finally, the *ctx::lac*Z gene was inserted as a *Bam*HI fragment into *Bam*HI-cleaved pLDR10 (Diederich *et al.*, 1992) oriented such that the transcription from the *ctx* promoter occurs in the opposite direction compared with the *bla* promoter of pLDR10.

Construction of pHKToxR′ was described elsewhere (Kolmar *et al.*, 1994). This phagemid vector contains the ToxR promoter/operator region plus the N-terminal 210 residues encoding the ToxR cytoplasmic domain plus TM region. The unique *Eco*RV and *Xba*I sites at the *tox*R′ 3′-terminus were used for introduction of various DNA fragments. In pHKToxR′(ΔTM) the ToxR TM region encoding DNA stretch was removed by directed mutagenesis using the oligonucleotide IMG436, which introduces a TGA codon after *toxR* codon 182.

For replacement of the ToxR′ TM region by the TM regions of the *neu* proto-oncogene-derived receptor and the interleukin receptor α chain, the ToxR′ TM region encoding DNA stretch was deleted by directed mutagenesis from a derivative of pHKToxR′REI (Kolmar *et al.*, 1994) with the mutagenic primer IMG385, which simultaneously introduces the restriction sites *Nhe*I and *Bam*HI (pHKToxR′-385). Both strands of the partially complementary oligonucleotide pair IMG388/IMG390 encoding residues 653 to 678 (according to SWISSPROT No. P06494; Bargmann *et al.*, 1986) of the *neu* proto-oncogene-derived receptor were elongated via PCR, the resulting duplex DNA was restricted with *Nhe*I and *Bam*HI and ligated with *Nhe*I–*Bam*HI-cleaved pHKToxR′-385, resulting in pHKToxR′(TM^{neu}). For construction of pHKToxR′(TM^{II4}) a DNA segment encoding residues 233–256 (according to SWISSPROT No. XP24394; Idzerda *et al.*, 1990) of the interleukin receptor α chain was amplified by PCR using the oligonucleotide pair IL4RTM1/Il4RTM2 and template DNA from plasmid pKCR–hIL4R (W.Kammer and K.Friedrich, unpublished results). This fragment was cleaved with *Nhe*I and *Bam*HI and ligated with the large *Nhe*I–*Bam*HI fragment of HKToxR′(TM^{neu}). Replacement of the ToxR′ TM helix encoding DNA stretch by a nucleotide sequence deduced from the TM region of membrane-anchored mouse IgM (residues 18–38 according to SWISSPROT No. P20769; Rogers *et al.*, 1980) was achieved by directed mutagenesis using the oligonucleotide IMG454. Directed mutagenesis with oligonucleotide IMG470 led to the substitution of *toxR′* proline codon 192 by alanine.

For the construction of pHKToxR′MalE derivatives the *mal*E gene was excised from vector pMal-p (New England Biolabs; Maina *et al.*, 1988) as a *Pvu*II–*Xba*I fragment and cloned into an *Eco*RV/*Xba*I-cleaved derivative of pMa5–8 (Stanssens *et al.*, 1989). The resultant construct was used for PCR amplification of the coding sequence for mature MalE using the primers IMG659 and RSPU. The amplified DNA fragment was cloned as an *Eco*RV/*Xba*I fragment into *Eco*RV/*Xba*I-cleaved pHKToxR′. An amber stop codon was introduced at the *mal*E 3′-terminus by *Xba*I cleavage, followed by fill-in reaction using T4 polymerase and religation.

For construction of pHKToxR′ZIP derivatives, the coding sequence for the GCN4 leucine zipper (ranging from nucleotide pair 1516–1623 according to GenEMBL accession No. K02205) was PCR amplified from *S.cerevisiae* chromosomal DNA using the primers IMG382 and IMG383. Variant genes *zip*^{L5P} and *zip*^{SEN} were obtained via PCR amplification using the primer combination IMG413/IMG383 and IMG382/IMG437, respectively. The resultant amplified DNA was restricted with *Eco*RV and *Hin*dIII and ligated into an *Eco*RV/*Hin*dIII-cleaved derivative of pHKToxR′. For the construction of pHKToxR′MalE/ZIP the ZIP encoding *Eco*RV/*Xba*I fragment from pHKToxR′ZIP was introduced into *Stu*I–*Xba*I-cleaved pHKToxR′MalE.

pMalZIP, pMalZIP^{L5P} were constructed from pMal-p (New England Biolabs; Maina *et al.*, 1988) by replacement of the 820 bp *Bgl*II–*Xba*I fragment of pMal-p, which encodes the 3′-proximal moiety of *malE* with a *Bgl*II–*Xba*I fragment from pHKToxR′MalE/ZIP or pHKToxR′MalE/ZIP^{L5P}, respectively, which encode the 3′ terminal *mal*E moiety plus the respective *zip* sequence. In the same way, the 920 bp *malE/zip* fragment was introduced into pMal-c (New England Biolabs; Maina *et al.* 1988) resulting in cMalZIP and cMalZIP^{L5P}. To obtain a resistance marker for transformation of FK12, which by itself confers ampicillin resistance, a tetracycline resistance gene was inserted into all three vectors. This was achieved by insertion of the *tet* gene encoding *Ssp*I–*Stu*I fragment from vector YIP5 (New England Biolabs) into the unique *Sca*I restriction site residing within the *bla* gene.

pHKToxR′PhoA was constructed by insertion of the 1.7 kb *Eco*RV/*Xba*I fragment from vector pHKREI (Kolmar *et al.*, 1992), which contains the coding sequence for mature alkaline phosphatase into *Eco*RV/*Xba*I-restricted pHKToxR′ followed by fusion of *phoA* (starting from nucleotide pair 361 according to GenEMBL accession No. X04586) to codon 210 of *toxR′* by site-directed mutagenesis using the mutagenic primer IMG097.

For production of ToxR′ devoid of a periplasmic module, an amber codon was introduced between *toxR′* and rei_v coding regions in pHKToxR′REI (Kolmar *et al.*, 1994) using the mutagenic oligonucleotide IMG249.

pBS-ToxR′ZIP^{SEN} and pBS-ToxR′MalE/ZIP^{SEN} were obtained by insertion of the ToxR′ZIP^{SEN} or ToxR′MalE/ZIP^{SEN} encoding *Sma*I–*Xba*I fragment from pHKToxR′ZIP^{SEN} and pHKToxR′MalE/ZIP^{SEN}, respectively, into *Sac*I–*Xba*I restricted pBluescript SK (Stratagene; GenEMBL accession No. 52325).

Electron microscopy studies

BMH71-18 carrying pBS-ToxR′ZIP^{SEN} or pBS-ToxR′MalE/ZIP^{SEN}, respectively, was grown overnight at 37°C in dYT medium containing 25 μg/ml ampicillin and 1 mM IPTG. For post-embedding immunogold labelling, cells from 200 ml culture were harvested by centrifugation and washed twice with 50 mM potassium phosphate, pH 7.0 and subsequently fixed with a mixture of 0.3% (v/v) glutaraldehyde and 0.2% (w/v) paraformaldehyde in the buffer described by Roth *et al.* (1981). The samples were embedded in Lowicryl K4M (Lowi, Waldkraiburg, Germany) as described (Roth *et al.*, 1981), the only

exception being that methanol rather than ethanol was used for dehydration.

Ultrathin sections were mounted onto formvar-covered nickel-grids, and free protein binding sites were blocked with 0.5% (w/v) skim milk (Duchamel and Johnson, 1985; Griffith, 1993). The samples were incubated overnight at 4°C or 3 h at room temperature with a series of dilutions of the primary monoclonal antibody VII-E-7 (Einberger *et al.*, 1990). Sections were washed by a spray of PBS–Tween [50 mM potassium phosphate, 0.9% (w/v) NaCl, 0.05% (v/v) Tween 20, pH 6.9]. The grids were then incubated with a series of dilutions of rabbit anti-mouse IgG gold complex (RAMG; Dakopatts, Hamburg) at room temperature for 30 min up to 1 h. Afterwards, the sections were rinsed as described above, followed by a washing step in H_2O. Post-staining was performed in 4% (w/v) aqueous uranyl acetate, pH 4.5 for 3–5 min. The specificity of the labelling was demonstrated by a control experiment using only the RAMG complexes. Micrographs were taken on a Philips EM 301 electron microscope at 80 kV acceleration voltage. Magnifications were calibrated with a cross-lined grating replica (Balzers Corp., Liechtenstein).

Assay of β-galactosidase activity

For determination of specific β-galactosidase activity in crude cell lysates of FHK12 transformed with the respective pHKToxR′ derivative, 20 μl of an overnight culture grown at 37°C in the presence of chloramphenicol (25 μg/ml) and IPTG (0.4 mM) were transferred into a microtitre well. After addition of 100 μl chloroform-saturated Z-buffer (100 mM sodium phosphate, 1 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7.0; Miller, 1972), the OD_{620} was determined using an ELISA reader (SLT340 ATTC, SLT Labinstruments, Crailsheim, Germany). Cells were lysed by addition of 50 μl Z-buffer containing 0.4% (w/v) SDS and incubation at 28°C for 10 min. 50 μl of Z-buffer containing 4 mg/ml *o*-nitrophenyl-β-D-galactopyranoside were added to the cell lysates and the optical density was recorded automatically every 30 s over a period of 60 min at 28°C. The specific β-galactosidase activity in the cell lysates was calculated from V_{max}, the maximum slope of each reaction curve and the optical density of the cell suspension as described (Miller, 1972): units = $1000 \times V_{max}/OD_{620}$.

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