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Fusion Proteins Containing the A2 Domain of Cholera Toxin Assemble with B Polypeptides of Cholera Toxin To Form Immunoreactive and Functional Holotoxin-Like Chimeras

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Cholera enterotoxin (CT) is produced by *Vibrio cholerae* and excreted into the culture medium as an extracellular protein. CT consists of one A polypeptide and five B polypeptides associated by noncovalent bonds, and CT-B interacts with CT-A primarily via the A2 domain. Treatment of CT with trypsin cleaves CT-A into A1 and A2 fragments that are linked by a disulfide bond. CT-B binds to ganglioside G_{M1}, which functions as the plasma membrane receptor for CT, and the enzymatic activity of A1 causes the toxic effects of CT on target cells. We constructed translational fusions that joined foreign proteins via their carboxyl termini to the A2 domain of CT-A, and we studied the interactions of the fusion proteins with CT-B. The A2 domain was necessary and sufficient to enable bacterial alkaline phosphatase (BAP), maltose-binding protein (MBP) or β-lactamase (BLA) to associate with CT-B to form stable, immunoreactive, holotoxin-like chimeras. Each holotoxin-like chimera was able to bind to ganglioside G_{M1}. Holotoxin-like chimeras containing the BAP-A2 and BLA-A2 fusion proteins had BAP activity and BLA activity, respectively. We constructed BAP-A2 mutants with altered carboxyl-terminal sequences and tested their ability to assemble into holotoxin-like chimeras. Although the carboxyl-terminal QDEL sequence of the BAP-A2 fusion protein was not required for interaction with CT-B, most BAP-A2 mutants with altered carboxyl termini did not form holotoxin-like chimeras. When holotoxin-like chimeras containing BAP-A2, MBP-A2, or BLA-A2 were synthesized in *V. cholerae*, they were found predominantly in the periplasm. The toxin secretory apparatus of *V. cholerae* was not able, therefore, to translocate these holotoxin-like chimeras across the outer membrane.

Cholera toxin (CT) is the prototype for the heat-labile enterotoxins of gram-negative bacteria (3, 10). CT consists of one A polypeptide (CT-A, 27 kDa) noncovalently associated with five B polypeptides (CT-B, 11.6 kDa). CT-A and CT-B are synthesized as cytoplasmic precursor polypeptides that are secreted across the plasma membrane and processed by removal of their signal sequences to mature periplasmic polypeptides (9). Mature CT-B monomers can assemble spontaneously into pentamers in the periplasm or in vitro, but assembly of CT-A and CT-B into holotoxin occurs faster than formation of B pentamers (9). CT-A and mature B pentamers do not interact under nondenaturing conditions in vitro to form holotoxin. Hence, CT-A is believed to interact with CT-B monomers or immature CT-B oligomers during assembly of holotoxin. *Vibrio cholerae*, but not *Escherichia coli*, can excrete holotoxin or B pentamers into culture medium by translocating them across the outer membrane (9, 22). The outer membrane of *V. cholerae* is postulated to contain a toxin secretory apparatus that specifically translocates CT from the periplasm to the exterior of the cell, but the nature and functions of the components of the toxin secretory apparatus have not yet been characterized.

CT-A in CT is cleaved by *V. cholerae* protease(s) or trypsin to form nicked CT, in which fragments CT-A1 (22 kDa) and CT-A2 (5 kDa) are linked by a disulfide bond (17). The B polypeptides of CT bind to ganglioside G_{M1} in the plasma membranes of target cells (4). CT-A enters the target cells, and the CT-A1 fragment catalyzes ADP ribosylation of Gsα, a GTP-binding protein that functions as a regulatory component of the plasma membrane adenylate cyclase system, and activates adenylate cyclase.

Although CT has been crystallized (33), the three-dimensional structure of CT at high resolution has not yet been reported. The homologous heat-labile enterotoxin LTp from *E. coli* has been crystallized both as the free holotoxin and as the complex with lactose, and X-ray diffraction has revealed the three-dimensional structures of the holotoxin and its receptor-binding site (31, 32). These recent studies demonstrate that CT-A interacts with CT-B primarily through its A2 domain, which inserts into the center of the ring formed by the five CT-B polypeptides in holotoxin. Earlier studies demonstrated that CT-A1 can be released by treatment of nicked CT in vitro with reducing agents and 4 M urea, leaving CT-A2 associated with B pentamer (20). This demonstrates that CT-A1 is not required to maintain the stability of the noncovalent interactions between CT-A2 and B pentamer. CT-A1 by itself is unable to assemble with CT-B, indicating that CT-A2 is required for assembly of holotoxin. The present study was undertaken to determine whether the presence of the CT-A2 domain in hybrid polypeptides is sufficient to enable them to assemble with CT-B to form holotoxin-like chimeras and to examine the effects of changes in amino acid sequence at the carboxyl-terminal end of A2 on formation of holotoxin-like chimeras.

(A preliminary report of this work was presented at the Fifth European Workshop on Bacterial Protein Toxins, Veldhoven, The Netherlands, June 30 to July 5, 1991 [13].)

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TABLE 1. Plasmids used and clones constructed in this study[a]

Plasmid	Phenotype and genotype	Characteristic(s)	Reference or source
pSKII−	Ap ss-ori	ColE1-derived phagemid cloning vector	Stratagene
pK184	Km p15a-ori	ColE1-compatible cloning vector	11
pK194	Km p15a-ori	ColE1-compatible cloning vector	11
pPhoA1-3	Ap BAP$^+$	*phoA* gene fusion cassette	15
pMAL-p	Ap MBP-*lacZα*	MBP fusion vector	New England Biolabs
pK18	Km	pUC-type vector	23
pUK21	Km ss-ori	Phagemid vector plac +2 bp	34
pMGJ60	Ap *ctxA*$^+$ *ctxB*$^+$	Wild-type CT operon clone, IPTG inducible in pSKII−	This work
pMGJ64	Ap *ctxA*$^+$ *ctxB*$^+$	pMGJ60 with an *Eco*RI site mutating CT-A K137Q (AAG → CAG)	This work
pMGJ67	Ap *ctxA*$^+$ *ctxB*$^+$	pMGJ64 Δ*Sma*I site in multiple cloning site	This work
pMGJ6701	Ap *ctxB*$^+$	pMGJ67 ΔCT-A A190–N197, *Eco*RI site added; T198S	This work
pMGJ6713	Ap *ctxB*$^+$	Derivative of pMGJ6701 with [*Eco*RI]	This work
pMGJ6730	Ap *ctxA*$^+$ *ctxB*$^+$	pMGJ67 with *Sma*I site CCGGGT → CCCGGG (P185–G186 unchanged)	This work
pMGJ19	Ap *ctxB*$^+$	Wild-type *ctxB* gene, *toxR* controlled	12
pMGJ11	Ap *ctxB*$^+$	Wild-type *ctxB* gene, IPTG inducible	12
pMGJ40	Tc *toxR*$^+$	RP4 derived (ColE1 and p15a compatible) clone expressing *toxR* gene	12
pMGJ1971	Ap *ctxB*1971	CT-B R35D mutant derivative of pMGJ19	12
pMGJ1972	Ap *ctxB*1972	CT-B R35E mutant derivative of pMGJ19	12
pMGJ1963	Ap *ctxB*1963	CT-B R35N mutant derivative of pMGJ19	12
pMGJ1110	Ap *ctxB*1110	CT-B K34D mutant derivative of pMGJ11	12
pMGJ37	Ap *ctxB*$^+$	*Sac*II (CCGCGG) linker in *Ssp*I site in CT-A2-coding region of pMGJ19	This work
pMGJ85	Km *ctxB*$^+$	Wild-type *ctxB* gene in pK184	This work
pMGJ86	Km BAP-A2$^+$	Fusion protein from pMGJ83 *Hin*dIII-[*Acc*I] cloned into pK194 *Hin*dIII-*Sma*I	This work
pMGJ83	Ap BAP-A2$^+$ *ctxB*$^+$	*Hin*dIII-*Eco*RV of pPhoA1-3 into pMGJ6712 *Hin*dIII-*Xmn*I (partial)	This work
pMGJ83-1	Ap BAP-A2$^+$	[*Acc*I] derivative of pMGJ83 creating frameshift in *ctxB*	This work
pMGJ84	Ap BAP-A2′	*Xba*I-*Eco*RI (partial) of pMGJ83 cloned into pSKII− (deleted for *ctxB*)	This work
pMGJ84-1	Ap BAP-A2′	12-bp *Nhe*I linker (CTAGCTAGCTAG) cloned into [*Kpn*I] site of pMGJ84	This work
pMGJ96	Ap MBP-A2$^+$, *ctxB*$^+$	*Sma*I-*Hin*dIII from pMGJ6729 cloned into pMAL-p *Stu*I-*Hin*dIII	This work
pMGJ98	Ap BAP-A2	[*Sac*II]-*Kpn*I from pMGJ37 (*ctxA2 ctxB*$^+$) cloned into pPhoA1-3 *Eco*RV-*Kpn*I	This work
pMGJ99	Km Ap ss-ori	*Nhe*I-*Bst*BI Km^r gene of pK18 cloned into pSKII− *Xba*I-*Acc*I	This work
pMGJ9901	Km (Ap^s)	His-Trp of *bla* mutated to Asp-Ile, *Eco*RV site introduced	This work
pMGJ102	Km (Ap^s)	*Ssp*I-*Eco*RV fragment of pMGJ9901 cloned into pK18 *Sma*I	This work
pMGJ104	Km BLA-A2 *ctxB*$^+$	*Cla*I-*Hin*dIII *ctxA2 ctxB* gene fragment of pMGJ64 cloned into pMGJ102 *Acc*I-*Hin*dIII	This work
pMGJ111	Km Ap BLA-A2 *ctx*B$^+$	Asp-Gly-(Pro) at *bla* fusion joint mutated back to wild-type His-Trp-(Gly)	This work
pMGJ112	Km Ap BLA-A2 *ctxB*$^+$	Insert from pMGJ111 cloned into pK18 *Kpn*I-*Hin*dIII	This work

[a] Symbols used in this table are defined as follows: ori, origin of replication; Δ, deletion; Ap, Km, Tc, resistance to ampicillin, kanamycin, or tetracycline, respectively; [ ], restriction site made blunt ended; ^{s}, sensitive; BAP-A2, MBP-A2, or BLA-A2, fusions of BAP, MBP, or BLA, respectively, to CT-A2 domain; $^+$, wild type; ′, mutant.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *E. coli* TG1 (*phoA*$^+$; Amersham Corp., Arlington Heights, Ill.), TX1 (streptomycin-resistant derivative of TG1, conjugated with XL1 blue [Stratagene, La Jolla, Calif.] to receive F′ *lacI*^{q} Tn*10* [this study]), and CC118 F′ Tc(Δ*phoA*) (18) were used as hosts for cloning of recombinant plasmids and expression of fusion proteins. Secretion of holotoxin-like chimeras was examined in *V. cholerae* CVD44, an *hly*::Tc derivative of JBK70 (Δ*ctxAB* [14]). Bacteria were grown in 2YT liquid medium (24) in Erlenmeyer flasks at 37°C with shaking (200 rpm) or plated on LB agar plates (21) with antibiotics as indicated for specific experiments (ampicillin, 50 μg/ml; kanamycin, 25 μg/ml; tetracycline, 10 μg/ml). Plasmids used in this study are described in Table 1.

Expression of fusion proteins. Expression of fusion proteins in logarithmic-phase cells (A_{600} = 0.8 to 1.0) was induced by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside) to 0.4 mM and then growth overnight. Crude periplasmic extracts were prepared by treating 5×- or 10×-concentrated suspensions of cells in phosphate- or Tris-buffered saline (21) with 1 mg of polymyxin B per ml for 5 to 30 min at 37°C. Cell debris was pelleted in a microcentrifuge for 5 min, and supernatants were collected and stored at 4°C. The localization of the holotoxin-like chimeras in strains of *V. cholerae* carrying specific plasmids was determined by growth in liquid medium and then assays of both the cell-free culture supernatants and 1× periplasmic extracts for hybrid antigen and CT-B pentamer.

DNA manipulations. Genetic fusions of the segment of *ctxA* that encodes the CT-A2 domain to the coding sequences for bacterial alkaline phosphatase (BAP, encoded by the *phoA* gene), maltose-binding protein (MBP, encoded by the *malE* gene), or β-lactamase (BLA, encoded by the *bla* gene) were done by standard molecular cloning techniques (24). Plasmids were introduced into *E. coli* by transformation (6) and into *V. cholerae* by electroporation (19), using the Bio-Rad Gene Pulser, 0.2-mm-wide cuvettes, 25 μF, 200 Ω, 2,500 V, a pulse length of approximately 4.5 ms, and 10% sucrose as an osmotic protectant. The nucleotide sequences of the fusion joints were verified by rescuing single-stranded DNA and sequencing by the dideoxy chain termination method as previously described (12) and are shown together with the corresponding amino acid translations in Table 2.

TABLE 2. Nucleotide sequences and deduced amino acid sequences of selected regions of *ctxA*, *phoA*, *malE*, *bla*, and gene fusion constructs

Protein and plasmid	Amino acid sequence and nucleotide sequence[a]	Manipulation and restriction site(s)
	187 199 240	
CT-A	...P G C G N A P R S S M S N T C D E...NRIQDEL	None
pMGJ67	...CCGGGTTGTGGGAATGCTCCAAGATCATCGATGAGTAATACTTCGGATGAA..	*Cla*I
	...P G C G ---------- N S C D E...NRIQDEL	G→T, Δ24 bp
pMGJ6701	...CCGGGTTGTGG----------GAATTCTTCGGATGAA..	*Eco*RI
	...P G C G N * + frameshift N S C D E...NRIQDEL	Fill in *Eco*RI
pMGJ6713	...CCGGGTTGTGGGAATT---------- AATTCTTCGGATGAA..	Creates *Xmn*I
	447	
BAP	...L G D I *	None
pPhoA1-3	...CTGGGGGATATCTAGA...	*Eco*RV, *Xba*I
	BAP-447 CT-199	
BAP-A2$_{197-240}$	...L G D N S C D E ...NRIQDEL	Ligate
pMGJ83	...CTGGGGGATAATTCTTGT...	[*Eco*RV-*Xmn*I]
	CT-235	
BAP-A2$_{197-236'}$	..R I R Y Q A Y R Y R R P A G G A R Y + 57 residues	
pMGJ84	...GAATTCGATATCAAGCTTATCGATACCGTCGACCTGCAGGGGGGGCCCGGTACC	*Eco*RI, *Kpn*I
	..L A S *	
	CT-235 .. A S *	
BAP-A2$_{197-236'}$	..R I R Y Q A Y R Y R R P A G G A R *	Add linker
pMGJ84-1	...GAATTCGATATCAAGCTTATCGATACCGTCGACCTGCAGGGGGGGCCCGCTAGCTAGCTAGC..	*Nhe*I
	223 240	
CT-A2	...I R G F SGYQSDIDTHNRIKDEL	Add linker
pMGJ37	..AATCCGCGGATTT...	[*Ssp*I]::*Sac*II
	BAP-447 CT-223 240	
PHOA-A2$_{222-240}$	...L G D G F SGYQSDIDTHNRIKDEL	Blunt and ligate
pMGJ98	...CTGGGGGATGGATTT...	[*Eco*RV-*Sac*II]
	CT-187 199	
CT-A	...P G C G N A P R S S M S N T C D E ...QDEL	Mutate C→G T→G
pMGJ6730	...CCCGGGTGTGGGAATGCTCCAAGATCATCGATGAGTAATACTTCGGATGAA ..	*Sma*I, *Cla*I
	366	
MBP-LacZα	...TNSSSVPGRGS I E G R P E F S K ...	
pMAL-p	...ATCGAGGGTAGGCCTGAATTCAGTAAA...	*Stu*I
	MBP-366 CT-186 199	
pMGJ96	...TNSSSVPGRGS I E G R G C G NAPRSSMSNTCDE ... QDEL	Ligate
malE	....ATCGAGGGTAGGGGGTGTGGG...	[*Stu*I-*Sma*I]
	260 263	
BLA	... I K H W *	
pMGJ99	... ATTAAGCATTGGTAA...	None
BLA-A2	... I K D I *	Mutate
pMGJ9901	... ATTAAGGATATCTAA...	*Eco*RV
BLA-LacZα	... I K D G D P L E S T C ...	Ligate
pMGJ102	... ATTAAGGATGGGGATCCTCTAGAGTCGACCTGCAG...	[*Eco*RV-*Sma*I]
	BLA-260 CT-193 240	
BLA-A2	... I K D G D P L E S M S N T C D E ...QDEL	Ligate
pMGJ104	... ATTAAGGATGGGGATCCTCTAGAGTCGATGAGTAATACTTCGGATGAA...	[*Acc*I-*Cla*I]
	240	
BLA-A2	... I K H W G P L E S M S N T C D E ...QDEL	Mutate
pMGJ111	... ATTAAGCATTGGGGTCCTCTAGAGTCGATGAGTAATACTTCGGATGAA...	Lose *Bam*HI

[a] [ ], restriction sites lost on ligation. Underline, restriction site sequence. Boldface bases or residues differ from or are not found in the wild-type gene or protein. Numbers above the residues correspond to the residue in the wild-type protein, and the last digit is directly over the residue. Dashes show deletions of residues or bases; dots indicate bases or residues not shown.

Addition of unphosphorylated oligonucleotide linkers to linear double-stranded DNA molecules was done as described previously (16).

Construction of BAP-A2 fusion clones. The source of the region encoding the CT-A2 domain and *ctxB* was plasmid pMGJ6713, derived from pMGJ6701 by filling in the *Eco*RI site between CT-A1 and CT-A2. Plasmid pMGJ6701 was constructed by site-directed mutagenesis from pMGJ67 (expressing a full-size CT operon) by deleting the coding region for eight amino acids within the disulfide loop of CT-A and introducing an *Eco*RI site (Table 2; Fig. 1A). The *Xmn*I site in pMGJ6713 (created by the filling in the *Eco*RI site) introduces a frameshift causing termination of translation after residue 189 in *ctxA*. The *Xmn*I cleavage site remains in frame with the coding region for the CT-A2 domain which also has a Thr-198-to-Ser-198 mutation. A *Hin*dIII-*Eco*RV cassette from pPhoA1-3, a vector containing an engineered *phoA* gene, was cloned into a *Hin*dIII-*Xmn*I (partial) digest of pMGJ6713 to create pMGJ83 (Fig. 1A), such that the *phoA* gene was fused in frame with the CT-A2 domain of *ctxA* (residues 197 to 240, Table 2). This fusion protein was designated BAP-A2.

To construct a fusion of BAP to residues 222 to 240 of CT-A2 (the extended-sheet domain), we made use of an *Ssp*I

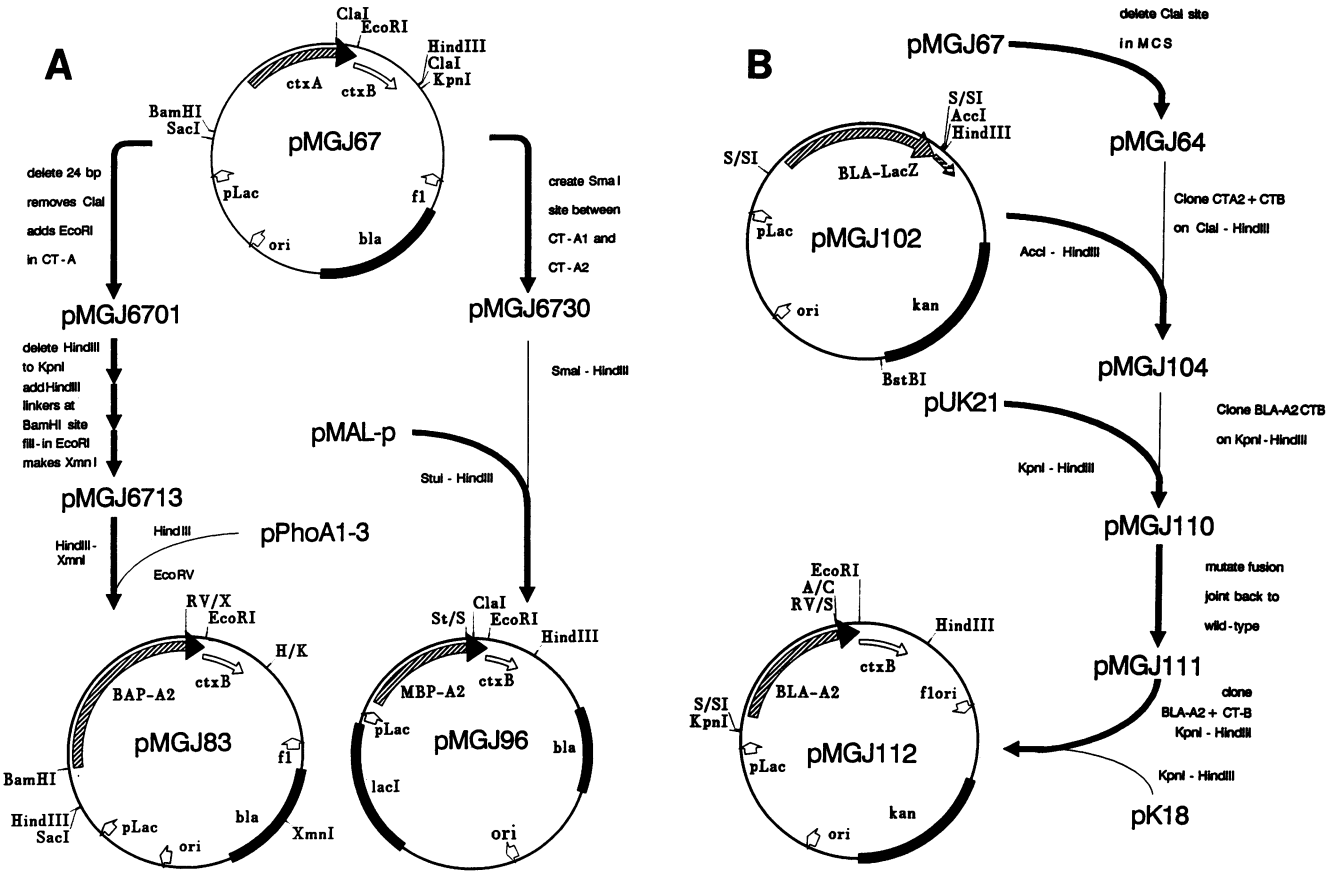

FIG. 1. Construction of fusion plasmids. (A) pMGJ83 (BAP-A2) and pMGJ96 (MBP-A2); (B) pMGJ112 (BLA-A2). Circular restriction maps show the starting constructs pMGJ67 and pMGJ102 and the final fusion clones pMGJ83, pMGJ96, and pMGJ112. Details of the cloning operations are described in Materials and Methods. Restriction sites are abbreviated as follows: RV, *Eco*RV; X, *Xmn*I; St, *Stu*I; S, *Sma*I; A, *Acc*I; C, *Cla*I; SI, *Ssp*I. A slash (/) indicates that the sites were lost upon ligation. The solid arrowhead shows the region of *ctxA* encoding the A2 domain, and the diagonally hatched blocks indicate the genes fused to A2. The small hatched arrow in pMGJ102 is the sequence encoding the *lacZα* peptide. Resistance genes indicated by the stippled gray blocks are *bla* (ampicillin) and *kan* (kanamycin). Plasmid replication origins, promoters, and f1 ss origins are shown as open arrowheads. In the flow charts, wide arrows denote the constructs providing the vector/origin and thin lines denote the sources of the restriction fragments cloned.

site present between the coding sequence for residues 221 to 222 of CT-A. A *Sac*II TAB linker (Pharmacia-LKB, Piscataway, N.J.) was introduced into the *Ssp*I site of pMGJ19, creating pMGJ37. The *Sac*II site was blunt ended, and the *ctxA2-ctxB* gene fragment was isolated by digestion with *Kpn*I and ligated into *Eco*RV-*Kpn*I-cut pPhoA1-3. The resulting construct (pMGJ98, Table 2) encoded a fusion polypeptide carrying residues 222 to 240 of CT-A at the carboxyl-terminal end of BAP ($BAP\text{-}A2_{222\text{–}240}$) as well as a wild-type *ctxB* gene.

To construct carboxyl-terminal alterations to the BAP-A2 fusion clone, we cloned the BAP-A2 fusion-coding sequence from pMGJ83 into pSKII− as an *Xba*I-*Eco*RI (partial) fragment (since the *phoA* gene has two internal *Eco*RI sites) to create pMGJ84; this construct expressed a BAP-A2 fusion with a 75-amino-acid carboxyl-terminal extension, since the CT-A2-coding region was linked to an open reading frame from the vector. A 12-bp termination linker (containing an *Nhe*I site and stop codons in all three reading frames) was introduced into a filled-in *Kpn*I site in the vector polylinker immediately 3′ to CT-A2 to create pMGJ84-1 (sequence and translation of the fusion joint are shown in Table 2). This clone produced a mutant BAP-A2 gene fusion deleted for the carboxyl-terminal QDEL residues with an additional 15 residues encoded by the vector polylinker. Derivatives of this clone were constructed by linearizing pMGJ84-1 with *Eco*RV at the CT-A2–linker junction and treating for variable times with exonuclease *Bal* 31. After repair with T4 DNA polymerase, the pool of molecules was self-ligated and transformed into *E. coli*. In a portion of the reactions, the terminating linker was juxtaposed to the CT-A2 sequences by digestion with *Apa*I and blunting the ends with T4 DNA polymerase before self-ligation and transformation. The DNA sequence of selected clones was determined, and together with the wild-type fusion (pMGJ83-1), these mutants were introduced into CC118(pMGJ85) to assess ability to complement with CT-B to form holotoxin-like chimeras. To enable complementation with various mutant *ctxB* genes (cloned in ColE1-type vectors), we cloned the BAP-A2 fusion alone from pMGJ83 on a *Hin*dIII-*Acc*I (filled-in) fragment into *Hin*dIII-*Sma*I-cut pK184, a vector compatible with the ColE1-derived mutant *ctxB* clones, to create pMGJ86.

Construction of MBP-A2 fusion clones. Plasmid pMal-p contains a functional *malE* gene fused at its 3′ end after codon 392 to a linker and a *lacZα* peptide. The intervening

linker DNA sequence contains a multiple cloning site, encodes a cleavage site for factor Xa protease (amino acids IEGR) in the resulting protein between the MBP and LacZα (or introduced polypeptide), and is followed by a *Stu*I restriction site. A fusion of MBP to CT-A2 (residues 186 to 240, Fig. 1A) to create pMGJ96 was constructed by cloning in a *Sma*I-*Hin*dIII fragment from pMGJ6730 into *Stu*I-*Hin*dIII-cut pMal-p. The *Sma*I site of pMGJ6730 was introduced by oligonucleotide-directed mutagenesis, silently mutating the codons for residues 185 and 186 (CCGGGT → CCCGGG, Pro-Gly). The fusion protein MBP-$A2_{186-240}$ encoded by pMGJ96 retains the complete cystine loop present between CT-A1 and CT-A2 (Table 2).

Construction of BLA fusions to CT-A2 domain. To enable mutation of the *bla* gene (in the phagemid vector pSKII−) without selection for ampicillin resistance (Ap^r), we cloned in a kanamycin resistance (Km^r) gene to provide an alternative means of selection. The Km^r gene from pK18 was cloned as an *Nhe*I-*Bst*BI fragment into pSKII− cut with *Xba*I and *Acc*I to create pMGJ99 (Ap^r Km^r, Fig. 1B). The single-stranded (ss) origin of replication enabled mutagenesis to be performed on ssDNA rescued by R408 helper bacteriophage as previously described (12). The His-Trp residues encoded at the carboxyl terminus of the vector-derived *bla* gene were mutated to Asp-Ile, creating an *Eco*RV site, with the oligonucleotide 5′ CTGATTAAGGATATCTAACTGT CAG 3′. A clone (pMGJ9901) containing an *Eco*RV site was obtained with the desired mutation. The mutated *bla* gene was subcloned as an *Eco*RV-*Ssp*I fragment into the *Sma*I site of pK18, creating pMGJ102, which expressed a BLA fusion to the LacZα peptide. A BLA-A2 hybrid holotoxin-containing clone (pMGJ104) was constructed by inserting a *Cla*I-*Hin*dIII fragment from pMGJ64 (*ctxA*$^+$ *ctxB*$^+$) containing the CT-A2-encoding domain and the *ctxB* gene into *Acc*I-*Hin*dIII-cut pMGJ102, such that the *bla* and CT-A2-coding sequences were joined by a five-codon linker derived from the multiple cloning site (Table 2), creating the fusion polypeptide BLA′-$A2_{193-240}$. To mutate the sequence coding for the Asp-Gly residues back to wild type (with respect to BLA, His-Trp), we transferred the insert from pMGJ104 to the vector pUK21 (Km^r, possessing an ss origin) to create pMGJ110. This clone was mutated with the oligonucleotide 5′ AGAGGACCCCAATGCTTAATCA 3′ to create pMGJ111, such that the amino acid translation of the resulting fusion joint was HWGLES (Table 2). The vector pUK21 has a mutated *lac* promoter which lowers activity to 10% of normal (34). We found this promoter to be only weakly active in *V. cholerae*, and consequently for high-level expression in *V. cholerae*, the insert from pMGJ111 was transferred to pK18 (a vector with a normal *lac* promoter) as a *Hin*dIII-*Kpn*I fragment, creating pMGJ112.

Detection of holotoxin-like chimeras. Holotoxin-like chimeras containing CT-A2 fusion proteins were detected and quantified with the modified ganglioside G_{M1}-solid-phase radioimmunoassay (G_{M1}-SPRIA) (12), using polyclonal rabbit antisera against BAP, MBP, or BLA as appropriate for specific fusion proteins and ^{125}I-labeled goat anti-rabbit immunoglobulin G (GARG). Data shown are representative of at least two assays on separate extracts from each strain. Individual datum points in figures represent results of single assays, and backgrounds obtained without extracts have been subtracted. Enzymatic activity of BAP-A2 holotoxin-like chimeras was determined by G_{M1} enzyme-linked immunosorbent assay (ELISA) by adding 100 μl of *p*-nitrophenylphosphate substrate in 0.1 M Tris (pH 8.0) to each microtiter well and measuring hydrolysis spectrophotometrically at 420 nm.

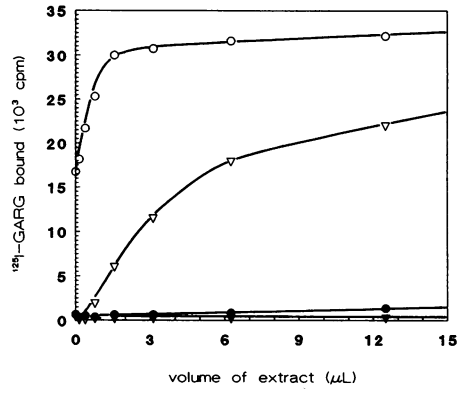

FIG. 2. Detection of BAP-A2 holotoxin-like chimeras by G_{M1}-SPRIA. Extracts tested were from *E. coli* CC118(pMGJ83) (BAP-A2 plus CT-B [○, ▽]) or CC118(pMGJ83-1) (BAP-A2 only [●, ▼]). Antigens were detected with rabbit polyclonal antisera against CT-B (○, ●) or BAP (▽, ▼).

Rabbit anti-BAP and rabbit anti-BLA antisera were purchased from 5Prime→3Prime (Boulder, Colo.), rabbit anti-MBP was from New England Biolabs (Beverly, Mass.), and rabbit anti-CTB 10 was prepared in our laboratory.

RESULTS

Characterization of BAP-A2 fusion protein. To determine whether the CT-A2 domain was able to confer on an unrelated protein the ability to associate with CT-B, we made a construct (pMGJ83) expressing both CT-B and a hybrid polypeptide consisting of BAP fused at its carboxyl terminus to CT-A2 (BAP-A2). The predicted mass of the mature BAP-A2 fusion protein is 52 kDa, compared with 27 kDa for CT-A. Assays of crude periplasmic extracts from *E. coli* CC118(pMGJ83) by G_{M1}-SPRIA (Fig. 2) demonstrated that immunoreactive alkaline phosphatase bound to the ganglioside-coated plates. In striking contrast, the BAP-A2 fusion protein produced by the related construct pMGJ83-1, which has a frameshift mutation in *ctxB* and does not make CT-B, failed to react in the G_{M1}-SPRIA. Detection of immunoreactive alkaline phosphatase by G_{M1}-SPRIA was dependent, therefore, on interaction of the fusion protein with CT-B to form a holotoxin-like chimera. Extracts of CC118(pMGJ83) and CC118(pMGJ83-1) both contained high levels of alkaline phosphatase activity (data not shown). The holotoxin-like chimeras also had alkaline phosphatase activity detectable by G_{M1}-ELISA (data not shown), indicating that the BAP portion of the fusion protein in the holotoxin-like chimera had formed enzymatically active dimers. None of the alkaline phosphatase activity in extracts from CC118(pMGJ83-1) bound to the ganglioside G_{M1}-coated plates, confirming that detection of alkaline phosphatase activity by G_{M1}-ELISA was dependent on the presence of holotoxin-like chimeras. Addition of pentameric CT-B to extracts of C118(pMGJ83-1) did not enable the BAP-A2 in the extracts to bind to ganglioside G_{M1}-coated plates, indicating that assembly of holotoxin-like chimeras required the interaction of BAP-A2 and CT-B during their synthesis and processing in vivo, as is the case in the assembly of native holotoxin.

Characterization of an MBP-A2 fusion protein. To show that the ability to form holotoxin-like chimeras was a general property of proteins containing the A2 domain of CT and not a unique property of the BAP-A2 fusion protein, we con-

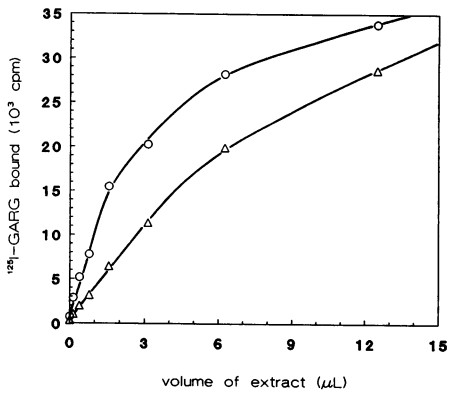

FIG. 3. Detection of MBP-A2 holotoxin-like chimeras by G_{M1}-SPRIA. Extracts of *E. coli* TX1(pMGJ96) were tested with rabbit anti-MBP (△) or rabbit anti-CT-B (○).

structed and characterized pMGJ96, a clone expressing both CT-B and a fusion of the maltose-binding protein to CT-A2 (MBP-A2). The predicted mass of the MBP-A2 protein was 46 kDa. Periplasmic extracts prepared from CC118(pMGJ96) also contained immunoreactive holotoxin-like chimeras, as demonstrated by G_{M1}-SPRIA, using rabbit polyclonal anti-MBP (Fig. 3).

Characterization of a BLA-A2 fusion protein. In order to create a hybrid A2-containing polypeptide whose size more closely approximated that of native CT-A, we constructed genetic fusions of the CT-A2 domain to the carboxyl terminus of BLA, a 29-kDa secreted protein. The predicted size of the BLA-A2 fusion protein was, therefore, 34 kDa. The carboxyl-terminal His-Trp dipeptide of wild-type BLA is replaced by Asp-Ile in the altered BLA protein and by Asp-Gly in the BLA-A2 fusion protein, because we introduced an *Eco*RV restriction at the 3′ end of the *bla* gene. We presumed that the carboxyl-terminal His-Trp dipeptide would not be necessary for BLA activity, since the carboxyl termini differ among related BLAs (1). We therefore replaced His-Trp by Asp-Ile, in a manner similar to that used for the engineered *phoA* constructs reported previously (15). However, both the mutant BLA and BLA-A2 fusion proteins containing the Asp-Gly dipeptide failed to confer resistance to ampicillin, and no immunoreactive holotoxin-like chimeras were detected in extracts of *E. coli* TX1(pMGJ104) (Fig. 1B and Table 2; data not shown). The fusion joint in pMGJ104 was then altered by site-directed mutagenesis so that the carboxyl-terminal sequence of BLA was restored to the wild type, creating pMGJ111 (Table 2). Cells containing this clone were resistant to ampicillin and produced holotoxin-like chimeras detectable by G_{M1}-SPRIA, using rabbit polyclonal anti-BLA (data not shown, but see Fig. 6). The His-Trp dipeptide at the carboxyl terminus of BLA is required, therefore, to permit the mutant BLA and BLA-A2 fusion proteins to fold into a conformation that permits expression of BLA activity and immunoreactivity.

Effects of alterations in the A2 domain on function of fusion proteins. On the basis of the recently published three-dimensional structure of LTp, we hypothesized that the extended sheet formed by residues 222 to 240 might be sufficient for A2-containing fusion proteins to interact with CT-B and form holotoxin. To test this hypothesis, we constructed pMJG98, encoding a fusion protein of BAP to residues 222 to 240 of CT-A2, designated BAP-$A2_{222-240}$

TABLE 3. Details of the CT-A2 portions of the fusions and mutants

Construct	Indicator polypeptide	CT-A2 polypeptide[a]	Formation of[b] holotoxin-like chimeras
pMGJ83	BAP	NSCDEKTQSLGVKFLDEYQSKVKRQIFSGYQSDIDTHN	RIQDEL	+
pMGJ98	BAP	GIFSGYQSDIDTHN	RIKDEL	−
pMGJ84-7	BAP	NSCDEKTQSLGVKFLDEYQSKVKRQIFSGYQSDIDTHNRIRCLSIPSTSRGGPLAS	±
pMGJ84-5	BAP	+++++++++++++++++++++++++++++++++RIKLIDTVDLEGGPAS	±
pMGJ84-4	BAP	+++++++++++++++++++++++++++++++++RIHQAYRRPRGGAR	±
pMGJ84-1	BAP	+++++++++++++++++++++++++++++++++RYQAYRRPRGGAR	±
pMGJ84-2	BAP	+++++++++++++++++++++++++++++++++RSLAS	±
pMGJ84-9	BAP	+++++++++++++++++++++++++++++++++RGGAR	+
pMGJ84-6	BAP	+++++++++++++++++++++++++++++++++PLAS	±
pMGJ84-3	BAP	+++++++++++++++++++++++++++++++++LLAS	−
pMGJ84-8	BAP	+++++++++++++++++++++++++++++++TVDLEGGPAS	−
pMGJ84-10	BAP	+++++++++++++++++++++++++++++++TVDAS	−
pMGJ86	MBP	GCGNAPRSSMSNTCDEKTQSLGVKFLDEYQSKVKRQIFSGYQSDIDTHN	RIQDEL	+
		— S	
pMGJ104	BLA	KDGDPLESMSNT	CDEKTQSLGVKFLDEYQSKVKRQIFSGYQSDIDTHN	RIQDEL	−
pMGJ111	BLA	KHWGPLESMSNT	CDEKTQSLGVKFLDEYQSKVKRQIFSGYQSDIDTHN	RIQDEL	+

[a] Single-letter notation; residues in boldface differ or are not present in wild-type CT-A. Solid lines link indicator polypeptide and CT-A2 sequence; plus signs denote residues that are identical to the sequence immediately above. S---S shows disulfide loop of CT-A2 domain.

[b] Determined by G_{M1}-SPRIA with polyclonal antibody against BAP, MBP, or BLA protein; +, wild type; ±, <10% of wild type; −, no detectable signal.

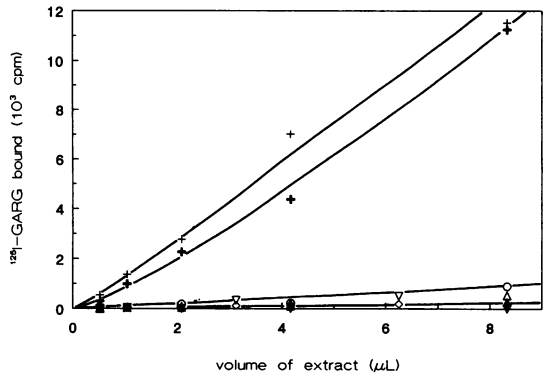

FIG. 4. Effects of C-terminal deletions of BAP-A2 on formation of holotoxin-like chimeras. The *ctxB* gene was provided on plasmid pMGJ85, and genes encoding the mutations of BAP-A2 were provided by the plasmids listed below. Extracts were assayed by G_{M1}-SPRIA with rabbit anti-BAP to determine the immunoreactivity of the holotoxin-like chimeras formed by the parental BAP-A2 fusion and by representative BAP-A2 mutants. Only pMGJ84-9 produces mutant holotoxin with immunoreactivity comparable to that of the wild-type fusion protein. Symbols: △, pMGJ84-2; ▼, pMGJ84-3; ○, pMGJ84-4; ▽, pMGJ84-5; ▲, pMGJ84-6; ◇, pMGJ84-7; +, pMGJ84-9; and +, pMGJ83-1 (wild-type fusion).

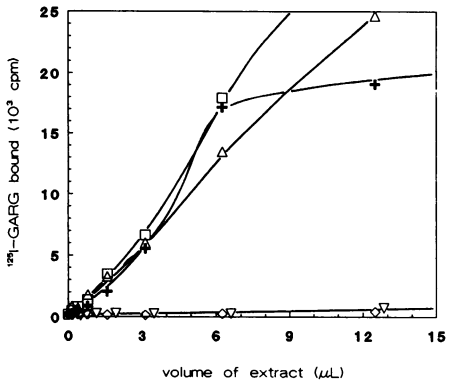

FIG. 5. Ability of BAP-A2 fusion protein to interact with wild-type and mutant CT-Bs to form holotoxin-like chimeras. The BAP-$A2_{197-240}$ fusion protein was expressed from pMGJ86 in the presence of various CT-B mutants. Holotoxin-like chimeras were detected by G_{M1}-SPRIA with rabbit anti-BAP. Symbols: △, pMGJ1110, CT-B[K34D]; ▽, pMGJ1971, CT-B[R35E]; ◇, pMGJ1972, CT-B[R35D]; □, pMGJ1963, CT-B[R35N]; +, pMGJ19, wild-type CT-B[R35, K34].

(Table 2). When syntheses of BAP-$A2_{222-240}$ and CT-B were induced from TX1(pMJG98), CT-B and active alkaline phosphatase were both secreted to the periplasm, but no holotoxin-like chimeras containing the fusion polypeptide were detected by G_{M1}-SPRIA (data not shown).

Next, we constructed additional variants of BAP-A2 to analyze the role of the carboxyl terminus of the A2 domain in assembly of holotoxin-like chimeras (Table 3). The parental plasmid for these constructs was pMGJ83, which contains an *Eco*RI site at the 3′ end of *ctxA*, resulting in a change of the carboxyl-terminal sequence of the A2 domain from KDEL in wild type to QDEL in pMGJ83. Control experiments demonstrated that introduction of the QDEL sequence into CT had no apparent effect on holotoxin assembly or toxicity (data not shown). Because the plasmids that expressed these modified BAP-A2 polypeptides did not encode *ctxB*, the ability of the modified BAP-A2 fusion proteins to form holotoxin-like chimeras was tested by complementation in *E. coli* with CT-B expressed from pMGJ85. All the mutants made active alkaline phosphatase, and the range of alkaline phosphatase activity in extracts containing the various mutants was approximately threefold (data not shown). The initial constructs pMGJ84 and pMGJ84-1 produced BAP-A2 fusion proteins with carboxyl-terminal extensions of 75 and 15 amino acids, respectively, and both were greatly impaired in their ability to form holotoxin-like chimeras (Fig. 4). Plasmids pMGJ84-2 through pMGJ84-10 were derived from pMGJ84-1 and encoded additional changes at the carboxyl terminus of the A2 domain. The mutant fusion protein encoded by pMGJ84-9 had RGGAR substituted for QDEL and formed holotoxin-like chimeras in amounts comparable to BAP-A2 from pMGJ83-1 (Fig. 4). Constructs that encoded mutant proteins with substitutions starting before the QDEL sequence (pMGJ84-3, -8, and -10) failed to assemble into holotoxin-like chimeras, and the mutants with QDEL replaced with sequences other than RGGAR that varied in length from 4 to 16 residues yielded only small amounts of immunoreactive holotoxin-like chimeras.

Effects of *ctxB* mutations on formation of holotoxin-like chimeras. Previous studies showed that mutant CT-B containing a negatively charged amino acid substituted for Arg-35 was unable to associate with native CT-A to form stable holotoxin (12). We tested several mutant CT-B polypeptides for their ability to associate with BAP-A2 in *E. coli* to form holotoxin-like chimeras (Fig. 5). Mutant CT-B polypeptides with Glu-35 or Asp-35 did not form holotoxin-like chimeras, but mutant CT-B polypeptides with Asn-35 or Asp-34 did. All these strains produced high levels of immunoreactive CT-B and BAP-A2 protein with alkaline phosphatase activity (data not shown). These observations indicate that residue 35 of CT-B is directly involved in, or is important for, the association of CT-B with the A2 domain of BAP-A2 in holotoxin-like chimeras.

Inability of *V. cholerae* to excrete holotoxin-like chimeras containing BAP-A2, MBP-A2, or BLA-A2. We expressed the BAP-A2, MBP-A2, and BLA-A2 hybrid toxin operons in *V. cholerae* CVD44, a strain deleted for the *ctx* operon, and examined the formation and localization of the holotoxin-like chimeras (Fig. 6). Because BLA-A2 was poorly expressed from the altered *lac* promoter of pMGJ111 in *V. cholerae*, we constructed pMGJ112 (Fig. 1B), in which BLA-A2 is expressed from the *lac* promoter of pK18, and used it for these experiments. In *V. cholerae* CVD44, the holotoxin-like chimeras were all localized predominantly in the periplasm and were not excreted into the culture medium. In contrast, pentameric CT-B produced simultaneously with the hybrid toxins in CVD44 was excreted into the culture medium. These results demonstrated that the toxin secretory apparatus in *V. cholerae* CVD44 was functional but unable to translocate the holotoxin-like chimeras across the outer membrane. The failure of the holotoxin-like chimeras to be excreted by *V. cholerae* was most likely determined, therefore, by the characteristics of their constituent BAP-A2, MBP-A2, and BLA-A2 hybrid polypeptides.

DISCUSSION

The present study demonstrated that three different hybrid proteins containing the A2 domain of CT can assemble with CT-B to form holotoxin-like chimeras. No portion of the A1 domain of CT was required for formation of holotoxin-like

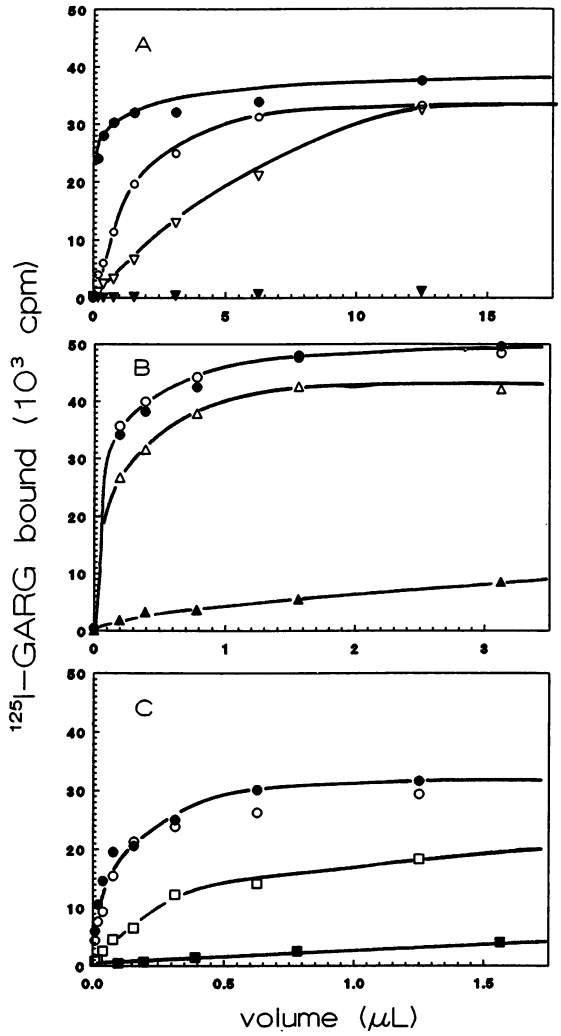

FIG. 6. Periplasmic localization of holotoxin-like chimeras in *V. cholerae* CVD44. (A) pMGJ83 (BAP-A2 plus CT-B); (B) pMGJ96 (MBP-A2 plus CT-B); (C) pMGJ112 (BLA-A2 plus CT-B). Open symbols show periplasmic extracts; filled symbols show culture supernatants. Antigen was detected by G_{M1}-SPRIA with rabbit antisera against CT-B (○, ●), BAP (▽, ▼), MBP (△, ▲), or BLA (□, ■).

chimeras. Our findings establish that the A2 domain is sufficient to enable hybrid proteins, and presumably also CT-A, to interact with CT-B during assembly. Duffy et al. (2) proposed that the amino terminus of A1 participates in binding of CT-A to CT-B. The possibility that the A1 domain may contribute to the assembly process for native CT is not excluded by our findings.

The crystal structure of CT is not yet published, but the conformation of CT is reported (5) to be almost identical to that of the related protein LTp from *E. coli* (32). The crystal structure of LTp showed that A2 contains an α-helix (residues 200 to 222) that interacts extensively with A1 and an extended chain (residues 222 to 228) which projects into the core of the LT-B pentamer and interacts with each of the LT-B polypeptides (31, 32). Residues 222 to 240 were not sufficient to mediate interaction with the B pentamer, since the fusion protein BAP-$A2_{222-240}$ described here did not assemble with CT-B into holotoxin-like chimeras. The inability of BAP-$A2_{222-240}$ to form holotoxin-like chimeras with CT-B could involve steric hindrance, altered conformation of residues 222 to 240, or other incompatible interactions between the BAP domain and CT-B. The α-helix in A2 at residues 200 to 222 may function as an essential spacer between A1 and the region of A2 that interacts most extensively with CT-B.

Among the BAP-A2 mutants with altered carboxyl termini, only those with QDEL (present in the parental construct used in this study) or RGGAR substituted for the C-terminal KDEL sequence of wild-type CT-A formed normal amounts of stable holotoxin-like chimeras with CT-B. These findings demonstrated that the sequence at the carboxyl-terminal end of A2 has significant effects on the assembly or stability of holotoxin-like chimeras, even though the wild-type KDEL sequence is not essential. Removal or substitution of nine or more residues at the carboxyl-terminal end of A2 resulted in complete loss of ability of BAP-A2 fusion proteins to assemble into holotoxin-like chimeras. Our BAP-A2 fusion proteins that were not truncated at the carboxyl-terminal end of A2 but were deficient in forming holotoxin-like chimeras had at least one hydrophobic (I or L) or bulky (Y) residue substituted for D or E in the KDEL sequence, and such substitutions may prevent the normal interaction of A2 with CT-B. In the original report on the three-dimensional structure of LTp, the positions of the last eight residues (233 to 240) of the A2 domain were not well resolved (32). Analysis of crystals containing LTp with galactose, and higher-resolution analysis of LTp alone, demonstrated that the carboxyl terminus of A2 extends out through the core of the B pentamer and includes a short, one-and-one-half-turn α-helix in the previously unresolved region (31). This structure suggests that mutants or fusion proteins with extensions from the carboxyl terminus of A2 should not necessarily be incapable of assembling into stable holotoxins. Indeed, a fusion of *E. coli* heat-stable enterotoxin to the carboxyl-terminal end of LT-A via a synthetic linker peptide was reported to assemble with LT-B into a holotoxin-like chimera (25, 27).

Our previous finding that mutant CT-B proteins with negatively charged residues substituted for Arg-35 were unable to form holotoxin with CT-A led us to consider the possibility that there might be a charge-charge repulsion between the CT-B mutants and CT-A (12). However, the three-dimensional structure of the related LTp holotoxin does not show any interaction of CT-B Arg-35 with the A2 domain, but Arg-35 does form a salt bridge with Glu-11 of the neighboring B polypeptide (30). If the same interaction occurs between the conserved Arg-35 and Glu-11 residues in CT-B, the Asp-35 and Glu-35 mutations could disrupt this salt bridge and cause a conformational change in the B pentamer that might prevent assembly or reduce stability of the holotoxin, while still permitting pentamerization, G_{M1}-binding activity, and immunoreactivity of the mutant CT-B polypeptides. Additional studies will be needed to define the effects of these mutations on the tertiary structure of the mutant CT-Bs.

Assembly of holotoxin-like chimeras required concurrent synthesis and secretion of the BAP-A2 fusion protein and CT-B, since addition of BAP-A2 to extracts containing pentameric CT-B did not result in formation of holotoxin-like chimeras. This is consistent with the hypothesis that CT-A acts as a nidus for interaction with CT-B during formation of holotoxin (7). The recent crystallographic studies of LTp, indicating that the extended-chain region of A2 passes through the narrow core of the B pentamer and is

flanked by α-helical regions of A2, are also consistent with the proposal that B pentamer assembles in vivo around the extended-chain region of A2. Our studies did not address quantitatively the kinetics of assembly of BAP-A2, MBP-A2, or BLA-A2 with CT-B, but substantial amounts of holotoxin-like chimeras were formed with each of these hybrid proteins.

The secretion apparatus of *V. cholerae* recognizes and exports to the culture medium native CT and *E. coli* LT (22) and holotoxin-like chimeras containing some but not all fusions of small polypeptides to CT-B (26) or LT (29). However, none of the holotoxin-like chimeras that we constructed in this study was excreted as an extracellular protein by *V. cholerae*. BAP (47-kDa monomer) and MBP (41 kDa) are much larger and BLA (29 kDa) is slightly larger than the A1 domain of CT-A (22 kDa). The holotoxin-like chimeras that we studied had predicted masses of 109 kDa for BAP-A2, 104 kDa for MBP-A2, and 92 kDa for BLA-A2, compared with 84 kDa for native CT. The holotoxin-like chimera containing BAP-A2 probably also dimerized via its BAP domain, as suggested by the alkaline phosphatase activity of the holotoxin-like chimera (28). Failure of *V. cholerae* to excrete the holotoxin-like chimeras could reflect steric hindrance within the toxin secretory apparatus, either because the holotoxin-like chimeras are larger than CT or because the conformations of the hybrid polypeptides are substantially different from that of CT-A. The crystallographic studies of LTp showed extensive interaction between A1 and the long α-helical region of A2 in LT-A. Similar interactions of the A2 region will probably not occur with the BAP, MBP, and BLA portions of the A2 hybrid polypeptides, and it is possible that the holotoxin-like chimeras containing BAP-A2, MBP-A2, and BLA-A2 are less compact than native CT. Because CT-B and LT-B can be excreted as pentameric B subunits into the culture medium by strains of *V. cholerae* that do not make the corresponding A polypeptides, but CT-A or LT-A cannot be translocated across the outer membrane of *V. cholerae* unless the corresponding B subunits are present, recognition of CT or LT by the toxin secretory apparatus is believed to be specific for the enterotoxin B subunits (8).

In conclusion, we constructed several fusion proteins consisting of BAP, MBP, or BLA linked at their carboxyl termini to the A2 domain of CT. The A2 domain of the fusion proteins renders them competent to associate with CT-B to form holotoxin-like chimeras. The structures of the A2 domain of the fusion proteins and of CT-B were modified in several ways by protein engineering to provide tools for analyzing the molecular basis for assembly of CT. We expect that the model described, based on analysis of holotoxin-like chimeras, will be useful for future studies of the toxin secretory apparatus of *V. cholerae* and for the development of hybrid neoantigens for use in studies of immunity to cholera and other microbial diseases.

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