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Mutational Analysis of Ganglioside GM_1-Binding Ability, Pentamer Formation, and Epitopes of Cholera Toxin B (CTB) Subunits and CTB/Heat-Labile Enterotoxin B Subunit Chimeras

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Variants of cholera toxin B subunit (CTB) were made by bisulfite- and oligonucleotide-directed mutagenesis of the *ctxB* gene. Variants were screened by a radial passive immune hemolysis assay (RPIHA) for loss of binding to sheep erythrocytes (SRBC). Variant CTBs were characterized for the formation of immunoreactive pentamers, the ability to bind ganglioside GM_1 in vitro, and reactivity with a panel of monoclonal anti-CTB antibodies. Substitutions at eight positions (i.e., positions 22, 29, 36, 45, 64, 86, 93, and 100) greatly reduced the yield of immunoreactive CTB. RPIHA-negative substitution variants that formed immunoreactive pentamers were obtained for residues 12, 33, 36, 51, 52 + 54, 91, and 95. Tyrosine-12 was identified as a novel residue important for GM_1 binding since, among all of the novel variants isolated with altered RPIHA phenotypes, only CTB with aspartate substituted for tyrosine at position 12 failed to bind significantly to ganglioside GM_1 in vitro. In contrast, CTB variants with single substitutions for several other residues (Glu-51, Lys-91, and Ala-95) that participate in GM_1 binding, based on the crystal structure of CTB and the oligosaccharide of GM_1, were not appreciably altered in their ability to bind GM_1 in vitro, even though they showed altered RPIHA phenotypes and did not bind to SRBC. Hybrid B genes made by fusing *ctxB* and the related *Escherichia coli* heat-labile enterotoxin *eltB* genes at codon 56 produced CTB variants that had 7 or 12 heat-labile enterotoxin B residue substitutions in the amino or carboxyl halves of the monomer, respectively, each of which which also bound GM_1 as well as wild-type CTB. This collection of variant CTBs in which 47 of the 103 residues were substituted was used to map the epitopes of nine anti-CTB monoclonal antibodies (MAbs). Each MAb had a unique pattern of reactivity with the panel of CTB variants. Although no two of the epitopes recognized by different MAbs were identical, most of the single amino acid substitutions that altered the immunoreactivity of CTB affected more that one epitope. The tertiary structures of the epitopes of these anti-CTB MAbs are highly conformational and may involve structural elements both within and between CTB monomers. Substitution of valine for alanine at positions 10 and 46 had dramatic effects on the immunoreactivity of CTB, affecting epitopes recognized by eight or six MAbs, respectively.

Cholera toxin (CT), an enterotoxin produced by *Vibrio cholerae*, is the prototype for the immunologically and structurally related family of heat-labile enterotoxins (LTs) produced by *Vibrio cholerae* and *Escherichia coli* (11). These toxins are composed of A and B subunits in a 1:5 molar ratio. Upon secretion to the periplasm in *E. coli* or *V. cholerae,* cholera toxin B (CTB) monomers spontaneously assemble into pentameric CTB and, in the presence of CTA, assemble into stable holotoxin. Free CTA and pentameric CTB, however, will not assemble into holotoxin. Delivery of the enzymatically active A subunit to the cytosol of sensitive cells is initiated by the binding of the pentameric CTB subunit to a cell surface receptor. For CT (and LT-I) this is ganglioside GM_1 (GM_1). There are five GM_1 binding sites on the pentamer (9).

The three-dimensional structures of LT-Ip (37, 38) and CT (44, 45) are known, and the structure of CTB complexed with the pentasaccharide of GM_1 (OS-GM_1) has also been determined (26). These studies identified more than a dozen residues in CTB that interact directly or indirectly with OS-GM_1 (see also Fig. 9). Included in this set are two residues (Trp-88 and Gly-33) that were previously identified as being essential for receptor binding by a variety of techniques (6, 16, 21, 41). The contribution that each of the other 10 residues makes to the GM_1-binding affinity of CTB is currently unknown. Similarly, little is known of how the monomers or the pentamer attain their native conformations. The intramolecular disulfide bond between Cys-9 and Cys-86 is essential (16), and in heat-labile enterotoxin B (LTB) substitution of the wild-type residue at position 64 (15) or deletion of the extreme carboxyl terminus (residues 100 to 103) prevents assembly of the pentamer (35). Each GM_1-binding site lies primarily within a single monomer, but Gly-33 of an adjacent monomer contributes a single solvent-mediated hydrogen bond, and is important for GM_1 binding. Pentamerization is therefore required for GM_1-binding activity (7).

CT and other family members are potent immunogens (29) and adjuvants (13). The pentameric B subunits contain the immunodominant antigenic determinants of the holotoxins (14). Several groups have made and partially characterized monoclonal antibodies (MAbs) directed against the B subunits of CT or LT (3, 4, 8, 12, 20, 39). More than 20 epitopes have been identified (31) on the basis of complex patterns of specificity and cross-reactivity, but the structures of individual epitopes have not yet been defined.

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We have generated here a collection of variants of CTB and hybrids between CTB and LTB by both random and site-directed means. We have characterized this collection to determine the effects of single amino acid substitutions on assembly of immunoreactive CTB pentamers and to delineate the contributions of specific residues to GM_1 binding. In addition, these variants were used to finely map the epitopes recognized by our collection of nine anti-CTB MAbs. We identified Tyr-12 as a residue important for GM_1-binding activity, and we show that most of our MAbs recognize highly conformational and structurally overlapping epitopes.

MATERIALS AND METHODS

Plasmids. Plasmids pMGJ8, pMGJ11, and pMGJ19 carry *ctxB* in pBluescript and have been described previously (16). Expression of *ctxB* from pMGJ8 and pMGJ19 is driven by the CT promoter and requires the presence of the positive regulatory protein ToxR, whose gene was provided in *trans* on plasmid pVM25 (28). Plasmid pMJK5 encodes *ctxB* expressed from the *lacUV5* promoter, using the leader sequence of the LTIIb-B gene (18). Strains carrying pMJK5 and pMGJ11 require induction with isopropyl-β-D-thiogalactopyranoside (IPTG) for the expression of *ctxB*. Derivatives of these plasmids with mutations in *ctxB* were numbered sequentially in the form pMGJ8xx, pMGJ11xx, pMGJ19xx, or pMJK5xx, where xx is a number from 01 to 99.

Mutagenesis. Bisulfite mutagenesis was done according to the method of Pine and Huang (30). Briefly, 3 μg of *Cla*I-*Eco*RI-cut parental vector was mixed with 1.5 μg of pMGJ8 or pMGJ19 single-stranded DNA and denatured by boiling and slow cooling to form gapped duplex molecules. Aliquots were treated with 1 or 3 M sodium bisulfite with 2 mM hydroquinone for 20 min to 3 h, and the reactions were terminated by dialysis at 4°C. Reactions were transformed into *E. coli* BW310(pVM25) (*ung toxR*$^+$) made competent by the method of Hanahan (10). *E. coli* CJ236 (*dut ung thi relA*, pCJ105) (Bio-Rad) was used to produce uracil-containing templates for oligonucleotide-directed mutagenesis, performed as described in the Bio-Rad MutaGene manual. The sequence of the oligonucleotides used were Y12X (TGTGCAGAANNSCACAACACA) and K91X (TG GAATAATSRCACGCCTCATG), where N, S, and R represent equal ratios of G, A, T, and C; G and C; or A and G, respectively. Oligonucleotide-directed synthesis products were transformed into *E. coli* TG1 (Amersham) or its derivative, TX1 (17). Insertion of small oligonucleotide linkers (TAB linkers) was performed as described in the TAB linker manual (Pharmacia).

The sequences of all mutated *ctxB* genes were determined by dideoxy chain termination by using Sequenase 2.0 T7 DNA polymerase as described by the manufacturer (USB Corp., Cleveland, Ohio).

Assays for CTB. Radial passive immune hemolysis assay (RPIHA) was done as previously described (16) with sheep erythrocytes (SRBC). Briefly, transformants expressing potential mutant *ctxB* genes were stabbed into an SRBC overlay on a selective Luria agar plate (27). After overnight growth at 37°C, a second overlay containing goat anti-CT serum and guinea pig complement (Gibco-BRL) was made and incubated for 1 h at 37°C, at which time clones producing wild-type (wt) CTB were surrounded by clear halos of hemolysis. Clones not producing CTB or producing CTB that does not bind to SRBC have no halo.

Bacterial extracts from clones expressing pMGJ8, pMGJ19, or derivatives were prepared from overnight cultures grown in 2YT medium (34) and concentrated 25-fold prior to treatment with 2 mg of polymyxin B sulfate/ml in phosphate-buffered saline (PBS) for 15 min at 37°C. Cell debris was removed by centrifugation, and extracts were stored at 4°C. Bacterial extracts from clones expressing pMGJ11 or PMJK5 and derivatives were prepared by inducing mid-log-phase cultures in TB medium (40) with 200 μM IPTG, followed by growth overnight at 37°C. Under these conditions, the majority of the CTB was present in the culture supernatant, which was cleared of cells by centrifugation, and the supernatant was stored at 4°C.

The production of mutant CTB was characterized and quantified by using solid-phase radioimmunoassay (SPRIA) as previously described (16). Total immunoreactive pentameric CTB was quantitated by sandwich SPRIA (S-SPRIA) by using goat anti-CT on the solid phase. Plates coated with 25 μl of 150 nM GM_1 (GM_1-SPRIA) per well were used to quantitate the GM_1-binding ability of mutant CTB. MAbs were further characterized by direct-SPRIA with plates coated with native CTB (at 1 μg/ml) or CTB denatured by boiling for 10 min (at 1 μg/ml). For all SPRIAs, nonspecific binding was blocked with 10% horse serum in PBS. Bound CTB was detected with rabbit anti-CTB specific serum, followed by [^{125}I]-goat anti-rabbit immunoglobulin G (GARG). Bound monoclonal antibody was detected with affinity-purified rabbit anti-mouse immunoglobulin G, followed by [^{125}I]GARG.

RESULTS

Isolation and characterization of variant CTB subunits. Plasmids pMGJ8 and pMGJ19 were used to induce C-to-T transitions in the coding and noncoding strands of *ctxB*, respectively, by bisulfite mutagenesis as described in Materials and Methods. Single colonies were tested by RPIHA, and transformants that lacked halos or showed altered halo morphology were selected and cloned. The DNA sequence of each mutant *ctxB* allele was determined. Variants were designated by the one-letter code for the wt amino acid, its position in the mature B subunit, and the one-letter code for the amino acid substitution (e.g., the mutant protein with leucine substituted for proline at position 2 was designated P2L). When multiple missense mutations were found, these were separated wherever possible by recloning restriction fragments or by using mutant restriction fragments as primers to introduce single mutations into a wt background.

Eighteen bisulfite-generated mutant alleles of *ctxB* with single missense mutations at 1 of 16 codons were found that resulted in an altered RPIHA phenotype (Table 1). Separation of multiply mutant alleles identified a further six single substitution variants, for a total of 24 variants at 22 positions. The closely linked mutations in pMGJ1981 (V52I and G54D) and pMGJ1988 (S55N and R73K) were not separately cloned. In total, this screen generated 36 new substitution variants and four linker insertion variants (see below and Table 1).

Periplasmic extracts from each of these mutant strains were tested by S-SPRIA for immunoreactive CTB. S-SPRIA profiles of representative mutant extracts are shown in Fig. 1. Three types of signal were obtained: one class giving a high, saturatable signal like that of the wt (e.g., E11K and H13Y), another class giving a low but measurable signal (E36K, P93S, S100N, and A46V), and a third class giving no detectable signal (G45D and E29K). Of the 22 bisulfite-generated variants that gave no halo by RPIHA, 18 gave very low (nine) or no (nine) signal by S-SPRIA, indicating that they produced little or no immunoreactive CTB pentamers. This group included 5 premature termination or amber mutants and 13 substitutions for Asp-22, Glu-29, Glu-36, Gly-45, Ala-64, Cys-86, Pro-93, and Ser-100. Only four halo-negative missense mutants produced wt levels of immunoreactive CTB. These were G33D, E36Q, E51K, and V52I+G54D. All except the G33D variant CTB bound as well as the wt CTB in a GM_1-SPRIA. Two mutants that gave turbid, incompletely cleared halos (E11K and D70N) produced normal amounts of immunoreactive CTB that also bound well by GM_1-SPRIA.

Small insertions of two to four residues were also made by inserting oligonucleotide linkers into naturally occurring restriction sites in *ctxB* (Table 1). All were negative by RPIHA. Insertion of a *Sac*II linker into the *Ssp*I site between codons four and five inserted two residues (PR) and prevented the resulting 105-residue CTB polypeptide from forming immunoreactive pentamers. Similarly, insertion of four residues (RDPD) after Tyr-27 by inserting a *Bam*HI linker into the filled-in *Acc*I site prevented formation of immunoreactive pentamers. In contrast, insertion of an *Apa*I linker into the *Rsa*I

TABLE 1. Phenotypes of bisulfite- or TAB linker-induced mutants of CTB made or used in this study

Substitution(s)[a]	pMGJ no.	Phenotype[b] determined by:			MAb epitope affected or linker added[c]
		RPIHA halo	S-SPRIA signal	GM_1-SPRIA signal	
Native CTB	8, 19	+++	+++	+++	NA[e]
P2L	814	++	++	++	None
A10V	802	++	++	++	32D3 15C11 (all others)
E11K	1984	(+++)	+++	+++	4C8
H13Y	824	++	+++	+++	None
Q16X	1992	−	−	−	NA
D22N, E29K	1982	−	−	−	NA
D22N	1990	−	−	−	NA
S26L, Q49X	811	−	−	−	NA
S26L	834	+++	+++	+++	None
T28I	807	++	+++	+++	None
E29K	1989	−	−	−	NA
G33D	1994	−	+++	−	(4C8) 40B10
E36Q	829	−	+++	+++	4C8 (40B10)
E36K	1983	−	+	+	ND[f]
T41I	803	++	+++	+++	None
G45D	1975	−	+	ND	ND
A46V	832	++	+++	+++	1E9 15C11 (4E2) 22C6 40D9 35G8
Q49X	831	−	−	−	NA
E51K	1976	−	+++	+++	(4C8 15C11) 40B10
V52I, G54D	1981	−	+++	+++	None
S55N, R73K, S100N	1974	−	+	ND	ND
S55N, R73K	1988	++	+++	+++	None
H57Y	826	++	+++	+++	None
S60L, P93S	821	−	+	+	ND
S60L	833	+++	+++	+++	None
Q61X	804	−	−	−	NA
A64V, S−7F, T−11I	818	−	+	+	NA
A64V	832	−	+	ND	NA
M68I	1985	+++	+++	+++	None
D70N	1980	(+++)	+++	+++	None
R73K	1991	++	+++	+++	None
C86Y	1979	−	−	−	NA
W88am	1978	−	−	−	NA
P93S	809	−	+	+	ND
P93L	805	−	+	+	ND
S100N	1986	−	+	+	ND
N4NPR	1909	−	−	−	NA (*Ssp*I::*Apa*I)[d]
Y27YRDPD	1114	−	−	−	NA ([*Acc*I]::*Bam*HI)[d]
V52VGP	6404	−	+++	+++	ND (*Rsa*I::*Apa*I)[d]
Q56PAE	901	−	+++	+++	None (*Hinc*II::*Sac*II)[d]

[a] X, termination codon; am, suppressible amber codon. A negative number indicates signal sequence residue.
[b] Parentheses indicate turbid (incompletely clearing) halos; +++, wt; ++, reduced size, −, no halo.
[c] Parentheses indicate binding only partially affected.
[d] [Filled-in] restriction site into which the indicated restriction site linker was inserted.
[e] NA, not applicable.
[f] ND, not done.

site or insertion of a *Sac*II linker into the *Hinc*II site generated immunoreactive CTB variants that were unaffected in their ability to bind to GM_1 in vitro but nevertheless were found to be halo negative by RPIHA. These linkers inserted GP after Val-52 or replaced Gln-56 with PAE. A further construct, pMGJ913, containing a polylinker sequence encoding 13 residues inserted into the *Hinc*II site formed immunoreactive CTB that also bound to GM_1 (not shown) and was found to be halo negative by RPIHA.

The RPIHA screening procedure described above identified residues previously implicated in GM_1 binding, either by mutagenic studies or X-ray crystallography (24, 25). It also showed that substitution of other CTB residues (e.g., Glu-36 and Glu-51) identified as interacting with GM_1 oligosaccharide in the three-dimensional crystal structure (26) did not abolish GM_1-binding activity in vitro. This prompted us to examine the roles of other CTB residues predicted to interact with GM_1 by creating specific oligonucleotide-directed mutants. Mutants alleles of *ctxB* were generated in IPTG-inducible clones (pMGJ11 or pMJK5), producing variants with single substitutions for Tyr-12, Lys-91, or Ala-95 (Table 2). All substitution variants except K91R were negative by RPIHA. K91R gave a wt halo. All mutants also produced normal levels of immunoreactive pentamers, except for aberrant mutants pMGJ1120 (which had deleted the Tyr-12 codon and mutated E11D) and pMGJ1121 (that also had an I96L substitution, in addition to A95G). These mutants produced no immunoreactive CTB. Yet all variant CTBs, except Y12D, that made immunoreactive pentamers also gave strong signals when tested by GM_1-SPRIA, showing that they retained substantial GM_1-binding activity. Extracts containing Y12D variant CTB gave a signal by GM_1-SPRIA only at a very high concentration of antigen (data

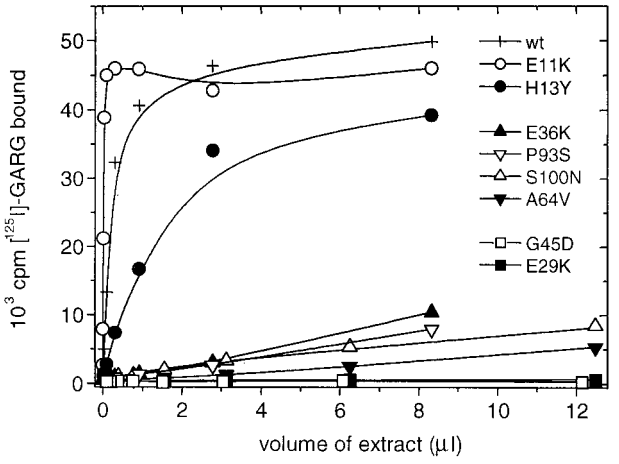

FIG. 1. Detection of antigen from strains producing selected variant CTBs. Serial dilutions of extracts were made in S-SPRIA wells, and antigen was detected with rabbit anti-CTB, followed by [^{125}I]GARG. Three types of mutant were detected: high-level producers such as wt, E11K, and H13Y; low-level producers such as E36K, P935, S100N, and A64V; and mutants that made no detectable antigen, such as G45D and E29K.

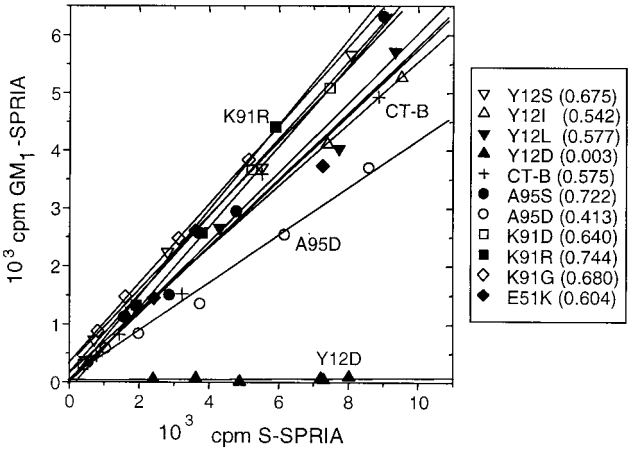

FIG. 2. Comparison of the relative GM_1-binding activities of wt and various variant CTBs. For a series of dilutions in a linear range, the signal obtained from GM_1-SPRIA was plotted against that from S-SPRIA. The correlation coefficients for the best-fit line ranged from a low of $r = 0.965$ for Y12L to $r = 1.000$ for K91G (except for $r = 0.298$ for Y12D). The slope of each line, in parentheses next to the relevant variant designation, is a measure of the relative binding activity of each CTB variant for GM_1.

not shown). By plotting the signals obtained from given dilutions of extract by S-SPRIA against the signals obtained by GM_1-SPRIA, we could measure the relative GM_1-binding ability of each variant CTB and compare it with wild-type (Fig. 2) (16). The E51K and K91D variant CTBs showed GM_1-binding ability comparable to that of wt CTB. In contrast, the A95D variant showed slightly reduced binding ability, and the Y12D variant lacked any significant GM_1-binding activity.

TABLE 2. Phenotypes of oligonucleotide-directed mutants of CTB made or used in this study

Substitution[a]	pMGJ or pMJK no.	Phenotype[c] determined by:			MAb epitope affected[d]
		RPIHA halo	S-SPRIA signal	GM_1-SPRIA signal	
Y12S	1115	−	+++	+++	NT
Y12I	1118	−	+++	+++	NT
Y12L	1119	−	+++	+++	NT
Y12D	1117	−	+++	−	4C8 40B10
E11D+Y12Δ	1120	−	−	−	NA
G33x	19xx[b]	−	+++	+++/−	(4C8)[e] 40B10
K34x	11xx	−	+++	+++	4C8 40B10
R35x	19xx	+++/−	+++	+++	4C8 (40B10)
W88x	19xx	−	++/+	++/+/−	(4C8 40B10)
K91R	501	+++	+++	+++	NT
K91G	502	−	+++	+++	NT
K91D	507	−	+++	+++	(15C11 40B10)
A95G	1122	−	+++	+++	NT
A95G+I96L	1121	−	−	−	NA
A95S	1123	−	+++	+++	NT
A95D	1124	−	+++	+++	(1E9 4C8 40B10)

[a] "x" indicates that several mutants were generated at this position; for G33 these are E, D, I, L, V, Q, S, T, A, K, and R. For K34 these are G, A, S, C, V, I, D, Y, and N; for R35 these are D, E, C, H, G, W, S, Y, I, M, N, and Q; and for W88 these are I, L, Q, V, H, K, N, E, M, P, S, G, T, and R (16).

[b] "xx" represents a number between 01 and 99 denoting the various mutants at this position.

[c] +++/− and ++/+/− represent the variable phenotypes associated with these mutants depending on the substitution.

[d] NT, not tested; NA, not applicable.

[e] Parentheses indicate partial reactivity

The data for this group of CTB variants are summarized in Fig. 3. While several of these variants affect elements structurally conserved among the B subunits of the LT family, a single novel variant, Y12D, completely disrupted interaction of the B pentamer with its receptor. A detailed comparison of these variants is presented in the Discussion. The distribution of the substitution variants is spread almost uniformly across the primary amino acid sequence of CTB, providing us with a useful group of variants with which to begin mapping the epitopes of our collection of anti-CTB MAbs.

Construction of hybrids between CTB and LT-Ip and an MAb epitope analysis with variant and hybrid CTBs. Both *ctxB* and *eltB* genes (encoding CTB and LT-IpB) have a *Hin*cII restriction site at the same position covering the codon for Gln-56, approximately in the center of the gene. A 0.5-kbp *Eco*RI-*Hin*dIII DNA fragment from pEWD299, a clone expressing *eltB* (5), was cloned into pSKII+ to make pMGJ62, which expresses *eltB* from the *lacUV5* promoter. Similarly, a *ctxB*-expressing clone was made by cloning a 464-bp *Eco*RI-*Hin*dIII fragment from the holotoxin-producing clone pMGJ67 (17) into pSKII− to create pMGJ63. We constructed hybrids in which the amino- or carboxyl-encoding half of *ctxB* in pMGJ63 was replaced by the corresponding portion of *eltB* from pMGJ62 and designated these clones pMGJ68 and pMGJ69, respectively. When analyzed by GM_1- and S-SPRIA with rabbit anti-CTB, both hybrids produced immunoreactive pentamers that retained the ability to bind to GM_1 (data not shown).

We now had available bisulfite-generated substitution mutants of *ctxB* at 21 positions and 10 new oligonucleotide-directed mutants at 3 new positions that produce immunoreactive CTB, in addition to the 38 of 46 oligonucleotide-directed mutants generated in our earlier study that also make immunoreactive pentamers (16). Altogether, 69 mutants at 28 of the 103 residues of mature CTB formed immunoreactive pentam-

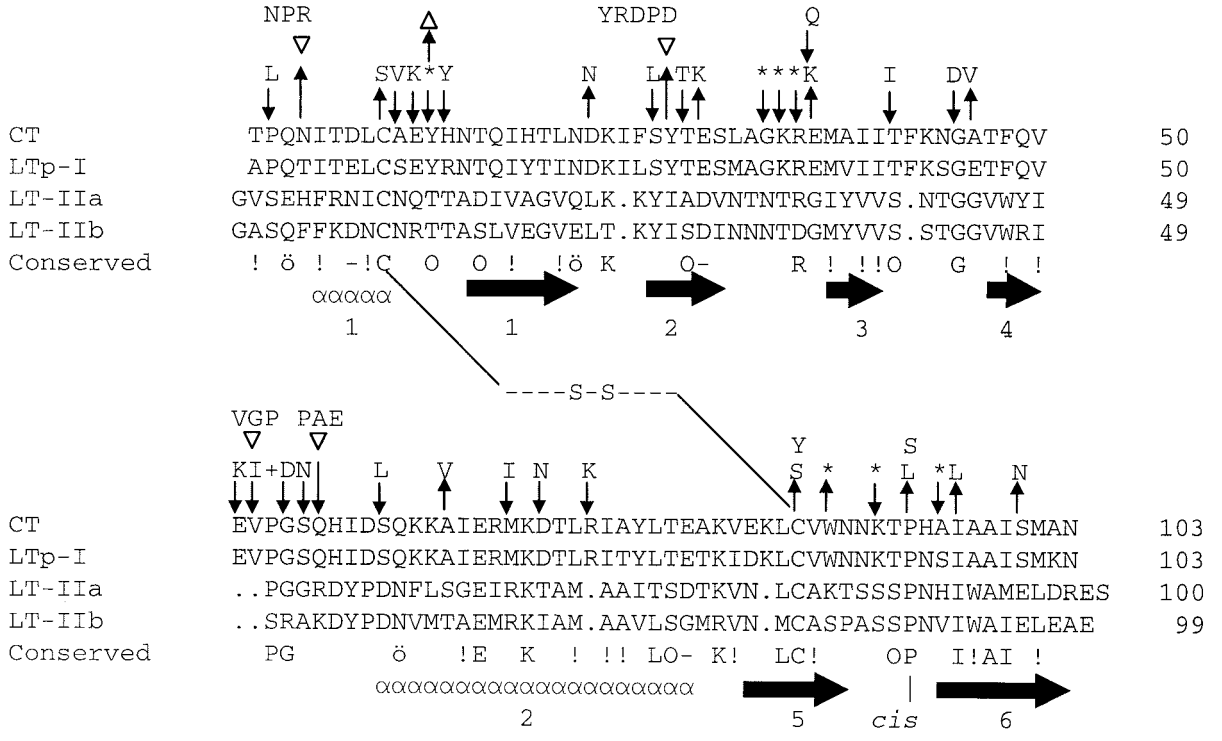

FIG. 3. Comparison of CTB/LTB family showing regions of secondary structure and location of substitutions in CTB. Gaps introduced to maximize alignment are shown by periods. The disulfide bond linking the two cysteines is shown by - - -S-S- - -. The consensus sequence, defined as those residues occurring in three of four of the family members shown, is shown beneath the aligned sequences. Similar residues are indicated by "ö" for amides (Gln, Asn); "−" for negative charges (Glu and Asp); "O" for hydroxyl groups (Ser, Thr, or Tyr) and "!" for hydrophobic (Ala, Leu, Ile, Val, Met, Phe, and Trp). Below the consensus sequence are shown the number and location of secondary structure elements determined for CT and LT-Ip ("α" symbols show the α-helix; arrows show the β-sheet). "*cis*" indicates the conserved *cis*-proline at position 93. LT-Ih differs from LT-Ip only at three or four of the residues T4S, E46A, T75A, and K102E. Substitutions preventing pentamer formation are indicated above the residue substituted (↑), inserted (▽), or deleted (△). Variants retaining ability to pentamerize are shown above downward arrows (↓). An asterisk indicates that three or more substitution mutants were generated by oligonucleotide mutagenesis.

ers. Including the LT-specific residues in the two CTB/LTB hybrids (7 in the CTB/LTB hybrid and 12 in LTB/CTB hybrid) in this collection of variants and hybrid forms of CTB, a total of 47 residues differ from the corresponding residues in native CTB. We have used this collection of CTB variants to further characterize the epitopes recognized by nine anti-CTB MAbs described in earlier studies from our laboratory (12, 21).

We first tested the CTB/LTB hybrids for reactivity with the anti-CTB specific MAbs 4C8, 32D3, 40D9, 4E2, 15C11, 22C6, 35G8, and 1E9, along with MAb 11E8, an anti-LTB that cross-reacts with CTB (4) (Fig. 4). MAbs reactivity was compared to CT from strains 569B (Classical) and U1 (El Tor). Both anti-CTB MAbs (4C8 and 32D3) and the anti-LTB MAb (11E8) that cross-react with the respective B subunits retained reactivity with both hybrids, as expected. However, the LT-non-cross-reactive MAbs 15C11 and 35G8 lost all reactivity against both hybrids, as did MAb 1E9. Similarly, MAbs 4E2 and 40D9 lost reactivity with the CTB/LTB hybrid, but retained reduced but detectable reactivity with the LTB/CTB. One or more of the 19 residues defined by the CTB/LTB and LTB/CTB hybrids are therefore part of the epitope recognized by MAbs 15C11, 35G8, 1E9, 4E2, and 40D9. MAb 22C6 that does not react with either LT-IpB or LT-IhB lost reactivity with the LTB/CTB hybrid but showed strong reactivity against the CTB/LTB hybrid, indicating that none of the 7 residues altered in this hybrid (residues 75, 80, 82, 83, 94, 95, and 102) are important in the 22C6 epitope. MAb 35G8 did not react with El Tor CT or with either hybrid. MAbs 22C6 and 15C11 were also unreactive to El Tor CT.

The locations of the 7 or 12 LT-specific substitutions in the hybrids are clustered on the upper and side surfaces of the pentamer in the three-dimensional crystal structure. The lower surface of the pentamer, in contact with the cell membrane when bound to GM_1, is highly conserved, and only His-13 of the variant LTB residues is visible from the lower surface view. In contrast, the bisulfite-generated mutants are in general clustered on the lower face of the pentamer, surrounding the receptor-binding site identified by Merritt et al. (26). The upper surface of the CTB pentamer, in contact with the A subunit in the holotoxin, is free of mutated residues.

A more detailed analysis of the functional epitopes of these MAbs was obtained from the pattern of cross-reactivity with the collection of CTB single substitution variants (Fig. 5 and 6, Table 3). All MAbs retained reactivity with mutants substituted for residues 2, 13, 26, 28, 42, 52+54, 55+73, 57, 60, 70, and 73 and with insertion mutants V52VGP and Q56PAE (Table 1). The A10V substitution affected the binding of every one of the MAbs (Fig. 5B) to various degrees, and it eliminated the binding of MAbs 32D3 and 15C11. A46V also affected reactivity with several of the MAbs, eliminating reactivity with

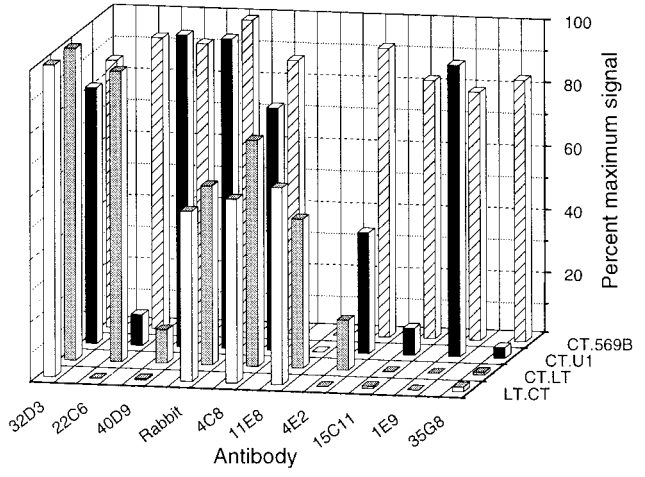

FIG. 4. MAb reactivity with CTB/LTB hybrids. All MAbs were raised against CT from *V. cholerae* 569B (Classical Inaba [12]) except for MAb 11E8 (anti-LT-Ih [3]). MAb 35G8 was isolated as an anti-CT MAb that did not cross-react with CT from El Tor strain 3083 (21). MAb 1E9 was raised against urea-denatured CT from 569B.

15C11, 22C6, and 35G8. MAbs 4C8 and 40B10 lost reactivity with many of the mutants that appear to be located in or near the GM_1-binding site (26). MAb 40B10 was isolated by its inability to bind CTB bound to GM_1 (21), but 4C8 was reported to react with CT or CTB as determined by GM_1-SPRIA (12). In a GM_1-SPRIA with 5 μg of CTB/ml as the test antigen, 4C8 gave only 10% (2,000 cpm) of the signal compared to MAbs 1E9, 4E2, 32D3, 22C6, and 40D9 (19,000 to 22,000 cpm; 13,000 for MAb 15C11). Raising the CTB concentration to 50 μg/ml increased the MAb 4C8 signal by 10 fold (19,000 cpm), while concomitantly reducing the signal for the other MAbs by 15 to 20% (except for MAb 15C11, which showed a twofold increase in signal). This suggested that MAbs 4C8 and, to a much lesser extent, MAb 15C11 were at least partially inhibited by GM_1 binding. Preincubation of CTB (at 50 ng/ml) with a 300-fold molar excess of GM_1 prior to analysis by S-SPRIA reduced MAbs 40B10 and 4C8 binding by 95 and 73%, respectively, while having only marginal effects on the binding of polyclonal rabbit B10 antiserum or MAbs 1E9 and 32D3 (reduced 20, 22, and 16%, respectively).

The epitope of MAb 40B10 was further analyzed as shown in Fig. 6. Lys-34 is a critical residue in this epitope since substitution with any of eight other residues eliminated reactivity with MAb 40B10. Charged or bulky substitutions for Gly-33 also eliminated the epitope, but G33A and G33S variants still bound MAb 40B10, albeit more weakly. Arg-35 was much less critical since only R35D and R35I reduced binding, and no substitution variant completely eliminated binding. E11K retained the ability to bind MAb 40B10, in contrast to MAb 4C8, with which binding was fully eliminated. Charged substitutions for Tyr-12, Glu-51, or Trp-88 eliminated reactivity with MAb 40B10, while charged substitutions for Lys-91 or Ala-95 only caused decreased reactivity.

These data demonstrate that, while each MAb has a unique pattern of reactivity with the available CTB variants, the epitopes recognized nevertheless overlap significantly. The location of each residue identified in these epitopes in the three-dimensional crystal structure indicates that these epitopes are highly conformation dependent and likely require CTB to adopt a quaternary structure, i.e., pentamerize, for the MAb to recognize its determinant.

Requirement for pentamer formation by CTB for MAb reactivity. All of the anti-CT MAbs described above, except 40B10, react strongly with CTB attached to the solid phase whether directly coated onto the plate, captured by GM_1 or by goat anti-CT, but binding of 40B10 to CTB is strongly inhibited in GM_1 capture assays. We have isolated two new MAbs, 1E9

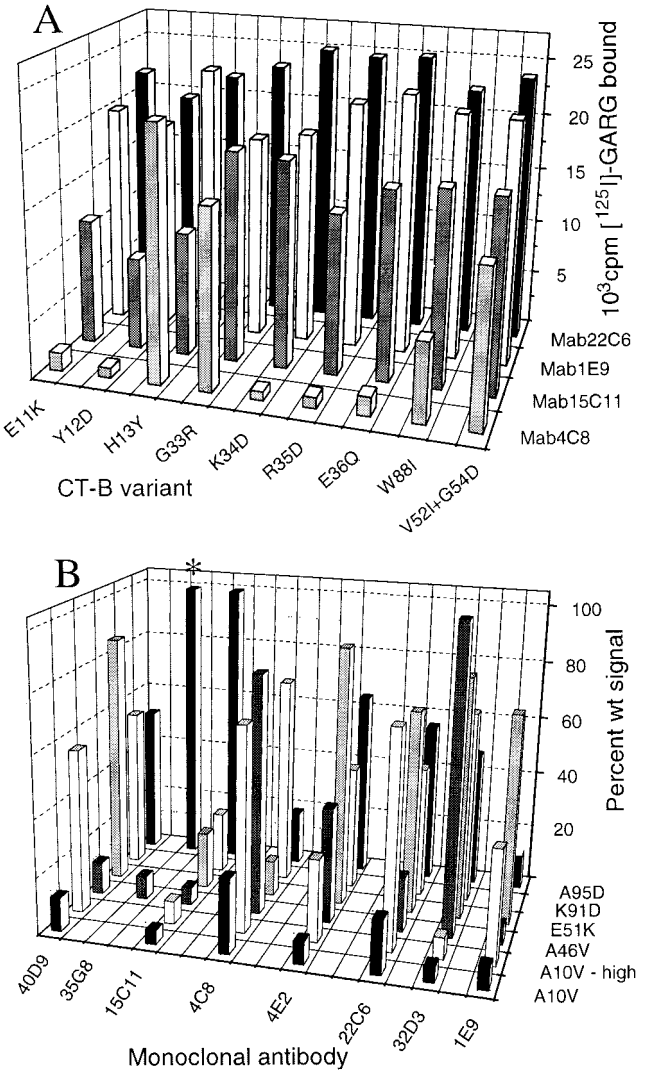

FIG. 5. MAb reactivity with CTB mutants. Antigen was analyzed by S-SPRIA by titration on goat G140 anti-CT-coated plates and detected with rabbit anti-CTB serum or MAb. The signal obtained for each MAb at the antigen dilution corresponding to the shoulder of the curve for reactivity with polyclonal antiserum was plotted. (A) Four MAbs were tested against each of nine mutant CTBs by S-SPRIA. (B) Eight MAbs were tested against each of five mutant CTBs by S-SPRIA. The signal for mutant A10V was also plotted at a 16-fold-higher antigen concentration to show the maximal reactivity obtained with these MAbs. MAb 1E9 was not tested against A95D, and MAb 35G8 reactivity is shown only for A46V and for V52I+G54D (marked with an asterisk in the rearmost row).

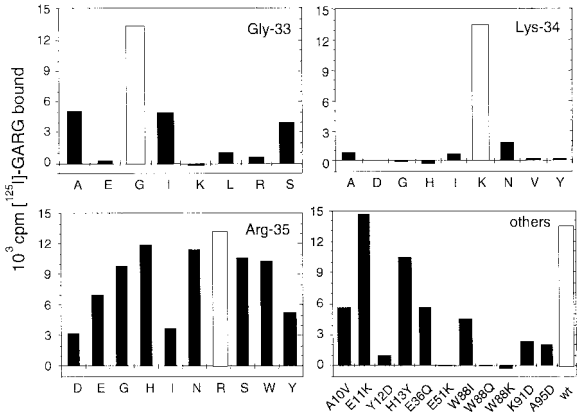

FIG. 6. Epitope analysis of MAb 40B10. Reactivity with mutants at positions 33, 34, and 35, tested by S-SPRIA (see Fig. 5 legend), is shown in separate panels as filled bars; that of the wt residue is shown as an open bar. The last panel shows the reactivity with selected single CTB variants and several variants for Trp-88.

and 12D2, raised against urea- or sodium dodecyl sulfate (SDS)-denatured CTB, respectively. We compared the reactivity of these new MAbs with MAbs 32D3 and 40D9 by GM_1-SPRIA, S-SPRIA, and direct-SPRIA, with either native CTB or CTB denatured by boiling, to coat the solid phase (Fig. 7). Each MAb detects native CTB directly coated onto plastic with similar efficiency (Fig. 7A). However, MAb 12D2 is totally unreactive in a GM_1-SPRIA, whereas MAb 1E9, like MAbs 32D3 and 40D9 (not shown), is strongly reactive (Fig. 7B). The failure to obtain detectable reactivity of MAb 12D2 in a GM_1-SPRIA was not due to blocking of the epitope of MAb 12D2 by GM_1, since MAb 12D2 was also unreactive by S-SPRIA, whereas MAbs 1E9 and 32D3 were strongly reactive (Fig. 7C). We conclude that MAb 12D2, which was raised against SDS-denatured CTB monomers, does not react with native CTB, whereas MAb 1E9, raised against urea-denatured CTB monomers, retains the ability to react with native CTB. Our results also establish that the goat anti-CT serum used to prepare the solid-phase for S-SPRIA has little, if any, specificity for nonnative (i.e., nonpentameric) CTB. All MAbs including 12D2 gave strong signals in direct-SPRIA where the plastic was coated with "native" CTB (Fig. 7A), suggesting that upon binding to the plastic at least a portion of the CTB is denatured, exposing an epitope that is not displayed in native CTB. In a direct-SPRIA with boiled CTB, MAb 32D3 was found to be totally unreactive, whereas MAb 12D2 was highly reactive (Fig. 7D). MAbs 1E9 and 40D9 show reduced but significant reactivity with boiled CTB. The ability to bind boiled CTB in a direct-SPRIA correlates with the ability to detect monomeric CTB in a Western blot (12; unpublished observations).

These data demonstrate that our collection of MAbs raised against native CTB react with highly conformation-dependent epitopes. MAb 1E9, raised against urea-denatured CTB, likely recognizes a determinant within a monomer that remains exposed in the pentamer, whereas MAb 12D2 recognizes a determinant within a monomer that is not exposed in the pentameric form.

DISCUSSION

The RPIHA was used to identify substitution variants of CTB that had lost the ability to bind to SRBC. We identified 20 halo-negative bisulfite-induced mutants and 16 halo-negative oligonucleotide-generated or linker insertion mutants of *ctxB*. Of the bisulfite-induced mutants, only 4 retained the ability to produce immunoreactive CTB pentamers, while 2 of the 4 insertion mutants and 10 of 12 oligonucleotide-generated mutants formed immunoreactive CTB pentamers. The other mutants with altered halo phenotypes (mainly smaller halos, but two with normal-sized incompletely clearing halos [E11K and D70N]) produced CTB that bound in a GM_1-SPRIA as well as the wt. Loss of a halo in RPIHA correlates with an inability to bind to SRBC but not necessarily with a loss of GM_1-binding activity in vitro or with biological activity on Y1 cells (see below). The major CT receptor on SRBC is reported to be a ganglioside similar or identical to GM_1 (32). It is possible that the GM_1 oligosaccharide is displayed differently in SRBC membranes versus on plastic, resulting in the different phenotypes for particular mutants in RPIHA versus GM_1-SPRIA. A precedent for this exists in that a variant of the related *E. coli* LT-IIa, which has lost the ability to bind to its preferred receptor GD_{1b} but still binds to GM_1 in vitro, nevertheless can-

TABLE 3. CTB variants affecting epitopes of anti-CTB MAbs

Wild-type CTB residue	Result[a] with MAb:								
	1E9	4C8	4E2	32D3	15C11	22C6	40B10	40D9	35G8
Ala-10	(A10V)	(A10V)	(A10V)	A10V	A10V	(A10V)	(A10V)	(A10V)	+
Glu-11	+	E11K	+	+	+	+	+	+	+
Tyr-12	+	Y12D	+	+	+	+	Y12D	+	+
Gly-33	+	(G33*)	+	+	+	+	G33*	+	+
Lys-34	+	K34*	+	+	+	+	K34*	+	+
Arg-35	+	R35*	+	+	+	+	(R35*)	+	+
Glu-36	+	E36Q	+	+	+	+	(E36Q)	+	+
Ala-46	(A46V)	+	(A46V)	+	A46V	A46V	+	A46V	A46V
Glu-51	+	(E51K)	+	+	(E51K)	+	E51K	+	+
Trp-88	+	(W88*)	+	+	+	+	(W88*)	+	+
Lys-91	ND	+	+	+	(K91D)	+	(K91D)	+	+
Ala-95	(A95D)	(A95D)	+	+	+	+	(A95D)	+	+

[a] Substitutions shown abolished reactivity with the MAb. Parentheses indicate partial reactivity retained. A plus sign indicates that the MAb retains full reactivity with all variants tested. An asterisk indicates more than one substitution tested affects reactivity. ND, not done.

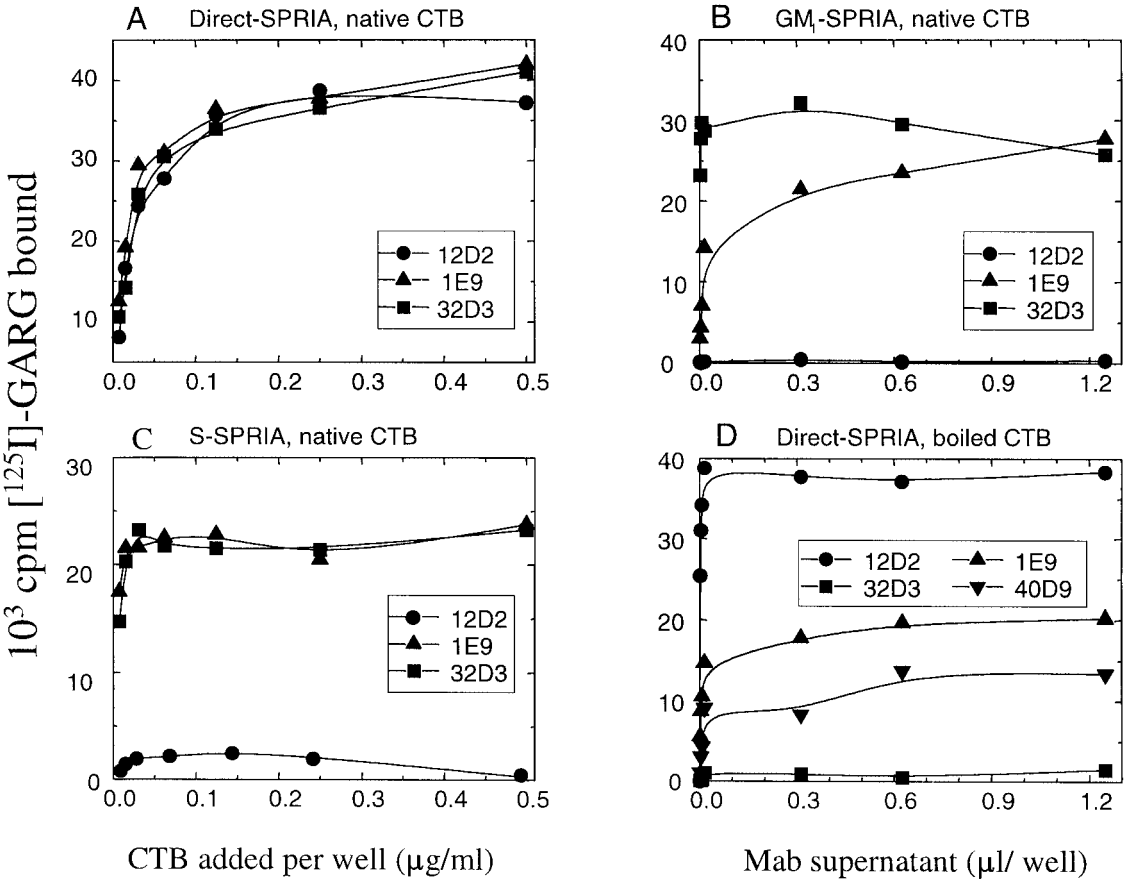

FIG. 7. Reactivity of MAbs with denatured or native CTB. MAb reactivity was determined by direct-SPRIA, S-SPRIA, and GM_1-SPRIA. For panels A and C, reactivity was determined against serial dilutions of native CTB (starting at 0.5 μg/ml). MAb culture supernatants were used at a 1/5 dilution for 12D2 and 1E9 and at a 1/30 dilution for 32D3. MAbs were titrated by GM_1-SPRIA (B) with CTB at 0.125 μg/ml and by direct-SPRIA (D) with CTB at 1 μg/ml denatured by boiling for 10 min.

not bind GM_1 in the context of the cell membrane of T84 cells (42). However, loss of ability to bind in a GM_1-SPRIA does correlate with loss of toxicity on Y1 adrenal cells for holotoxins containing these CTB variants (16). Additionally, slight alterations in GM_1 binding that are not detected under our in vitro conditions may be important in interaction with GM_1 in a cell membrane.

GM_1-binding activity of variants. The residue substitutions in CTB variants with halo-negative phenotypes (E11K, E36Q, E51K, V52I+G54D, V52VGP, Q56PAE and substitutions for Tyr-12, Lys-91, and Ala-95, in addition to the substitutions for Gly-33, Lys-34, Arg-35, and Trp-88 that we identified previously [Fig. 8 and reference 16]) are located very close to the actual GM_1-binding site as defined by crystallography (Fig. 9) (26). In total, only two of the 15 halo-negative mutants identified here that made immunoreactive CTB completely lost the ability to bind to GM_1 by SPRIA. The bisulfite-induced G33D variant was identified in an earlier study (16), and the oligonucleotide-directed Y12D variant was novel. Three other mutants with substitutions for Tyr-12 (Y12S, Y12I, and Y12L) made variant CTBs that bound GM_1 in vitro and yet were found to be negative by RPIHA, and only the Y12D variant CTB showed a severe defect in GM_1 binding in vitro. In the crystal structure of CTB bound to OS-GM_1, the phenyl ring of Tyr-12 has hydrophobic interactions with the acetyl group of sialic acid (Fig. 9) (26). It appears that Leu, Ile, or Ser are compatible with or do nor prevent this interaction and that the negatively charged asp is sufficient to prevent GM_1 binding.

Substitutions for residues on either side of Tyr-12 (Glu-11 and His-13) produce an altered phenotype in RPIHA but did not appear to affect CTB binding to GM_1 as determined by SPRIA. In the crystal structure of CTB with OS-GM_1, the sialic acid moiety makes hydrogen bonds with only the peptide backbone of these residues (26) (Fig. 9), and thus substitutions would not necessarily interfere with these interactions, unless other factors, such as charge effects or steric hinderance, have dominant effects. A precedent for such effects exists in that certain substitutions for Gly-33, whose only interaction with receptor is via its carbonyl oxygen and backbone nitrogens, dramatically affect receptor binding (24, 25). Recently, a CTB variant with an E11R substitution was characterized (43) and found to retain GM_1 binding in vitro but to have decreased biological activity in vivo. In contrast, holotoxin produced with our E11K CTB variant retained significant biological activity on Y1 cells (data not shown).

In the crystal structure of CTB bound to OS-GM_1, hydrogen bonds are also formed between side chains of Glu-51 and Lys-91 and the terminal galactose of GM_1 and contribute to

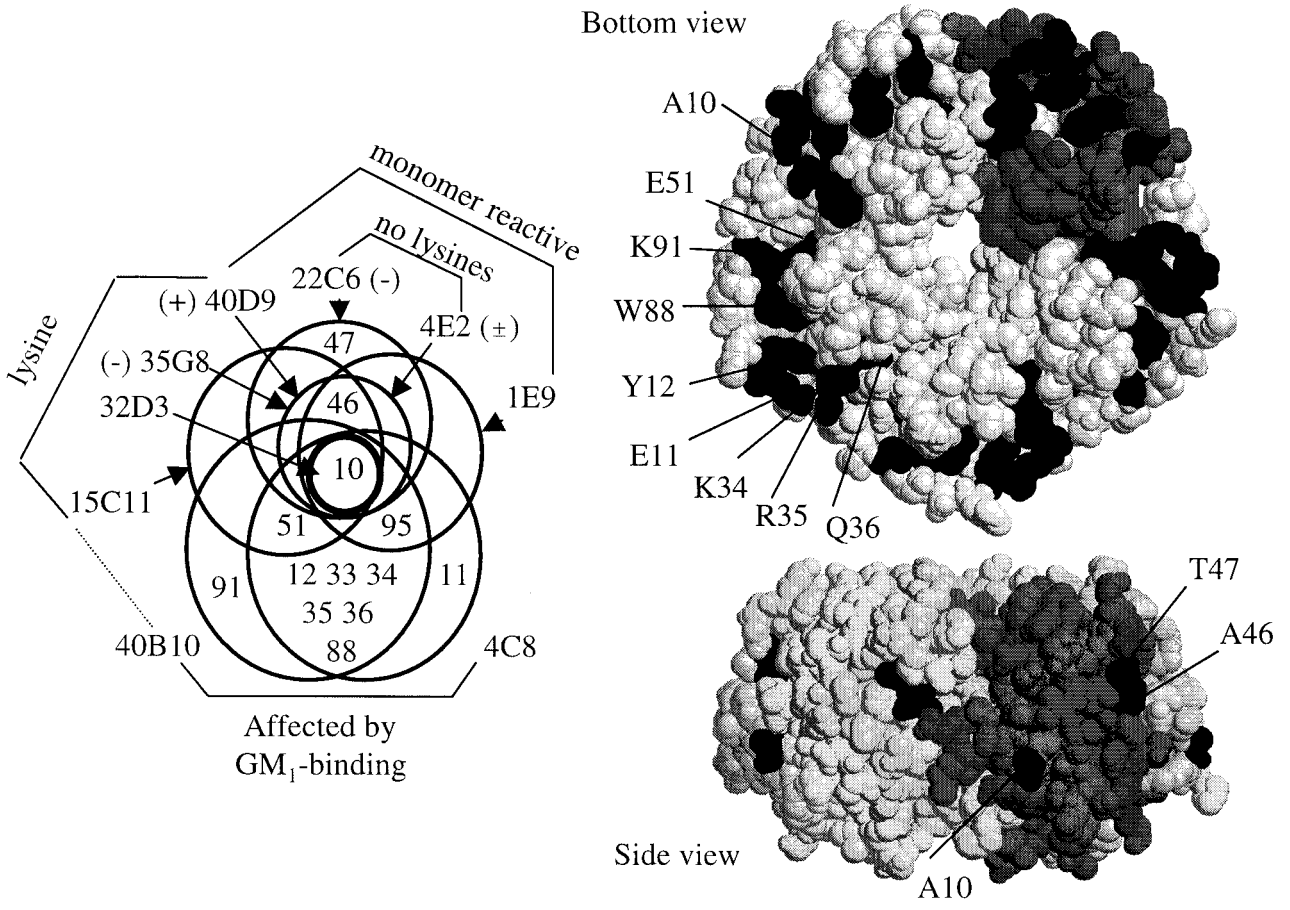

FIG. 8. Location of substitutions in CTB affecting MAb reactivity. (Left) Venn diagram showing residues (numbered) defining each MAb epitope. Presence or absence of lysines and monomer reactivity are taken from (12). The symbols "±" and "−" refer to weak or no reactivity, respectively, with El Tor toxin as described in the text. (Right) Space-filling view of the CTB pentamer shown from the lower surface or side views. A CTB monomer is shown shaded gray, and residues affecting MAb epitopes are shown in black and identified by one-letter code and residue number.

the binding energy of the CTB-receptor interaction (Fig. 9). Substitutions for either of these residues that disrupt some or all of the hydrogen bonds might be expected to weaken this interaction. K91R formed a halo detected by RPIHA and thus still bound to SRBC. The K91G, K91D, and E51K mutant CTBs were determined to be halo negative by RPIHA and yet displayed normal GM_1 binding as seen by SPRIA. Apparently, the loss of the hydrogen bonds between the terminal galactose and either Glu-51 or Lys-91 does not dramatically affect GM_1 binding in vitro. The interactions between Glu-51 and Lys-91, together with His-57 (all conserved between CT and LT-I), have been proposed in LT-I to be involved in a pH-dependent conformational change of the pentamer (33), a change that also occurs with CTB (22, 33). Efforts in those studies to make site-directed mutations for Glu-51 and Lys-91 in the B subunit of LT-I were unsuccessful, leading to the suggestion that these mutations were toxic to the *E. coli* host. This was not so for CT, since our E51K and K91D variants of CTB were produced at similar levels to wt CTB and showed no evidence of being detrimental to the *E. coli* host cells.

Pentamerization of CTB. The failure of many mutants to form immunoreactive CTB can be rationalized by studying the crystal structure of the pentamer (23, 26). The substitution variants E29K and S100N could introduce steric clashes with the side chains of residues in the central α-helix and a β-strand of an adjacent monomer, respectively, and may severely affect the ability of these variants to pentamerize. Alterations at the carboxyl terminus of LTB were reported to cause a temperature-sensitive defect in pentamerization (35); however, lowering the temperature did not increase the amount of immunoreactive CTB recovered for S100N (data not shown). The insertion mutant Y27YRDPR also disrupts β2 and pentamerization. The other insertion mutant that fails to assemble (N4NPR) adds a second proline to the amino terminus and probably alters the folding of the first α-helix. Two substitutions of Asp or Ser/Leu for fully conserved residues (Gly-45 and Pro-93), situated in critical turns between β-sheets also dramatically affected these variants ability to pentamerize.

Ala-64 forms part of the long α-helix that lines the pore of the pentamer. Substitution with Val in CTB prevented formation of immunoreactive CTB and gave no detectable signal by GM_1-SPRIA. A similar A64V variant of LT-IpB does not form pentamers stable to SDS treatment but is detected by GM_1 enzyme-linked immunosorbent assay with 10-fold less sensitivity (15). Sixma et al. (38) suggest that a steric clash between A64V and Met-31 of the neighboring monomer disrupts pen-

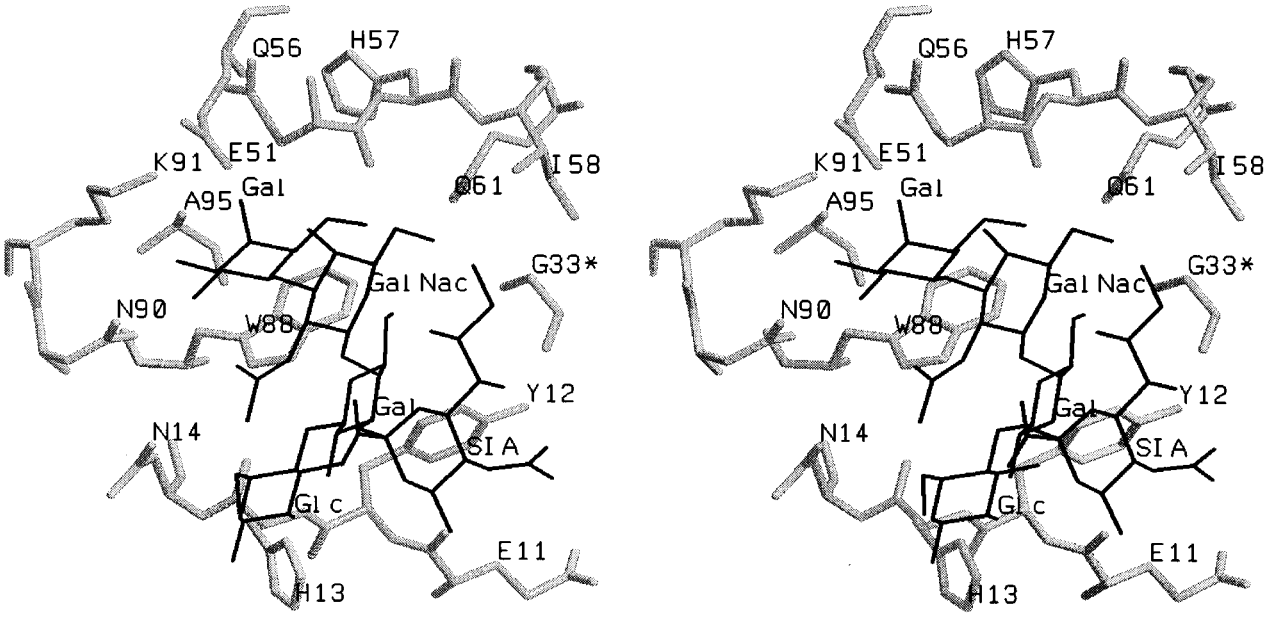

FIG. 9. Stereo representation of the CTB residues involved in GM_1 interaction. Coordinates for CTB-GM_1 are from Merritt et al. (26). The oligosaccharide of GM_1 is shown in stick form (black), and individual sugars are identified [Gal-GalNac-Gal-(SIA)-Glc]. CTB residues of a single CTB monomer are shown as wire frame representations (gray). G33* shows the sole contribution of the neighboring monomer to the binding site. Variants for all residues shown except N14, I58, Q61, and N90 were isolated in this study.

tamerization. In CTB, residue 31 is a bulkier Leu, and an A64V CTB mutant may have a more severe defect in pentamerization than the corresponding LTB mutant, resulting in our inability to detect any GM_1 binding by this variant. Residue 31 is also involved in the second shell interaction in the receptor-binding site (26), as are Ala-95 and His-13. Most single substitutions for Ala-95 (except A95D) or His-13 identified in this study showed negligible differences in binding to GM_1 in vitro, although all Ala-95 variants failed to form halos by RPIHA. A study substituting the corresponding residues in LT-IB (Met-31, Ser-95, and Arg-13) with those of CTB (19) showed no apparent differences in binding to GM_1 in vitro but did show alterations in binding to Bio-gel A-5m, suggesting subtle differences in binding to receptors other than GM_1.

The two insertion variants that do not disrupt the pentamer, V52VGP and Q56PAE, are situated in the large loop between β4 and the long α-helix on the undersurface of the pentamer. Both mutants were determined to be halo negative by RPIHA and, although Gln-56 is closely associated with the terminal Gal of GM_1, neither mutant was affected in GM_1 binding in vitro. Indeed, a mutant with a polylinker encoding 13 amino acids replacing Gln-56 still produced CTB pentamers that bound GM_1 (data not shown), as did a variant of CTB with a 10-residue epitope of human immunodeficiency virus type 1 replacing 8 residues (that is, residues 56 to 63) of CTB (2). The carbonyl oxygen of Gln-56 may form a hydrogen bond to the terminal galactose of GM_1 (36), and the 50-to-64 loop adopts a more rigid conformation upon binding of CTB with GM_1. However, our data show that individual residues and the number of residues in this loop are not critical for binding to GM_1 in vitro. Very recently, alanine-scanning mutagenesis of this loop identified a CTB variant, H57A, that retained GM_1-binding activity in vitro but that nevertheless had lost its ability to bind to host cells and as holotoxin showed no activity on intestinal T84 cells (1). Interestingly, holotoxin produced with the H57Y variant retains toxic activity on mouse Y1 cells (preliminary data [not shown]), suggesting the reduced activity of the H57A variant on T84 cells is specific for that substitution.

Epitope analysis. Several other studies have demonstrated at least five or six unique epitopes of CTB and LTB (4, 12, 21, 39), generally without identifying the specific residues recognized by the MAbs. We used this collection of CTB mutants to determine the nature, extent, and composition of the epitopes recognized by individual MAbs. Based on the pattern of reactivity with individual CTB mutants, variants, and hybrids and the effects of GM_1 binding on reactivity, each of the nine MAbs studied was shown to possess a unique epitope. Nevertheless, some structural elements of CTB were shared by two or more of these epitopes.

The best-defined epitopes belong to the MAbs that show partial (4C8) or complete (40B10) inhibition by GM_1, since most of our CTB mutants were obtained by a screening procedure that was designed to identify residues involved in receptor recognition. These two epitopes are highly conformational, recognizing residues in peptide loops linking α1 to β1 (Ala-10 to Tyr-12), β4 to α2 (Glu-51), and β5 to β6 (Trp-88, Glu-91, and Ala-95) of one monomer and β2 to β3 (Gly-33 to Gln-36) of the adjacent monomer (Fig. 8). Thus, these MAbs require the integrity of the quaternary structure of the pentamer to be reactive. The epitopes of MAbs 4C8 and 40B10 are highly cross-reactive, and both are affected to various degrees by substitutions at 9 of the 11 residues shown to contribute to their epitopes. Glu-11 is in the 4C8 but not the 40B10 epitope, and the opposite is true for Lys-91. Both epitopes absolutely require Tyr-12 and Lys-34 but differ in their sensitivity to

substitutions for residues 33, 35, 36, 51, and 88. The inability of 40B10 to react with any CTB variants containing substitutions for Gly-33, while 4C8 can at least partially tolerate such substitutions, is probably the basis for the complete and partial inhibition by GM_1, respectively, of MAb binding to native CTB. Interestingly, both can tolerate substitutions for Trp-88, which is intimately involved in GM_1 binding, but not Tyr-12, here identified as also contributing significantly to receptor recognition. Trp-88, being located at the base of the GM_1-binding cleft, may be less likely to contribute to surface-exposed epitopes. MAb 15C11, which is partially affected by substitutions for Glu-51 and Lys-91 showed evidence of slight inhibition by GM_1 binding in that the maximum signal obtained by S-SPRIA was reduced 50% by prior incubation of bound CTB with GM_1 (not shown), suggesting that its binding site also overlaps with the GM_1-binding cleft.

The effects of the A10V and A46V substitutions on MAb binding are also of considerable interest. The A10V substitution affects, but in most cases does not eliminate, reactivity with all MAbs tested, suggesting a significant change in monomer conformation that does not prevent assembly of CTB pentamers. Possibly, this distorts the way the first α-helix packs against the body of the pentamer. The reactivity of MAbs 32D3 and 15C11 is completely lost. A10V is the only variant to affect the reactivity of 32D3, which retained reactivity with all other mutants, as well as LT-Ip, LT-Ih, and the chimeric B pentamers. In all, variants at 47 other positions had no effect on this epitope. The epitope defined by MAb 32D3 is highly dependent on conformational integrity of the pentamer since it showed no reactivity to denatured CTB (Fig. 7D). In contrast, MAbs 1E9 and 40D9 showed binding to denatured CTB but to a significantly lesser degree than 12D2. We propose that the epitopes of MAbs 1E9 and 40D9 are conformation dependent but located within the CTB monomer. MAb 12D2 most likely recognizes a linear epitope of CTB, since it is the only MAb that is strongly reactive with denatured CTB and that binds to a tryptic digest of CTB in a Western blot (data not shown). This epitope is not exposed in the native CTB pentamer, and thus it could be located at the monomer-monomer interface or within the globular core of the monomer.

A46V defines another immunologically important residue, since the mutant affects reactivity with six of the nine MAbs, four completely. Qu and Finkelstein (31) showed that residue 46 was involved in several of the 20 different patterns of reactivity they observed among a panel of anti-CTB MAbs and LTB subunit variants. Residue 46 also differs between CTB and the B pentamers from LT-Ih (Ala) and LT-Ip (Glu). The lack of reactivity of MAb 40D9 with A46V also explains why 40D9 cross-reacted with the B pentamers of LT-Ih (Ala-46) but not LT-Ip (Glu-46). All other MAbs (except 40B10) that failed to cross-react with LTB also showed reduced binding to the A46V CTB variant. The epitopes of most of these MAbs also have other important residues that differ between CTB and LTB in both the amino and carboxyl halves of the monomer since they also fail to react with either hybrid. Exceptions are MAb 22C6, which bound the CTB/LTB hybrid, therefore eliminating any of the seven carboxyl-terminal residues as contributing to its epitope, and MAbs 4E2 and 40D9, which bind weakly to the CTB/LTB hybrid. These findings indicate that one or more of these same seven variant residues in the carboxyl-terminal half of CTB contributes partially to their epitopes, along with one or more of the 12 variant residues in the amino-terminal half of CTB. Since MAbs 15C11, 22C6, or 35G8 do not react with CT from *V. cholerae* U1 (El Tor, differing from classical CTB by H18Y, T47I, and possibly G54S substitutions) but do bind the V52I+G54D mutant, either His-18 or Thr-47 are also required components of their epitopes. Since MAb 22C6 does bind the CTB/LTB hybrid (H18Y), this implicates T47I as the U1 CTB variant residue that prevents its binding. Similarly, the H18Y substitution may be the cause of reduced reactivity of MAb 4E2 with both U1 CT and the CTB/LTB hybrid.

In summary, we have shown that the RPIHA is a sensitive assay to test for CTB binding to receptor in the context of a eukaryotic cell membrane, and we have used this assay to identify Tyr-12 as a novel essential residue for GM_1 binding by CTB, in addition to the Gly-33 and Trp-88 residues previously identified. Other CTB variants with substitutions for other residues that are known to interact with GM_1 in the crystal structure still retain the ability to bind GM_1 by SPRIA, even though they show an altered RPIHA phenotype, which suggests that the multivalent nature of the GM_1-receptor binding site interaction may be tolerant of several single substitutions. Using the variants isolated in this study, together with novel CTB/LTB hybrids, we have begun to determine the tertiary structure of epitopes of anti-CTB MAbs, which we show are highly conformational and may involve structural elements both within and between CTB monomers. Fully defining the epitopes of these MAbs should provide additional insights into the immunological properties of CT and related enterotoxins and may aid in the design of future vaccines against cholera and related diarrheas.

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