INFECTION AND IMMUNITY, Jan. 1991, p. 114–118
0019-9567/91/010114-05$02.00/0

Vol. 59, No. 1

Localization of Protective Epitopes within the Pilin Subunit of the *Vibrio cholerae* Toxin-Coregulated Pilus

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Received 3 July 1990/Accepted 28 October 1990

From a collection of monoclonal antibodies (MAbs) that recognize the native structure of the toxin-coregulated pilus of *Vibrio cholerae*, two protective MAbs (16.1 and 169.1) were used to localize the corresponding epitopes on the pilus. These MAbs were shown to specifically recognize the carboxyl half of the TcpA pilin subunit, as determined by their recognition of proteolytic fragments and hybrid pilin proteins. The positions of the epitopes were precisely determined through the use of overlapping synthetic peptides corresponding to this region of the pilin. The MAbs were found to recognize adjacent peptides, delineating a region between residues 157 and 199. Since the protective nature is specific for these two antibodies, the findings suggest that this region defines a domain that participates in toxin-coregulated pilus-mediated colonization and therefore represents a target for studies of its potential as an immunogen for incorporation into a component cholera vaccine.

Despite attempts at vaccination and antibiotic therapy, cholera continues to be a source of high morbidity and mortality in regions of the world where the causative organism, *Vibrio cholerae* O1, remains endemic. Much of the effort towards formulating an improved vaccine focuses on the induction of mucosal immunity stimulated by various surface antigens (14). As a general strategy, bacterial colonization fimbriae (pili) represent a group of surface organelles with high potential in this regard (1, 13). In the case of *V. cholerae*, several molecules with a possible role in adhesion and colonization have been characterized by biochemical, morphological, or genetic means and include surface proteins and hemagglutinins in addition to several types of pili (4, 8, 24, 26, 28). While the mechanism of *V. cholerae* colonization is likely to be a complex process, involving a temporal expression of several factors, thus far only two of these molecules, the mannose-sensitive hemagglutinin expressed by strains of the El Tor biotype (6) and toxin-coregulated pilus (TCP), which is expressed by both El Tor and classical biotype strains (21, 23, 26), have been genetically demonstrated to be essential to the process. Of the two molecules, the role of TCP is the more extensively characterized, with the colonization requirement having been demonstrated for humans (9). This colonization defect is paralleled in the infant mouse experimental cholera model, with *tcpA* pilin mutants showing a 5- to 6-log-unit increase in 50% lethal dose (26).

TCP is a thin filamentous pilus forming characteristic bundles on the surface of *V. cholerae* when cultured under high toxin-expressing conditions. This regulation reflects the dependence of both toxin and TCP expression on ToxR (16, 17, 26). At least 16 genes that are part of the global ToxR virulence regulon and require ToxR for their expression have been identified (18). Eight of these genes encode products required for TCP biogenesis and function (23), but the mechanism by which TCP mediates colonization is not yet known. For example, it has not been completely determined whether the pilus domains that participate in colonization are found exclusively on TcpA or whether there are additional ancillary pilus subunits that participate in the colonization process. Unlike a number of pilus types, none of the corresponding gene products have thus far been shown to be directly associated with the pilus structure, implying that TCP may be a homopolymer of TcpA pilin subunits. This possibility is supported by a recent study that included screening 120 monoclonal antibodies (MAbs) directed against TCP; all of them recognized the major subunit (22). The likelihood that domains functional in TCP-mediated colonization are located within the TcpA pilin is supported by the finding that only a fraction of the MAbs with comparable titers against intact TCP were significantly protective in passive immunization experiments using an infant mouse cholera model (22). The fact that only a few of these antibodies that recognize TcpA tertiary structure are protective has been taken to indicate that they act by binding domains of the pilus that are critical for function rather than via nonspecific steric hindrance of binding by generally coating the pilus. In this study we have mapped the locations of the epitopes recognized by two of these protective MAbs.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *V. cholerae* O395 (classical, Ogawa) was used throughout this study. Derivatives carrying *tcpA-phoA* gene fusions were constructed as previously described (25, 26). The locations of fusion joints determined by DNA sequencing are shown in Fig. 1. To promote TCP expression, *V. cholerae* strains were grown in Luria broth (pH 6.5) at 30°C for 18 h with aeration. *Escherichia coli* DH5α (Bethesda Research Laboratories, Gaithersburg, Md.) was used as the host strain for cloning experiments and was grown in Luria broth (20).

Pilus purification. TCP pili were purified from strain O395 as previously described (21).

Antisera. The polyclonal antibodies and MAbs directed against purified TCP have been previously described (21, 22). Polyclonal antibody in rabbit hyperimmune antiserum exhibited a titer of 25,600 (reciprocal of the highest dilution with peroxidase-conjugated sheep anti-rabbit immunoglobu-

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lin G as the secondary antibody in an enzyme-linked immunosorbent assay) against TCP and was used at a 1:2,000 dilution for Western immunoblots and undiluted for dot blot analysis in this study. Ascitic fluid samples containing MAbs 16.1 and 169.1 had titers of 64,000 and 32,000, respectively, against TCP and were used at dilutions of 1:1,000 and 1:500, respectively, for Western blots and undiluted for dot blot analysis. Polyclonal antibody against TcpA peptides was generated after the peptides were conjugated to the carrier protein keyhole limpet hemocyanin by glutaraldehyde-mediated cross-linking. New Zealand White rabbits were immunized with 150 μg of peptide-keyhole limpet hemocyanin conjugate in Freund's complete adjuvant and boosted twice with the same amount in Freund's incomplete adjuvant. The titers of antibodies against TCP1 and TCP6 are 800 and 3,200, respectively, and they were used at 1:500 and 1:1,000 dilutions, respectively, for Western blot analysis.

Chemical and enzymatic cleavage of TcpA pilin. Chemical cleavage of TCP with CNBr (Pierce, Rockford, Ill.) was performed according to the method of Gross and Witkop (7). In the case of staphylococcal V8 protease digestion, TCP was found to be somewhat resistant to this enzyme under standard conditions described by Cleveland et al. (2). Better digestion was achieved by excluding sodium dodecyl sulfate (SDS) and incubating the reaction mixture for a longer time at a higher temperature as follows. Pili were dissolved at a concentration of 100 μg/ml in polyacrylamide gel electrophoresis (PAGE) sample buffer lacking SDS, heated at 100°C for 2 min, and digested for 1 h at 37°C by the addition of protease to a concentration of 20 μg/ml. The reaction was stopped by the addition of SDS, and in some cases 2-mercaptoethanol, to concentrations of 2% and 10%, respectively, and heating for 2 min at 100°C.

Analysis of TcpA cleavage products. The pilus preparation or cleavage products were diluted twofold in 2× protein sample buffer, with or without 2-mercaptoethanol, and separated by SDS-PAGE (11) with an 18% acrylamide gel. The proteins were then electrophoretically transferred onto polyvinyl fluoride (PVDF) membranes (Millipore, Bedford, Mass.) according to the method of Xu and Shively (29). After transfer, the paper was cut into strips, some of which were used for immunoblotting and some of which were used for amino-terminal sequence analysis of the cleavage products. Reaction with polyclonal antibodies or MAbs was detected with goat anti-rabbit immunoglobulin G or goat anti-mouse immunoglobulin G peroxidase-conjugated antibodies, respectively, and then visualized by adding 4-chloro-1-naphthol. For sequence analysis, the strips were stained with 0.2% Coomassie blue R250 and the band corresponding to the one detected by the immunoblot was cut out and sequenced by automated Edman degradation on an Applied Biosystems 4778 gas phase sequencer.

Dot blot analysis of synthetic peptides. Peptides were synthesized on an Applied Biosystems model 430A synthesizer by using the HOBt esters of tBoc amino acids in an NMP solvent coupling system as described by the manufacturer. Ten microliters of synthetic peptide (1 mg/ml) was immobilized onto an Immobilon AV affinity membrane by following the manufacturer's (Millipore) suggested protocol. The reactions with antibody were visualized as described above.

Construction and analysis of gene fusions expressing the carboxyl end of TcpA. The *Ssp*I site located near the middle of *tcpA* (Fig. 1) was utilized to fuse the 3′ half of the gene into the open reading frame that is expressed from the *lac* promoter in plasmid vector pUC18 (30). Plasmid pRTG7H3, carrying *tcpA* on a 2-kb *Hin*dIII fragment (19), was the source of *tcpA* DNA and was digested with *Ssp*I and at a *Hin*dIII site downstream of the gene. The resulting fragment was ligated into pUC18 that had been digested with *Sma*I and *Hin*dIII. The ligated molecules were transformed into *E. coli* DH5α. Plasmid DNA was extracted (20) from Ap^r isolates that yielded white colonies in the presence of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and analyzed for the correct insert by restriction enzyme digestion and agarose gel electrophoresis (15). The hybrid protein was visualized by Western analysis of total protein extracts (20, 27) after expression was induced in an early-log-phase culture with 0.1 mM IPTG (isopropyl-β-D-galactopyranoside) for 3 h.

RESULTS

Analysis of chemical cleavage and proteolytic products of TcpA. The TcpA pilin primary sequence shown in Fig. 1 was used to predict the products resulting from the chemical or enzymatic cleavage of the protein. To first approximate the region of the TcpA pilin recognized by the protective MAbs, a pilus preparation was subjected to treatment with CNBr. Analysis of the resulting products by SDS-PAGE and immunoblotting after transfer to Immobilon-PVDF membranes revealed a single band with a molecular mass of approximately 10 kDa that was recognized by both MAbs (Fig. 2). Under reducing conditions, a smaller band was detected by MAb 16.1, indicating that the original larger band spanned a disulfide bond. This suggests that the CNBr fragment detected under reducing conditions results from cleavage of the Met residues at positions 93 and 130, which produces a fragment spanning the region from residue 93 to the pilin C terminus under nonreducing conditions which is divided in two upon reduction, with the 6.9-kDa C-terminal band likely being the one recognized by MAb 16.1 (Fig. 1). We have been unable to confirm this interpretation because the visualized fragment has been refractory to several attempts at N-terminal sequence analysis. However, the two Cys residues at positions 120 and 186 have previously been shown to be disulfide bonded, resulting in a slower migration of the intact TcpA under reducing conditions than under nonreducing conditions (17a), further suggesting that the increased migration of the detected CNBr fragment upon reduction in lane B of Fig. 2 was due to its separation from the cleavage product in lane A. Also of interest is the finding that the reduced CNBr product was no longer, or sometimes barely, detectable with MAb 169.1, suggesting a conformational specificity in the epitope recognized by this antibody.

In a second determination, the products of a staphylococcal V8 protease digestion were analyzed as described above, except that under reducing conditions a portion of the membrane was stained with Coomassie blue so that the corresponding fragment could be cut out of the membrane and its N-terminal sequence could be determined. The staphylococcal V8 digestion yielded a single band that was recognized by both antibodies and migrated with an apparent molecular mass of about 11 kDa, slightly larger than the fragment identified by CNBr treatment. This fragment also migrated faster upon reduction indicating that, like the CNBr fragment, it spans the disulfide bond (data not shown). The smaller fragment was again readily detectable by MAb 16.1 but not by MAb 169.1. Sequence analysis of the N terminus of the staphylococcal V8 fragment revealed that it was digested at the Glu residue at position 83 (Fig. 1). The larger-than-expected apparent molecular weight of approxi-

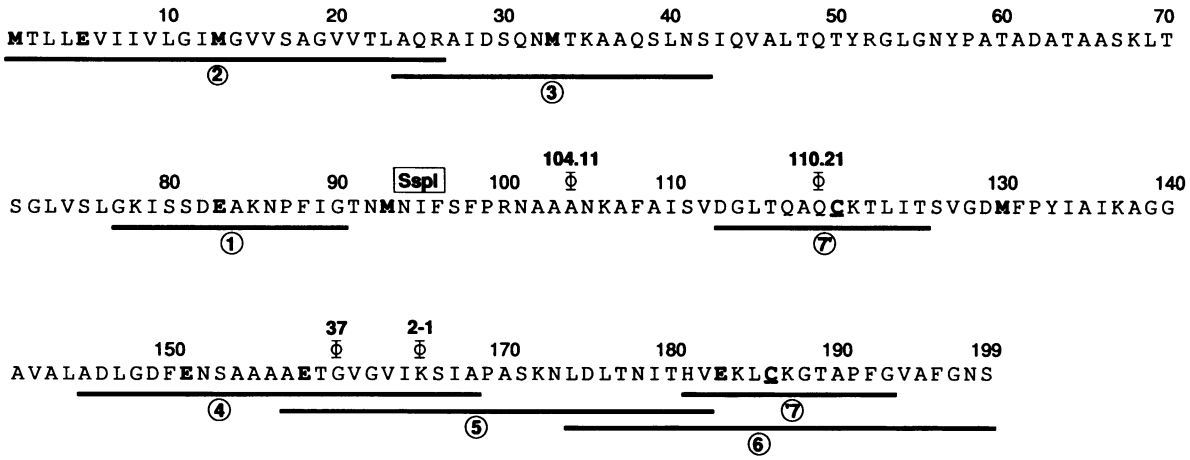

FIG. 1. Primary sequence of the mature form of TcpA pilin. The locations of relevant restriction enzyme recognition sites, in-frame *phoA* fusion joints determined by DNA sequence analysis (Φ), and synthetic peptides (circled numbers) are indicated. Methionine (M) and glutamate (E) residues (potential sites for CNBr and staphylococcal V8 protease cleavage, respectively) are in boldface type. The two cysteine (C) residues that participate in intrachain disulfide bond formation are in boldface type and underlined.

mately 10 kDa for the reduced fragment makes it likely that it extends to near the C terminus of the protein (perhaps the potential site at residue 183), suggesting that not all of the potential staphylococcal V8 sites were accessible to the enzyme.

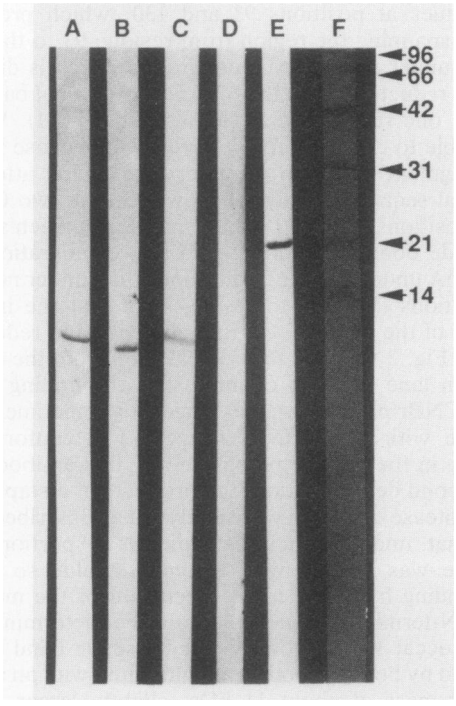

FIG. 2. Immunoanalysis of proteolytic fragments after treatment of TcpA with CNBr. The pilin was treated as described in the text, and the products were separated by SDS-PAGE on an 18% acrylamide gel without (lanes A and C) or with (lanes B, D, and E) 2-mercaptoethanol. The samples were then transferred to PVDF membranes, incubated with the indicated antibodies, and developed. Lanes A and B, MAb 16.1; lanes C and D, MAb 169.1; lane E, polyclonal anti-TCP antibody; lane F, molecular mass standards (given in kilodaltons on the right).

While the results of the proteolytic analysis suggested that the recognition sites for both MAbs are located in the C-terminal half of TcpA, additional approaches were undertaken to support this interpretation and to more precisely localize the epitopes.

Antibody recognition of hybrid proteins. To genetically confirm the C-terminal location of the epitopes recognized by the antibodies, two experiments were performed. The first was to analyze hybrid protein products expressed by available O395 *tcpA-phoA* gene fusion strains. The fusion joints are indicated in Fig. 1. The four hybrid proteins, as well as TcpA itself, could all be distinguished on Western blots by using anti-TcpA antibodies or antibodies directed against synthetic peptide 1 (also indicated in Fig. 1). None of the hybrid proteins were recognized by the two MAbs 16.1 and 169.1 (data not shown), again indicating that the epitopes that these antibodies recognize lie in the C-terminal portion of TcpA and likely are localized downstream of or spanning the most distal fusion joint represented by fusion 2-1.

To genetically confirm the C-terminal location of the epitopes recognized by the monoclonal antibodies, a hybrid protein was constructed by using a DNA fragment extending from the *Ssp*I site in *tcpA* at a position corresponding to residue 95 of the protein to a *Hin*dIII site in the coding region of the downstream *tcpB* gene. This fragment was inserted between the *Sma*I and *Hin*dIII sites of plasmid vector pUC18 such that the *Sma*I-*Ssp*I junction in the resulting plasmid, pSSP1, retained an open reading frame that expresses a hybrid protein composed of the C-terminal half of TcpA fused to the coding region that spans the multiple cloning site of pUC18. Western immunoanalysis of a whole-cell protein extract of *E. coli* expressing this product showed a band of approximately 12 kDa, as predicted from the sequence, that was recognized by polyclonal anti-TcpA antibody and by both MAbs (Fig. 3). This hybrid protein was recognized by antibodies raised against peptide 6 but not by antibodies against peptide 1.

Localizing the epitopes with overlapping synthetic peptides. Since both the TcpA cleavage products and the genetic constructs demonstrated that the epitopes recognized by the protective MAbs lay in the C-terminal domain of the protein, probably spanning or downstream of the 2-1 fusion joint, a

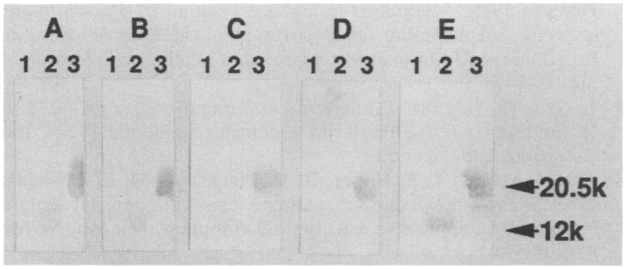

FIG. 3. Immunoanalysis of the hybrid protein expressed by plasmid pSSP1. Western analysis was performed on total cell lysates prepared from strain DH5α carrying either pUC18 or pSSP1 after induction with IPTG for 3 h and compared with TcpA isolated from *V. cholerae*. Lanes 1, DH5α(pUC18); lanes 2, DH5α(pSSP1); lanes 3, TcpA. The antibody probes are polyclonal anti-TcpA (A), MAb 16.1 (B), MAb 169.1 (C), polyclonal anti-peptide 1 (D), and polyclonal anti-peptide 6 (E). k, Kilodaltons.

series of overlapping peptides corresponding to this region was synthesized in order to more precisely define the regions recognized by the antibodies. Several peptides corresponding to other regions of TcpA were synthesized and utilized as controls. The regions corresponding to these peptides are shown in Fig. 1. The peptides were immobilized on Immobilon AV affinity membranes and then reacted with either polyclonal anti-TcpA antibodies or each of the MAbs. The polyclonal antiserum recognized a number of the peptides, while MAb 16.1 was seen to be specific for peptide 6 and MAb 169.1 was specific for peptide 5 (Fig. 4).

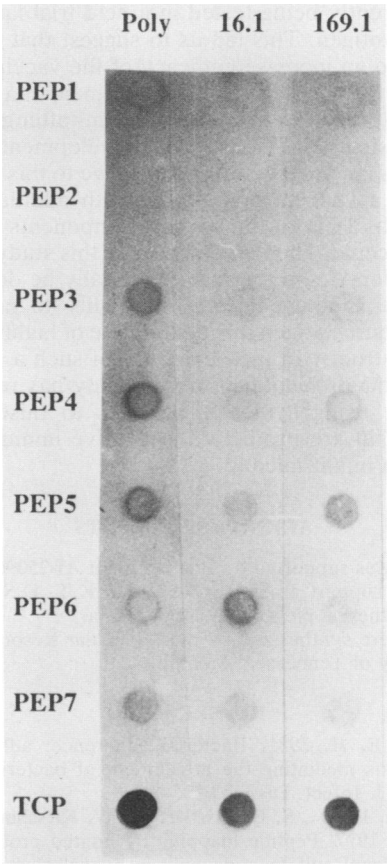

FIG. 4. Dot blot analysis of synthetic peptides. Peptides (PEP1 to PEP7) or TCP was immobilized on Immobilon AV affinity membranes and reacted with polyclonal anti-TCP antibodies (Poly) or either of the indicated MAbs.

DISCUSSION

The mechanism by which TCP mediates colonization is not fully understood. In this study we utilized two previously described MAbs that provide passive immunity in an infant mouse cholera model and specifically recognize TcpA (22) to localize a region within the pilin that participates in the colonization process. By using immunodetection of various hybrid *tcpA* gene products, proteolytic fragments, and overlapping synthetic peptides, it was determined that the epitopes recognized by the antibodies lie near each other and map to the carboxyl end of TcpA. The positions of the overlapping peptides used to finally localize the epitopes are shown in Fig. 1. The specific recognition of peptide 5 by MAb 169.1 places the position of the epitope it recognizes between residues 157 and 182. Since it does not recognize peptide 4 or peptide 6, the region of overlap between these peptides is not sufficient to form the epitope. The epitope recognized by MAb 16.1 maps between residues 174 and 199, as evidenced by its recognition of peptide 6. Again, a complete epitope is not contained within either of the overlapping regions between peptide 6 and peptides 5 and 7, since neither of these last two peptides is recognized by the antibody. The positions of the epitopes recognized by the antibodies include a portion of the disulfide loop and the region immediately adjacent on the carboxyl-terminal side.

Recent studies have utilized a combination of peptide binding and competitive inhibition studies, as well as antibodies directed against synthetic peptides, to determine the position of a domain that mediates the recognition of buccal epithelial cells by the related *Pseudomonas* PAK pilus (10, 12). The PAK pilus is one of a number of type 4 pili that are elaborated by a variety of diverse bacteria that have the common feature of colonizing mucosal surfaces (5). TCP is highly related to this type of pilus and has recently been included in this pilus class (19, 26). The position of the functional domain within the PAK pilin is strikingly similar to what we report here for TCP, being located within and adjacent to the carboxyl side of the cystine loop. Additional studies that have examined the ability of MAbs against PAK to inhibit *Pseudomonas* binding have determined that the most effective MAbs again mapped to the C terminus (3). Interestingly, the most broadly active MAb was partially dependent on intact intrachain disulfide bridges, similar to our MAb 169.1. The similarity between the overall predicted structures of type 4 pilins and the similar localization of a functional domain within this structure make it likely that there is a common molecular basis for the mechanism by which these pili mediate colonization. We should stress, however, that while a putative cell surface receptor has been identified for PAK pili (10), no such receptor has yet been identified for TCP and its exact mechanism for mediating colonization has yet to be determined. An alternative function that the antibodies might be inhibiting could be a specific bacterial interaction that promotes microcolony formation. High levels of TCP expression are known to promote bacterial autoagglutination in vitro (26).

In a previous study, we demonstrated the protective potential of TcpA-specific antibodies in passive immunization by using an infant mouse cholera model (21). As part of these studies, we determined that the killed whole-cell

vaccine currently being tested in a field trial lacked detectable TcpA antigen. This led us to suggest that its inclusion might lead to an increased efficacy of the vaccine (21). One way in which TCP could be included in such a formulation would be by preparing appropriate strains after growth under TCP-expressing conditions. The development of such a formulation is in progress. An alternative to this method is to supplement a vaccine preparation with additional components, such as TCP, or to use such components to formulate a defined vaccine. The identification in this study of domains within the TcpA pilin that can potentially be developed for this purpose should enhance our ability to provide large amounts of pure antigen through the use of highly expressive genetic constructs. In fact, evidence of such a potential for the pilin domains identified in this study has recently been obtained by using synthetic peptides to raise polyclonal antibodies that are protective in passive immunization experiments in infant mice (21a).

ACKNOWLEDGMENTS

This work was supported by USPHS grant AI-25096 and biomedical research support grant RR05423 to R.K.T. D.S. is a visiting scholar from the People's Republic of China.

Peptides were synthesized by the Molecular Resource Center at the University of Tennessee, Memphis.

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