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Interaction between Functional Domains of *Bacillus thuringiensis* Insecticidal Crystal Proteins

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Interactions among the three structural domains of *Bacillus thuringiensis* Cry1 toxins were investigated by functional analysis of chimeric proteins. Hybrid genes were prepared by exchanging the regions coding for either domain I or domain III among Cry1Ab, Cry1Ac, Cry1C, and Cry1E. The activity of the purified trypsin-activated chimeric toxins was evaluated by testing their effects on the viability and plasma membrane permeability of Sf9 cells. Among the parental toxins, only Cry1C was active against these cells and only chimeras possessing domain II from Cry1C were functional. Combination of domain I from Cry1E with domains II and III from Cry1C, however, resulted in an inactive toxin, indicating that domain II from an active toxin is necessary, but not sufficient, for activity. Pores formed by chimeric toxins in which domain I was from Cry1Ab or Cry1Ac were slightly smaller than those formed by toxins in which domain I was from Cry1C. The properties of the pores formed by the chimeras are therefore likely to result from an interaction between domain I and domain II or III. Domain III appears to modulate the activity of the chimeric toxins: combination of domain III from Cry1Ab with domains I and II of Cry1C gave a protein which was more strongly active than Cry1C.

Bacillus thuringiensis produces a crystalline inclusion body containing highly specific insecticidal proteins, also called δ-endotoxins, which may vary, depending on the bacterial strain, in both number and type (5, 19, 43). Their mode of action involves a cascade of events including solubilization of the crystal, activation of the toxins by gut proteases, and recognition of a binding site on the midgut brush border membrane, followed by pore formation, membrane transport disruption, and cell lysis, ultimately leading to insect death (12, 17, 23, 43).

The three-dimensional structures of three insecticidal crystal proteins, Cry1Aa, Cry3A, and CytB, have been elucidated by X-ray diffraction analysis (18, 31, 32). The activated Cry1Aa and Cry3A proteins are remarkably similar with respect to their overall structures, and they share three structural domains (18, 31). Domain I, located at the N-terminal end of the activated protein, is made of a bundle of eight hydrophobic and amphipathic α-helices, whereas domains II and III are made mostly of β-sheets. Domain I is thought to be responsible for pore formation in the epithelial cell membrane. A number of mutations in domain I abolish or reduce the toxicity of the protein without affecting its binding properties significantly (1, 7, 21, 60). N-terminal fragments of Cry1Ac (57) and Cry3B (56) have been shown to form channels in liposomes and planar lipid bilayer membranes. Experiments in which fragments from different closely related toxins were exchanged have localized the specificity-determining and receptor-binding domains of a number of toxins to domain II (15, 16, 27, 33, 35, 50, 58). Site-directed mutagenesis experiments have further stressed the importance of three domain II surface-exposed loops situated at the apex of the molecule in receptor recognition (28, 34, 38–40, 48, 49, 61). Domain III is also involved in specificity, since mutations in this domain which affect toxicity and binding have been described (2). In addition, full insecticidal activity often requires the presence of sequences from both domains II and III of an active toxin (15, 20, 44). Exchange of domain III between different insecticidal proteins also often results in host range modifications and changes in the membrane proteins to which the toxins bind (3, 10, 11, 29, 35).

Models have been proposed to explain channel formation (14, 23, 24, 31, 46), but experimental evidence is lacking to clearly demonstrate the exact involvement of each toxin domain in the overall toxic process. The different domains appear to influence each other to some extent, since mutations in domain I which affect binding have been described (60), as well as mutations in domain II which affect toxicity without significantly affecting overall binding (37, 48). Mutant Cry3A with an altered domain II loop 3 even had reduced binding but increased toxicity (61). Mutations in highly conserved block 4 of domain III which affect toxicity and pore formation have also been described (8, 47, 59). Some of these were, in addition, shown to have no significant effect on initial binding (8).

We report here the effect of domain swapping on pore formation and membrane permeability in Sf9 cells. These cultured insect cells have proven to be particularly useful for studying the formation and properties of channels induced by *B. thuringiensis* toxins (49, 52, 53), and their high sensitivity to Cry1C has been shown to correlate with specific binding to a 40-kDa plasma membrane protein (26). Domain II from a toxic parental protein is clearly required for activity, but it is not sufficient and must interact properly with domain I for the chimeras to be active. Exchange of domain III can lead to an increase in toxicity, whereas exchange of domain I may result in total loss of toxicity. The size of the pores was also affected by the exchange of domain I but was unrelated to the level of toxicity.

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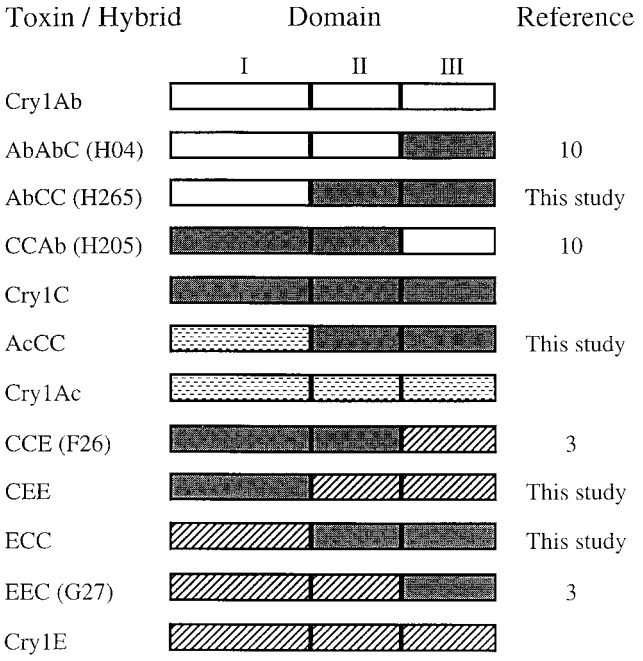

FIG. 1. Schematic representation of the domain compositions of the parental and hybrid toxins used in this study. The names originally given to previously described hybrid toxins are in parentheses (3, 10).

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* DH5α and crystal-negative *B. thuringiensis* subsp. *kurstaki* HD1 Cry$^-$ B were used as host strains for transformation. pAlter-1 (Promega) was used for site-directed mutagenesis. pHT3101 was used as an *E. coli-B. thuringiensis* shuttle vector (30). *cry1C* from *B. thuringiensis* subsp. *entomocidus* 60.5, *cry1E* from *B. thuringiensis* subsp. *kenyae* 4F1, and *cry1Ac* from *B. thuringiensis* subsp. *kurstaki* HD1 were subcloned from plasmids p60.5G31 (54), pEM14 (55), and pMP37 (36), respectively.

Molecular techniques. Standard recombinant DNA techniques were used as described by Sambrook et al. (41). DNA fragments containing the *B. thuringiensis cry1Ac*, *cry1C*, and *cry1E* toxin genes were subcloned into pAlter-1 prior to oligonucleotide-mediated site-directed mutagenesis conducted by using the Altered Sites II kit (Promega). Mutated clones were analyzed by DNA sequencing by using the chain termination technique (42) in an Applied Biosystems 370A nucleotide sequence analyzer. DNA and protein sequence alignments were obtained through the CLUSTAL method by using the Megalign software from the DNAStar package.

Construction of chimeric genes. The structures of the toxins used in the present study are represented schematically in Fig. 1. Construction of the chimeras EEC (G27), CCE (F26), AbAbC (H04), and CCAb (H205) has been described previously (3, 10, 11). The AbCC hybrid (H2C5) was selected after in vivo recombination from the *cry1Ab-cry1C* tandem plasmid pRM7 (10). These chimeric genes were expressed in *E. coli* XL-1 blue, and the proteins were purified as described previously (3, 10, 11). Domain I chimeras AcCC, CEE, and ECC were constructed by exchanging domain I at the level of the highly conserved QLTRE sequence separating domain I from domain II as shown by the three-dimensional structure of Cry1Aa (18). An *Xho*I site was created by mutagenesis of codons 264 and 265 ($_{264}$ACA AGA GAA→$_{264}$ACT CGA GAA [*Xho*I site underlined]) of *cry1Ac* and codons 263 and 264 ($_{263}$ACA AGG GAA→$_{263}$ACT CGA GAA [*Xho*I site underlined]) of *cry1C* and *cry1E*. Mutagenesis and frame conservation were verified by DNA sequencing prior to cloning. This *Xho*I site was located over similar codons in *cry1Ac*, *cry1C*, and *cry1E*, thus allowing the replacement of the domain I coding region without any frameshift. In addition, this mutagenesis did not change the amino acid sequence of the toxin and the resulting chimeric proteins therefore carried a QLTRE bridge identical to that of the parental proteins. By use of a unique *Sac*I site located upstream from the promoter region in *cry1Ac*, *cry1C*, and *cry1E* along with the newly created *Xho*I site, an *Xho*I-*Sac*I fragment containing the region encoding domain I and the first 28 amino acids of the protoxin, as well as the promoter sequence, was generated. The *cry1Ac-cry1C* (AcCC) chimeric gene was constructed by replacing the *Xho*I-*Sac*I fragment of the mutated *cry1C* gene with the corresponding *Xho*I-*Sac*I fragment from the mutated *cry1Ac* gene. Similarly, the chimeric *cry1C-cry1E* (CEE) and *cry1E-cry1C* (ECC) genes were constructed by replacing the *Xho*I-*Sac*I fragment from the mutated *cry1E* and *cry1C* genes with that from the same mutated *cry1C* and *cry1E* genes, respectively. The chimeric *cryAc-cry1C* (AcCC), *cry1C-cry1E* (CEE), and *cry1E-cry1C* (ECC) genes were transferred to the shuttle vector pHT3101 (30) and expressed in a crystal-negative *B. thuringiensis* strain.

Electroporation of *B. thuringiensis* cells. Transformation was performed as described by Lereclus et al. (30). Electroporated cells were plated onto solid brain heart infusion medium with erythromycin (25 μg/ml). The presence of the expected construct in selected recombinant *B. thuringiensis* strains was verified by extracting *B. thuringiensis* plasmid DNA and transforming *E. coli* with the extracted DNA. Recombinant *E. coli* strains were selected with erythromycin as for screening of recombinant *B. thuringiensis* strains. Plasmid DNA was extracted from *E. coli* and mapped with restriction enzymes. Maps were compared to those of similar constructs extracted from the original recombinant *E. coli* strains obtained prior to electroporation of *B. thuringiensis*.

Purification of parasporal inclusions. A 125-ml liquid culture, on the "usual medium" of de Barjac and Lecadet (9) supplemented with glucose and erythromycin, was inoculated with 2.5 ml of an overnight preculture and grown at 28°C under vigorous agitation until complete lysis, as monitored by light microscopy. Cultures were harvested by centrifugation, and parasporal inclusion bodies were purified as described previously (22). Protein concentration was determined by the method of Bradford (4).

Solubilization and activation of protoxins. Inclusion bodies were solubilized and protoxins were activated as described previously (13). Activated toxins were centrifuged at 14,000 × *g* for 30 min at 4°C, and the supernatant was purified by low-pressure liquid chromatography through a Q-Sepharose anion-exchange column (Pharmacia) equilibrated with 40 mM Na_2CO_3, pH 10.7. Activated toxins were eluted with a 50-to-500 mM NaCl gradient. Fractions containing the eluted proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. They were dialyzed for 48 h at 4°C against sterile double-distilled water, and precipitated protein was lyophilized.

Cell cultures. Sf9 cells (ATCC CRL 1711) were grown at 27°C in Grace's insect cell culture medium (Gibco) supplemented with 350 mg of sodium bicarbonate per liter, 3.33 g of yeastolate (Difco) per liter, 3.33 g of lactalbumin hydrolysate (Difco) per liter, 50 mg of sodium ampicillin (Gibco) per liter, and 10% (vol/vol) heat-inactivated fetal bovine serum (Gibco). Cultures (100 to 150 ml) were grown in spinner flasks inoculated with 1.0×10^5 cells/ml with constant stirring at 50 to 60 rpm. Experiments were carried out with cells in the midlogarithmic phase of growth.

Solutions. Osmotic-swelling experiments were carried out with 50 mM KCl–21 mM NaCl–14 mM $MgCl_2$–11 mM $MgSO_4$–6.8 mM $CaCl_2$–3.9 mM glucose–2.2 mM fructose–120 mM sucrose–10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES)-Tris, pH 6.5 (G* medium) (51). The composition of this medium was modified as required for individual experiments by either replacing the KCl with 50 mM *N*-methyl-D-glucamine hydrochloride, increasing the KCl concentration from 50 to 100 mM, or adding 100 mM glucose, sucrose, or raffinose. The osmolarity of all solutions was measured with a DigiMatic model 3D2 osmometer (Advanced Instruments) and adjusted with sucrose.

Viability assays. The viability of Sf9 cells was evaluated by determining their ability to exclude trypan blue after different periods of exposure to the toxins. Cell culture aliquots were incubated at room temperature (24°C) with gentle agitation in the presence or absence (controls) of 50 μg of toxin per ml. Samples (80 μl) of the cell suspension were then mixed with 20 μl of 0.4% (wt/vol) trypan blue and mounted immediately in the counting chamber of a hemocytometer. Over 200 cells were counted in duplicate for each sample, and the proportion of cells remaining translucent was taken as a measure of cell viability.

Intracellular pH measurements. Intracellular pH was monitored in individual cells with a previously described microspectrofluorimetric technique (51, 52), with slight modifications. The PIPES-Tris concentration of the medium was increased from 10 to 50 mM. Cells were loaded by incubation with a 5 μM concentration of the membrane-permeant acetoxymethyl ester derivative of 2,7-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF) (Molecular Probes) in G* medium for 20 min at room temperature and rinsed with BCECF-free G* medium. Experiments were carried out at room temperature in a custom-made coverslip holder fitted to the stage of a Zeiss inverted microscope coupled to a Spex CMIII spectrofluorimeter. Fluorescence intensities were measured at excitation wavelengths of 450 and 500 nm and an emission wavelength of 530 nm. Intracellular pH was calculated from the ratio of the fluorescence intensities measured at 500 and 450 nm as described earlier (51, 52).

Video imaging analyses. Osmotic volume changes of individual Sf9 cells were analyzed as described previously (53). Briefly, cells were observed under ×150 magnification with an Olympus IMT-2 inverted microscope. Images were recorded every 1 s with a monochrome CCD-72 camera (DAGE-MTI) connected to a 486 DX2 PC computer equipped with an IP8/AT videographics system board (Matrox). The surface area occupied by each cell was measured, and the cellular volume was estimated with a program made by us.

RESULTS

Influence of domain exchange on cell viability. The activities of the various toxins were first tested with a trypan blue exclusion viability assay (Fig. 2). Among the parental toxins (Fig. 2A), only Cry1C was strongly active against Sf9 cells, killing

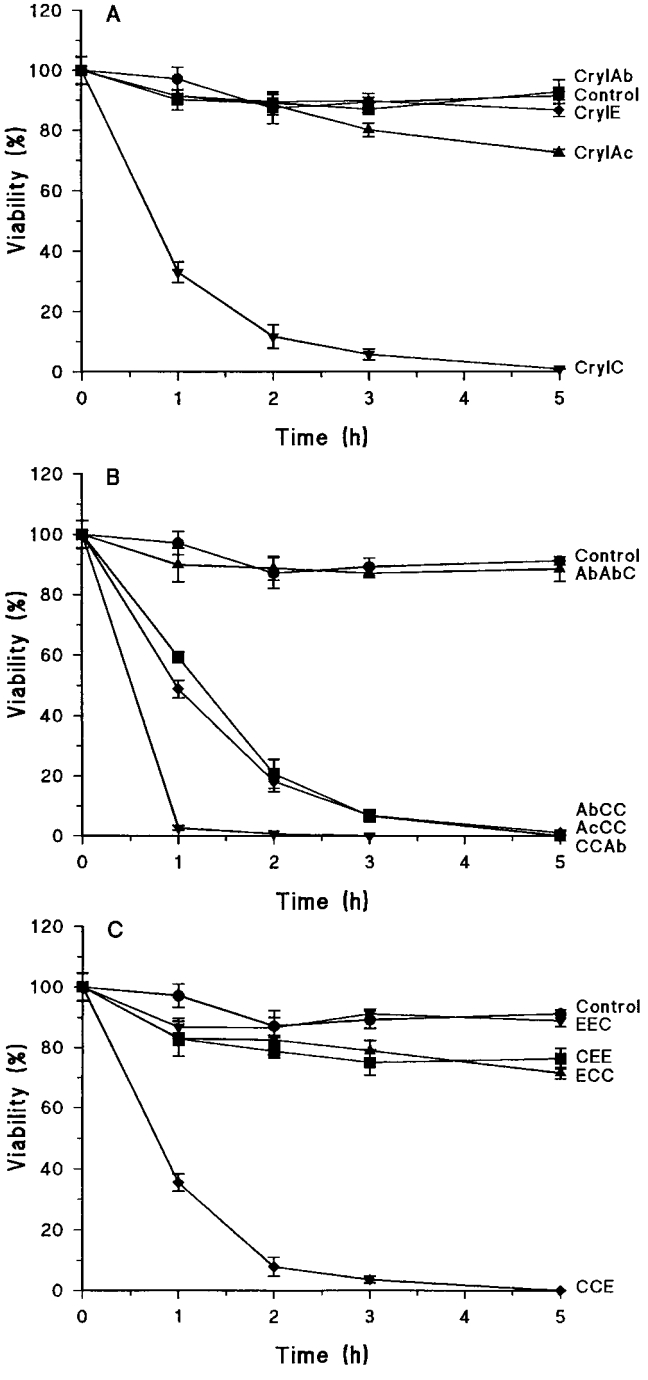

FIG. 2. Effects of parental and chimeric toxins on the viability of Sf9 cells. The cells were grown in Grace's insect cell culture medium. Their viability was evaluated by determining their ability to exclude trypan blue after different periods of exposure to 50 μg of the indicated parental toxins per ml. (A) Symbols: ●, control; ■, Cry1Ab; ▲, Cry1Ac; ▼, Cry1C; ◆, Cry1E. (B) Cry1Ab-Cry1C and Cry1Ac-Cry1C hybrids. Symbols: ●, control; ■, AbCC; ▲, AbAbC; ▼, CCAb; ◆, AcCC. (C) Cry1E-Cry1C hybrids. Symbols: ●, control; ■, CEE; ▲, ECC; ▼, EEC; ◆, CCE. The data are means ± the standard errors of the means of three experiments, each done in duplicate.

over 85% of the cells after 2 h of incubation. A slight sensitivity to Cry1Ac was observed after prolonged incubation, however, with about 20% mortality after 5 h. The viability of cells incubated with Cry1Ab and Cry1E, on the other hand, was not significantly affected in comparison with that of controls incubated without toxin. Of the Cry1Ab-Cry1C and Cry1Ac-Cry1C hybrids, only those containing domain II from Cry1C were active (Fig. 2B). The toxicity of AbCC and AcCC was comparable to that of Cry1C, whereas AbAbC had no detectable activity and CCAb was more highly toxic, killing over 95% of the cells within 1 h. In contrast, among the Cry1C-Cry1E hybrids, only CCE was strongly active against Sf9 cells, causing above 90% mortality after 2 h (Fig. 2C). ECC, another toxin which contained domain II from Cry1C, was inactive along with EEC and CEE (Fig. 2C).

Effects of chimeric proteins on intracellular pH variations. As was shown previously, the intracellular pH of Sf9 cells is strongly dependent on the extracellular K^+ concentration (51). Removal of this ion from the external medium resulted in reversible acidification of the cells (Fig. 3A) due to the presence of a strong K^+-H^+ exchange activity in the plasma membrane (51). In the presence of an active toxin, such as the AcCC hybrid, the intracellular pH equilibrated with that of the

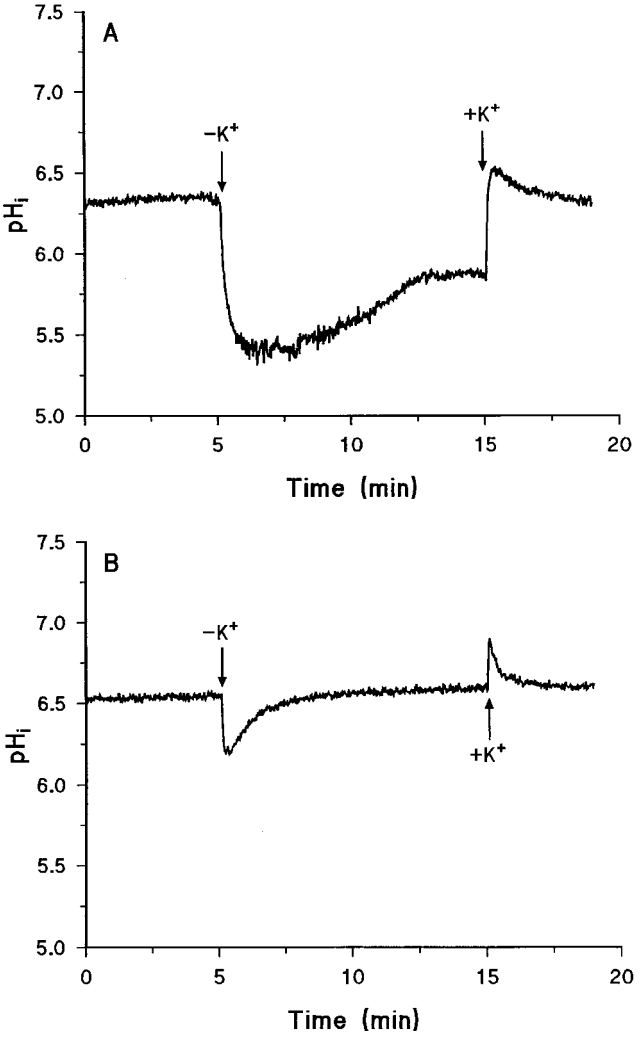

FIG. 3. Effect of the extracellular K^+ concentration on the intracellular pH (pH_i) of Sf9 cells. Sf9 cells were preincubated for 15 min without (A) or with (B) 25 μg of the AcCC chimeric toxin per ml in G* medium containing 50 mM KCl at pH 6.5. The bath was rinsed with toxin-free G* medium, and the intracellular pH was monitored for 5 min. Extracellular KCl was then replaced with *N*-methyl-D-glucamine–HCl for another 10 min, after which the cells were returned to G* medium. The traces shown are representative of five experiments.

medium, i.e., 6.5 (Fig. 3B). The cell acidification resulting from removal of external K^+ was transient, and the pH returned rapidly to its original level. Restoration of the extracellular K^+ caused an alkalinization of the cells which was also transient.

Experiments similar to that illustrated in Fig. 3B were carried out to evaluate the ability of the parental and chimeric toxins to abolish pH gradients across the plasma membrane of Sf9 cells (Fig. 4). Only toxins which were strongly active in the trypan blue exclusion assay (i.e., Cry1C, AbCC, CCAb, AcCC, and CCE) caused the pH to equilibrate across the plasma membrane, both in the presence and in the absence of extracellular K^+ (Fig. 4). With any of the other toxins, the intracellular pH remained between 6.23 and 6.35 in the presence of K^+ and stabilized at about 5.9 after removal of K^+, as was observed in the absence of toxin (Fig. 4).

Permeability of the pores induced by parental and chimeric proteins. The effects of the different toxins on the plasma membrane permeability of Sf9 cells were further analyzed with a recently described video imaging technique (53). Cells were preincubated for 15 min in G* medium with either nothing (controls) or one of the toxins, and the KCl concentration of the medium was rapidly raised from 50 to 100 mM. In the absence of toxin, this hypertonic shock caused Sf9 cells to shrink by 8 to 12% of their original volume (Fig. 5A). This was also observed with the inactive toxins Cry1Ab, Cry1Ac, Cry1E (Fig. 5A), AbAbC (Fig. 5B), EEC, CEE, and ECC (Fig. 5C). The active toxins Cry1C, AbCC, AcCC, CCAb, and CCE, on the other hand, allowed KCl to diffuse across the plasma membrane, and the cells rapidly recovered their original volume (Fig. 5). In the presence of the relatively high toxin concentration used in these experiments (10 μg/ml), the permeability to KCl was, in fact, sufficiently high to completely abolish the effect of the hypertonic shock with most of the active toxins (Fig. 5).

To estimate the sizes of the pores formed by the active chimeras, a comparison of toxin-induced permeability to various sugar molecules was done (Fig. 6). Cells exposed to Cry1C (Fig. 6B) and CCAb (Fig. 6D) became permeable to glucose and sucrose but remained impermeable to raffinose. CCE (Fig. 6F) allowed the diffusion of glucose but did not increase the permeability of the membrane to sucrose. Finally, whereas cells preincubated with AbCC (Fig. 6C) and AcCC (Fig. 6E) became highly permeable to KCl, those exposed to AbCC, in contrast with those exposed to AcCC, acquired a slight permeability to glucose, evidenced by the fact that their volume stabilized at a higher level than that of control cells (Fig. 6A) (53). Based on the hydrodynamic radii of these sugar molecules (25, 45), the diameters of the pores formed by Cry1C and CCAb are between 1.0 and 1.2 nm and those of the pores made by AbCC and CCE are between 0.8 and 1.0 nm. The pore generated by AcCC has a diameter of less than 0.8 nm.

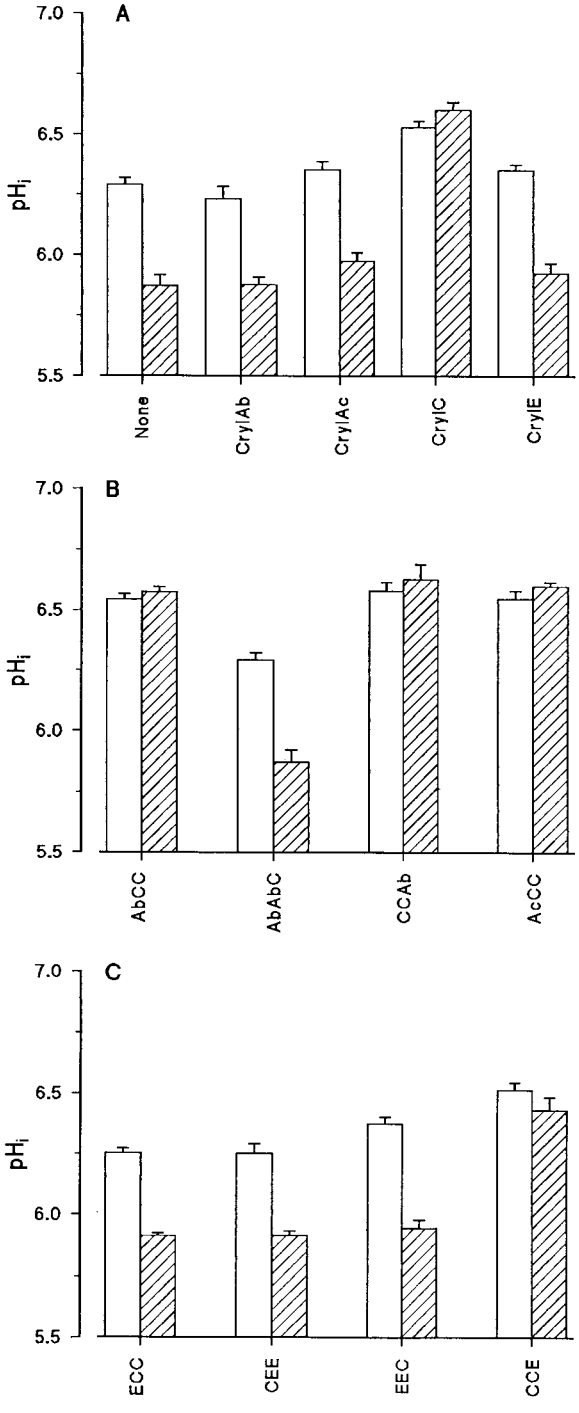

FIG. 4. Effects of parental and chimeric toxins on the intracellular pH (pH_i) of Sf9 cells. Sf9 cells were preincubated with 25 μg of the indicated parental toxins per ml (A), Cry1Ab-Cry1C and Cry1Ac-Cry1C hybrids (B), or Cry1E-Cry1C hybrids (C). Intracellular pH was recorded in experiments similar to those illustrated in Fig. 3 in the presence of KCl (open bars) and 10 min after replacement of the medium with a K^+-free solution (hatched bars). The extracellular pH was 6.5 throughout the experiments. The data are means ± the standard errors of the means of five experiments.

DISCUSSION

Results of the present study stress the importance of domain II in the specificity of *B. thuringiensis* Cry toxins, in agreement with previous reports (see the introduction). However, they also provide evidence of interactions essential for toxin activity. Sf9 cells were sensitive to only one of the parental toxins, Cry1C, and all hybrid proteins that were active had domain II of this toxin. A similar conclusion was drawn from the results obtained with all three of the techniques used to characterize the different toxins. Nevertheless, the presence of domain II from Cry1C was not sufficient to confer activity against Sf9 cells on the hybrid proteins, as evidenced by the inactivity of the ECC hybrid. The reason for this apparent incompatibility between domain I from Cry1E and the rest of the molecule from Cry1C remains to be established. It suggests, however, that interactions between domain I and the other two domains are probably important for the activity of the toxin. Since a thor-

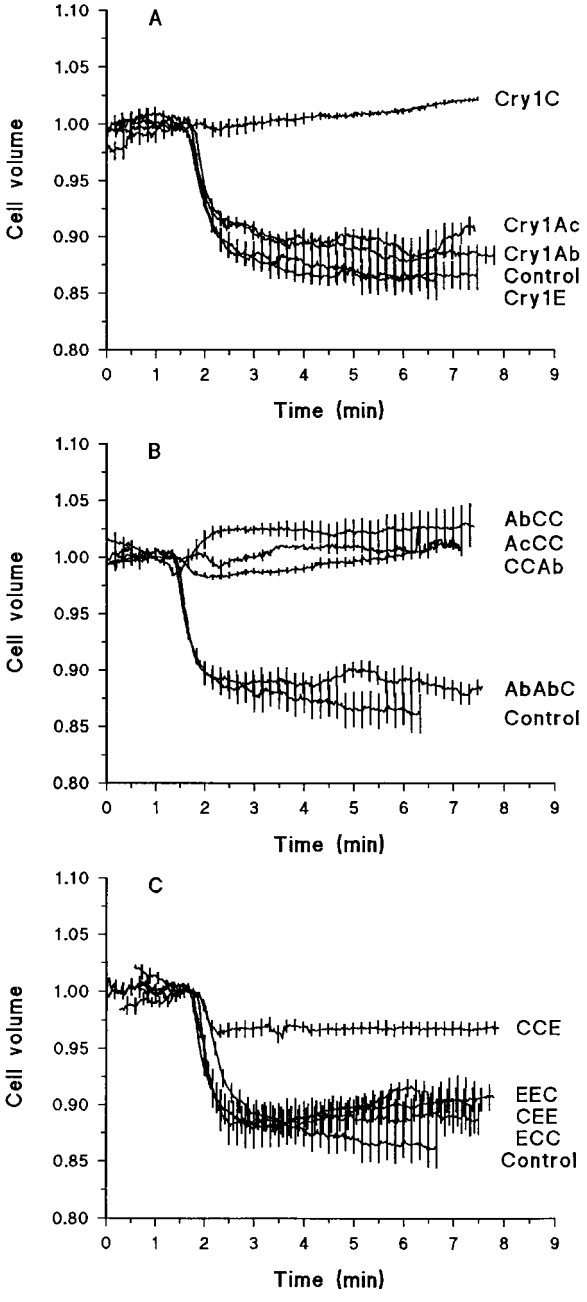

FIG. 5. Video imaging analysis of the pore-forming abilities of parental and chimeric toxins in Sf9 cells. The cells were preincubated for 15 min with 10 μg of the indicated parental toxins per ml (A), Cry1Ab-Cry1C and Cry1Ac-Cry1C hybrids (B), or Cry1E-Cry1C hybrids (C). Pore formation was assessed from the rate of osmotic swelling of the cells. These were submitted to a hypertonic shock by replacement of the bathing solution with a solution with a similar composition but enriched with 50 mM KCl. Cell volume was recorded every 1 s. The data are means ± the standard errors of the means of four to eight experiments. For clarity, error bars are shown at every 10th experimental point.

ough effort was made to avoid the introduction of additional changes within the hinge region separating domains I and II, as well as within the domains themselves, these data suggest that the lack of activity of ECC is related to a structural incompatibility of domain I from Cry1E and domains II and III from Cry1C. A number of interdomain salt bridges and hydrogen bonds have been identified in the crystal structure of Cry1Aa (18). It remains to be seen whether this incompatibility is due to the absence of some of these interactions or whether new interactions which interfere with toxin activity were generated in ECC.

Further evidence of functional interactions between the different toxin domains was obtained from the comparison of the sizes of the pores formed by the different hybrid proteins. Pore size depended, in part, on the toxin from which domain I originated. The pores formed by AbCC (diameter, 0.8 to 1.0 nm) and AcCC (diameter, <0.8 nm) were smaller than those formed by Cry1C (diameter, 1.0 to 1.2 nm). However, it is uncertain whether the smaller size of the pores formed by AbCC and AcCC reflects the properties of Cry1Ab and Cry1Ac since the sizes of the pores formed by very few *B. thuringiensis* toxins have been reported. Cry1Ac has nevertheless recently been shown to form much larger pores (diameter, 2.4 to 2.6 nm) which allow the diffusion of raffinose and various polyethylene glycol molecules into *Manduca sexta* brush border membrane vesicles (6). Although this comparison may suggest a strong influence of domains II and III on the size of the pores formed by AcCC, the possibility cannot be excluded that the properties of the pores formed by *B. thuringiensis* toxins also depend on the membrane receptor to which they bind (24, 53). The sizes of the pores formed by given toxins could therefore differ, depending on the biological membrane in which they are formed. Unfortunately, the inactivity of Cry1Ab and Cry1Ac toward Sf9 cells precludes a direct comparison of the properties of the pores formed by AbCC and AcCC with those formed by the parental toxins in these cells. On the other hand, the size of the pores can clearly be affected by domain III since, in contrast to Cry1C and CCAb, which allowed rapid diffusion of sucrose, CCE allowed, at best, very poor diffusion of this molecule.

Although differences in the sizes of the pores formed by different toxins were readily detected, these differences did not appear to be directly related to toxicity, at least at the relatively high doses used in the present study, since Cry1C, AbCC, AcCC, and CCE killed Sf9 cells at similar rates. On the other hand, even though Cry1C and CCAb had similar pore sizes, CCAb was clearly more active than Cry1C. The level of toxicity therefore appears to be more strongly affected by the number of pores formed by a given toxin than by relatively small differences in pore size such as those reported here.

Several cases in which substitution of domain III increased the toxicity of a *B. thuringiensis* toxin have been reported previously (3, 10, 11, 29, 35). For example, EEC is highly toxic to *Spodoptera exigua* and *Mamestra brassicae* larvae even though these insects are not sensitive to Cry1E (3). Similarly, AbAbC is much more active against *S. exigua* than is Cry1Ab (10). Although the presence of domain III from Cry1C was insufficient to confer activity against Sf9 cells on these hybrid proteins, it remains probable that the CCAb hybrid can bind to another receptor in addition to that which recognizes Cry1C, even though Sf9 cells have repeatedly been reported to be insensitive to Cry1Ab (52, 53). Previous ligand blot studies have indeed revealed that CCAb appears to bind to one of the proteins which bind Cry1Ab in brush border membrane vesicles of *S. exigua* but to a different minor Cry1Ab-binding protein in those of *M. sexta* (10, 11). Further work is required, however, to establish whether a similar mechanism operates in Sf9 cells.

In agreement with a number of studies involving mutagenesis of *cry* genes (see the introduction), the results of the present study indicate that relatively minor modifications of the primary structures of the toxins can lead to considerable changes in their properties. AbCC formed slightly larger pores than did AcCC despite the fact that Cry1Ab and Cry1Ac have

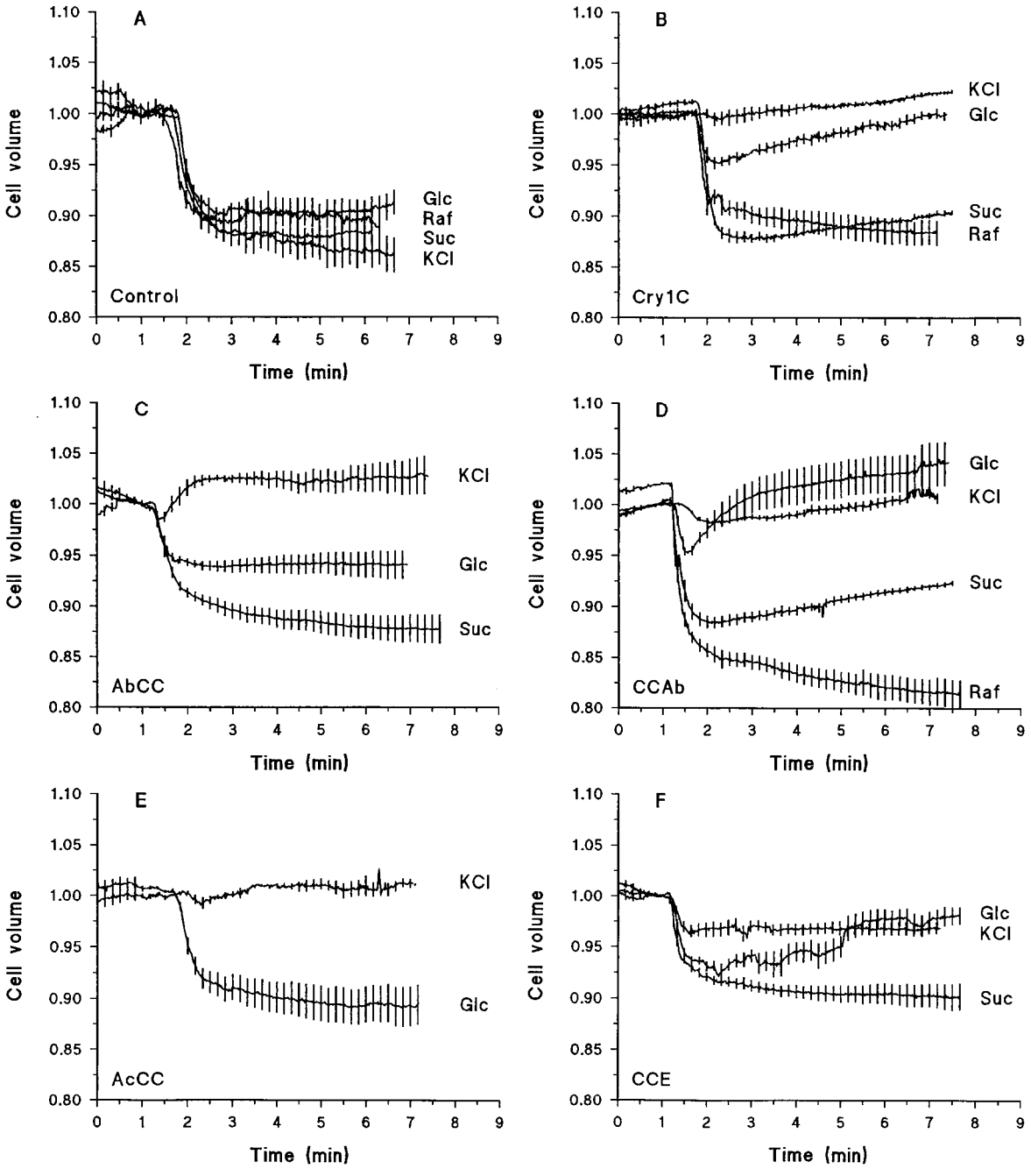

FIG. 6. Estimation of the sizes of pores formed by *B. thuringiensis* toxins in the plasma membrane of Sf9 cells. Sf9 cells were preincubated for 15 min without toxin (A) or with 10 μg of Cry1C (B), AbCC (C), CCAb (D), AcCC (E), or CCE (F) per ml. They were submitted to a hypertonic shock by replacement of the bathing solution with a solution with a similar composition but enriched with either 50 mM KCl or 100 mM glucose (Glc), sucrose (Suc), or raffinose (Raf). Cell volume was recorded every 1 s. The data are means ± the standard errors of the means of four to eight experiments. For clarity, error bars are shown at every 10th experimental point. The estimated pore diameters were 1.0 to 1.2 nm for Cry1C and CCAb, 0.8 to 1.0 nm for AbCC and CCE, and less than 0.8 nm for AcCC.

almost identical primary structures in domain I. In addition, because Cry1Ac and Cry1E are highly homologous in domain I, 90% of the amino acid residues are identical in AcCC and ECC. Nevertheless, the pore-forming ability of AcCC was comparable to that of Cry1C whereas ECC was completely inactive.

Although this study provides further support for the notion that domain I is directly involved in pore formation and domains II and III play a critical role in specificity, it also demonstrates that the toxicity, as well as the properties, of the pores formed by the hybrid proteins cannot be simply predicted from the properties of the toxins from which the different domains being combined originated.

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