Clostridium botulinum C3 ADP-ribosyltransferase Gene

CLONING, SEQUENCING, AND EXPRESSION OF A FUNCTIONAL PROTEIN IN ESCHERICHIA COLI

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C3 ADP-ribosyltransferase is an exoenzyme produced by certain strains of Clostridium botulinum types C and D, which specifically ADP-ribosylates rho and rac proteins in eukaryotic cells. The enzyme was purified from a culture filtrate of C. botulinum type C strain 003-9, and the amino acid sequence from the amino-terminal Ser to Asn emerged was determined by Edman degradation. Using a set of degenerate primers based on the sequence, we amplified a part of the gene for this enzyme by polymerase chain reaction. A 2.1-kilobase pair HincII fragment of C. botulinum DNA containing the whole structural gene was then identified by Southern analysis with the polymerase chain reaction product as a probe, and the complete nucleotide structure of the gene together with flanking regions was determined by cloning and DNA sequencing the HincII fragment. The gene encodes a protein of 244 amino acids with a Mr of 27,362 which begins with a putative signal peptide of 40 amino acids. Escherichia coli carrying this gene produced the active enzyme, and about 60% of it was found in the culture medium. Immunoblot analysis with antiserum against the enzyme revealed the presence of two immunoreactive proteins of 27 and 23 kDa in the cytoplasmic/membrane fraction and only the 23-kDa protein in the periplasm and the medium, suggesting that the enzyme expressed is processed in the E. coli, exported into the periplasm and released into the culture medium.

Bacterial mono-ADP-ribosyltransferases catalyze the transfer of an ADP-ribose moiety of NAD to acceptor proteins in eukaryotic cells, thereby affecting cellular functions (for recent reviews, see Ref. 1). C3 ADP-ribosyltransferase secreted by certain strains of a Gram-positive bacterium, Clostridium botulinum, is the latest member of this group of enzymes. In 1987 we reported an ADP-ribosyltransferase in preparations of C1 and D botulinum toxins which catalyzes ADP-ribosylation of a 21-kDa eukaryotic protein (2, 3). Current with our report, Aktories et al. (4, 5) purified an ADP-ribosyltransferase from the culture filtrate of C. botulinum type C and named it C3 ADP-ribosyltransferase to distinguish it from the C1 and C2 toxins produced by the same bacterium. This enzyme also ADP-ribosylates a 21-kDa protein in eukaryotic cells. The relation of the ADP-ribosyltransferase activity in the toxin preparations to C3 ADP-ribosyltransferase reported by Aktories et al. was confusing at first, but it is now clear that the enzyme activity in the toxin preparations is carried by C3 ADP-ribosyltransferase and not by the toxins per se (6-9).

Several groups of investigators purified the ADP-ribose acceptor proteins in this reaction and identified them as members of rho gene products (10-16). Very recently, another group of proteins were also shown to serve as substrates of C3 ADP-ribosyltransferase and named rac proteins (17, 18). Both rho and rac proteins belong to a growing family of small molecular weight GTP-binding proteins with ras proteins as prototypes (19). The importance of ras proteins in cell growth and transformation has been well documented (20), and accruing evidence suggests that other small molecular weight GTP-binding proteins serve as regulators in numerous biological processes including signal transduction, protein trafficking, secretion, and cell growth (19). Several findings suggest that rho and rac proteins also play important roles in cell physiology. For example, diploid strains of yeast Saccharomyces cerevisiae carrying the mutant of RH01 could not sporulate (21). Mouse NIH 3T3 cells with overexpression of a rho protein showed the transformed phenotype, and the transformed cells were tumorigenic in nude mice (22). The molecular mechanisms of these phenomena, however, remain obscure. C3 ADP-ribosyltransferase may be a useful tool to analyze the action mechanisms of the rho and rac proteins, because the ADP-ribosylation occurs at asparagine residues in their putative effector binding domains and alter their functions (23). Several groups have already shown that the introduction of C3 ADP-ribosyltransferase into various culture cells and Xenopus oocytes caused morphologic changes with concomitant modification of the acceptor proteins (24-26). However, use of the purified enzyme as a probe has several drawbacks, because permeation of the protein into cells requires special techniques or takes several hours or days of incubation with µg/ml concentrations of the enzyme. The C3 ADP-ribosyltransferase gene, when isolated, could overcome these difficulties and might be a better tool to probe the functions of rho and rac proteins. Knowledge of the gene structure is also desirable to understand better the catalytic mechanism of C3 ADP-ribosyltransferase, the enzyme which catalyzes a unique C3-glycosidation reaction of an ADP-ribose to an asparagine residue (23).

We undertook the cloning of the gene for C3 ADP-ribosyltransferase for these reasons. Here we report the complete
FIG. 5. Alignment of the peptide sequences obtained by protease digestions of C3 ADP-ribosyltransferase. The peptides obtained by digestions of purified C3 ADP-ribosyltransferase were aligned according to their sequences and the amino-terminal sequence of the purified protein. Solid lines indicate residues identified by Edman degradation. X denotes an unidentified amino acid residue. The prefixes K, E, D, and C represent peptides derived from digestions with lysyl endopeptidase, V8 protease, endoproteinase Asp-N and α-chymotrypsin, respectively. They are numbered sequentially from the amino terminus. The sequences used for the design of PCR primers are shown by double underlines.

nucleotide structure of the gene for this enzyme. We also show that E. coli carrying the gene produced the active enzyme, and a substantial amount of the enzyme was released into the culture medium.

EXPERIMENTAL PROCEDURES

RESULTS

Amino Acid Sequencing of C3 ADP-ribosyltransferase—C3 ADP-ribosyltransferase was purified from the culture filtrate of C. botulinum type C strain 003-9 as described under "Experimental Procedures." After desalting and denaturation by passing through a reversed-phase column, the purified enzyme was either directly subjected to Edman degradation to determine the amino-terminal structure, or digested with lysyl endopeptidase, V8 protease, endoproteinase Asp-N or α-chymotrypsin. The generated peptides were purified by reversed-phase HPLC and sequenced. The HPLC profiles of each protease digest of the protein are shown in Figs. 1-4. By aligning the obtained sequences, we could determine a part of the primary structure of C3 ADP-ribosyltransferase as a stretch of 192 amino acid residues from its amino terminus (Fig. 5).

Identification and Cloning of DNA Fragment Containing C3 ADP-ribosyltransferase Gene—Based on the obtained amino acid sequence of C3 ADP-ribosyltransferase, a pair of degenerate oligonucleotides corresponding to Glu60-Gly86 (sense) and Tyr67-Glu73 (antisense) were synthesized. Using these oligonucleotides as primers and C. botulinum DNA as a template, PCR was performed to amplify a part of the C3 ADP-ribosyltransferase gene flanked by these primers. As shown in Fig. 6, a DNA fragment of the predicted size was amplified. DNA sequencing of this PCR product confirmed that the amplified product (482 bp) was indeed a part of the C3 ADP-ribosyltransferase gene (data not shown).

Southern analysis of C. botulinum DNA with this PCR product as a probe is shown in Fig. 7. The PCR product hybridized to a 2.1-kb fragment in the HincII digest (lane 1), to 1.2- and 0.6-kb fragments in the SspI digest (lane 2) and in the HincIIISspI digest (lane 3) and to a 0.6-kb fragment in the Dral digest (lane 4) and in the HincII/Dral digest (lane 5) and to a 0.5-kb fragment in the Dral/SspI digest (lane 6). By comparing these data with the nucleotide sequence of a part of the C3 ADP-ribosyltransferase gene obtained by PCR, we deduced that the whole structural gene of this enzyme is most likely to be contained in the 2.1-kb HincII fragment, while the gene is divided into the 1.2- and 0.6-kb SspI fragments and the region coding for its amino-terminal half is localized in the 0.6-kb Dral fragment.

1 Portions of this paper (including "Experimental Procedures" and Figs. 1-4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

2 The abbreviations used are: HPLC, high performance liquid chromatography; bp, base pair(s); kb, kilobase pair(s); PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; rabbit anti-C3 antiserum, rabbit antiserum raised against C3 ADP-ribosyltransferase.

FIG. 6. Agarose gel electrophoresis of the PCR product. PCR was carried out and the product was subjected to 2% agarose gel electrophoresis as described under "Experimental Procedures." Lane 1, φX174/HaeIII DNA size markers; lane 2, the PCR product; lane 3, φX174/HincII DNA size markers.

FIG. 7. Southern analysis of C. botulinum DNA with this PCR product as a probe. The PCR product was subjected to 2% agarose gel electrophoresis as described under "Experimental Procedures." Lane 1, φX174/HaeIII DNA size markers; lane 2, the PCR product; lane 3, φX174/HincII DNA size markers.
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The DNA sequence of the 2.1-kb insert in pBTC3-1 was determined completely on both strands by sequencing the subclones of various fragments derived from the insert and sequencing from synthetic primers as depicted in Fig. 8. The inserts in pBTC3-2 and pBTC3-3 were also sequenced by essentially the same strategy. These three plasmids were found to contain the 2,067-bp inserts of the identical sequence, providing confidence that the DNA sequence is correct and no artifactual mutations were introduced into the sequence. The complete nucleotide sequence of the C3 ADP-ribosyltransferase gene and its flanking regions, together with the deduced amino acid sequence of the gene, is shown in Fig. 9. There is an open reading frame of 732 bp, starting at the ATG codon at nucleotide 435-437 and terminating at a TAG codon at nucleotide 1,167-1,169, which encodes a protein of 244 amino acids with a $M_\text{r}$ of 27,362. The translational start of the C3 ADP-ribosyltransferase gene is assigned to the ATG codon at nucleotide 435-437, because this is the only possible start codon between the TCA codon for serine at nucleotide 555-557 which has been shown to be the amino-terminal residue in the secreted form of the enzyme and an upstream in-frame termination codon TAG at nucleotide 417-419. In addition, a possible Shine-Dalgarno sequence (43, 44), AGGAGGGG, is present at 4 bp upstream of the ATG codon, which is very similar to the ribosome binding sequences found in other Gram-positive organisms (45-47).

The 192 amino acid residues following the serine at nucleotide 555-557 of the deduced amino acid sequence match completely with that determined by Edman degradation. The first 40 amino acid residues encoded by the open reading frame share several major structural features with a prokaryotic signal sequence (45, 48-51) such as (a) 1-3 basic amino acid residues at the amino terminus, (b) a stretch of hydrophobic amino acid residues following directly the positively charged amino terminus, (c) proline or glycine residues located in the hydrophobic domain, (d) serine and/or threonine residue(s) following the hydrophobic core and close to the carboxyl terminus, and (e) an alanine or a glycine residue at the carboxyl-terminal end (cleavage site). Thus, these 40 amino acid residues are likely to represent the signal peptide of C3 ADP-ribosyltransferase. Removal of the putative signal peptide would generate the mature C3 ADP-ribosyltransferase consisting of 204 amino acids with a $M_\text{r}$ of 23,119, which is in good agreement with the molecular weight of the purified C3 ADP-ribosyltransferase estimated from SDS-polyacrylamide gel electrophoresis analysis.

Examination of the sequence has revealed several regions with homology to the −35 and −10 elements for a Gram-positive promoter (46, 47, 52) (e.g. TTTACA at nucleotide 376-381 and TAAAGT at nucleotide 398-403 with a reasonable spacing of 16 bp, shown by double underlines in Fig. 9). However, a precise location is difficult due to the high A + T content in the 5' noncoding region of this gene (84.1%). The A + T content of the coding region of the C3 ADP-ribosyltransferase gene is also high (69.9%), in conformity with the base analysis of C. botulinum DNA (53). Consequently, the codon usage for the preprotein of C3 ADP-ribosyltransferase as summarized in Table I shows a strong preference for A/T bases. Downstream of the TAG termination codon, there are three regions of dyad symmetry, the last of which is capable of forming the most stable hairpin structure consisting of an 11-bp stem separated by a 6-bp loop ($\Delta G = -22$ kcal at 25 °C (Ref. 54)), which probably constitutes a transcriptional terminator (55).

With expression of C3 ADP-ribosyltransferase in E. coli—When we transformed E. coli JM 109 cells with the pBTC3 plasmids, we noticed that the transformant grew very slowly, taking 48 h at 37 °C to form 0.5-mm colonies on LB agar plates containing 100 µg/ml ampicillin. During the sequence determination we subcloned various restriction fragments derived from the 2.1-kb insert, and we found that the pBluescript containing any fragment without a nucleotide segment 1-239

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**Fig. 7.** Southern blot analysis of C. botulinum DNA probed with the $^{32}$P-labeled PCR product. C. botulinum DNA digested with HindII (lane 1), SspI (lane 2), HindII and SspI (lane 3), DraI (lane 4), HindII and DraI (lane 5), and DraI and SspI (lane 6) was subjected to Southern blot analysis as described under "Experimental Procedures." DNA fragment sizes are shown in kb at left.

**Fig. 8.** Restriction map and sequencing strategy used for determining the C3 ADP-ribosyltransferase gene and flanking regions in pBTC3-1. The thick arrow represents the coding region of C3 ADP-ribosyltransferase gene with the transcription orientation indicated by the direction of the arrow. The open box denotes the 482-bp PCR product used as the hybridization probe in Southern analysis. Each small arrow represents the length and orientation of a contiguous sequence determination. The closed circle denotes the regions for which synthetic oligonucleotide primers were prepared for sequencing.
Supernatant of activity. As shown in Fig. 10, both the cell extract and culture could dictate the synthesis of C3 ADP-ribosyltransferase in fractions from JM 109. We, therefore, constructed plasmids pBTC3D-1 and pBTC3D-2 (D for deletion) by deleting a fragment containing the insert DNA sustained the normal growth of E. coli. Enzyme activity which specifically ADP-ribosylates rho A protein (compare lanes 2, 3, 6, and 7), while no activity was detected in the preparations from E. coli JM 109 transformed with the vector pBluescript (lanes 10 and 11). The enzyme activity in the cell extract and the culture supernatant of E. coli JM 109(pBTC3D-1) was precipitated by rabbit anti-C3 antiserum (lanes 4 and 8) but was not affected by normal rabbit serum (lanes 5 and 9). Similar results were obtained with the cell extract and culture supernatant from E. coli JM 109(pBTC3D-2) (data not shown). About the same amount of the enzyme activity was detected in the corresponding supernatants of JM 109(pBTC3D-1) and JM 109(pBTC3D-2). The distribution of C3 ADP-ribosyltransferase activity found in the cell extract and culture supernatant of these preparations was 40% and 60%, respectively. Assuming that the C3 ADP-ribosyltransferase expressed in E. coli has the same specific activity as the enzyme from C. botulinum...
has (8), the E. coli-expressed C3 ADP-ribosyltransferase would comprise approximately 1% of the total cellular protein and be present at 2 μg/ml in the culture supernatant.

The localization of the expressed C3 ADP-ribosyltransferase was further examined by immunoblot analysis on fractions obtained by the osmotic-shock method (Fig. 11). The major immunoreactive components in the culture supernatants and the periplasmic fractions of E. coli JM 109(pBTC3D-1) and JM 109(pBTC3D-2) were seen as a doublet at a molecular weight similar to that of the C3 ADP-ribosyltransferase secreted by C. botulinum (23,000). In the whole cell extracts and the cytoplasmic/membrane fractions, another major immunoreactive component was observed at an apparently higher molecular weight of 27,000. Neither of these components were observed in the absence of the C3 ADP-ribosyltransferase gene. The enzyme activity determined in these fractions correlated well with the intensity of the lower molecular weight protein species (data not shown). On the basis of these results, we assume that the lower molecular weight protein represents the active enzyme, and the higher molecular weight protein detected in the whole cell extract and the cytoplasmic/membrane fraction is a precursor form of C3 ADP-ribosyltransferase which retains the signal peptide (see "Discussion").

**DISCUSSION**

The strain of C. botulinum used in this study is a producer of C1 and C2 botulinum toxins along with C3 ADP-ribosyltransferase. Although C3 ADP-ribosyltransferase itself was reported to have no toxicity toward animals (5, 6, 8), we first determined a large part (192 amino acid residues) of the primary structure of the enzyme by protein chemistry techniques to avoid cloning toxic genes in E. coli. Using primers synthesized on the sequence data, a part of the enzyme gene flanked by these primers was amplified by PCR. With the PCR product as a hybridization probe, a 2.1-kb HincII fragment containing the whole C3 ADP-ribosyltransferase gene was then definitely identified by Southern analysis and cloned. The cloned 2.1-kb HincII fragment contained an open reading frame for a preprotein of the enzyme composed of 244 amino acids including the putative signal peptide of 40 amino acids. Removal of the signal peptide would generate the mature C3 ADP-ribosyltransferase of 204 amino acids with a M, of 23,119, which is consistent with the value estimated upon SDS-polyacrylamide gel electrophoresis of the enzyme purified from the culture filtrate of C. botulinum. Assuming C3 ADP-ribosyltransferase receives no post-translational processing at its carboxyl-terminal region, our initial determination of the primary structure was nearly complete, lacking at most only 12 COOH-terminal amino acids.

The C3 ADP-ribosyltransferase gene was cloned in the opposite orientation in the two plasmids, pBTC3D-1 and pBTC3D-2. E. coli JM 109 cells harboring the two plasmids produced about the same amount of C3 ADP-ribosyltransferase as judged by the enzyme assay and immunoblot analysis. The fact that the expression of the C3 ADP-ribosyltransferase gene was independent of the orientation with respect to the lac promoter within the vector suggests that the gene was transcribed from its native promoter of Clostridial origin, although it was not precisely located.

Many proteins that are synthesized with signal peptides and secreted in other organisms have been expressed in E. coli.
The enzyme and the botulinum neurotoxins of known structure have not been thoroughly studied in this paper and remains as a future task, although the release by wholesale cell lysis could be negated by the absence of the putative preprotein in the medium. Practically, our results open up the possibility for preparing active C3 ADP-ribosyltransferase from E. coli, a more amenable and safer bacterium than the natural producer.

The primary structure of C3 ADP-ribosyltransferase was compared with those recorded in the GenBank and EMBL databases, with particular attention to other bacterial mono-ADP-ribosylases of known structure such as cholera toxin (58), E. coli heat-labile enterotoxin (59, 60), pertussis toxin (61), diphtheria toxin (62, 63), Pseudomonas exotoxin A (64), and dinitrogenase reductase ADP-ribosyltransferase from Rhodospirillum rubrum (65). However, no significant homology was found between C3 ADP-ribosyltransferase and these toxins/enzymes. Thus, the assignment of catalytically important residues in the ADP-ribosylation reaction awaits future study. In addition, there is no homology between this enzyme and the botulinum neurotoxins of known structure (66-68).

While our research was in progress, the partial nucleotide sequence of the C3 ADP-ribosyltransferase gene was reported by Popoff et al. (69). The partial amino acid sequence deduced from their study differs by 40\% from that determined in this study. The discrepancy might be due to the difference of the source of DNA used for cloning. They used a bacteriophage DNA isolated from a South African strain of C. botulinum type D (70), while we used total DNA from C. botulinum type C strain 903-9. There might be several isoforms of C3 ADP-ribosyltransferase produced by different strains of C. botulinum. If this is the case, comparative study on them would help to identify the catalytically important residues in C3 ADP-ribosyltransferase. However, Popoff et al. have not reported the expression of their gene, and whether that gene encodes functional C3 ADP-ribosyltransferase is not yet known. At present, the gene cloned in this study is the only one that has been verified to encode the enzymatically and immunologically active C3 ADP-ribosyltransferase. This gene should, therefore, be a basis for further study on the mechanism of enzymatic catalysis for the ADP-ribosylation of rho and rac proteins. This gene would also be a useful tool for getting insight into the hitherto unrevealed functions of rho and rac proteins in eukaryotic cells.

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REFERENCES

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EXPERIMENTAL PROCEDURES

Nucleotide sequence analysis of C3 ADP-ribosyltransferase cDNA

Amino acid sequencing of C3 ADP-ribosyltransferase

Proteinase digestion was carried out with four different pro- teases. For digestion with endoproteinase V8 from Staphylococcus aureus, the enzyme was added and the incubation continued at 30°C for 12 h. For digestion with ALChromobacter tunicus lysyl endopeptidase or endoproteinase Asp-N from Pseudomonas stutzeri, the sample (1.0 pmol) was incubated with 10 pmol of the enzyme in 100 pl of 25 mM Tris-HCl, pH 8.0, containing 1% (v/v) acetonitrile in 0.1% SDS at 37°C for 12 h. For digestion with o-chymotrypsin from Bovine pancreas, the sample (1.0 pmol) was incubated with 10 pmol of o-chymotrypsin in 100 pl of 50 mM MgCl₂, pH 8.0, at 37°C for 12 h.

Proteolysis was followed by reversed-phase HPLC using an Aquapore RP-18 column (250 mm x 4.6 mm i.d.). The enzyme was injected onto a column and eluted with a linear gradient of 0.1% (v/v) trifluoroacetic acid in distilled water and eluted with a linear gradient between 0 and 80% acetonitrile in 0.1% (v/v) trifluoroacetic acid over 40 min. Elution was monitored at 210 nm with a UV detector. Peak fractions were lyophilized with acetonitrile in 0.1% trifluoroacetic acid and subjected to amino acid analysis.

Amino acid analysis was performed using an Applied Biosystems model 120A protein sequencer equipped with a Beckman 990 amino acid analyzer.
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**Gene cloning and expression:**

Genes coding for C3 ADP-ribosyltransferase were isolated from a library of Botulinum C3 ADP-ribosyltransferase (C3 ADPRT) ADP-ribosyltransferase, as described elsewhere (1). The recombinant C3 ADPRT was expressed in E. coli DH5α. Purified C3 ADPRT was assayed for its ADP-ribosyltransferase activity.

**Expression of the gene:**

Expression of the gene in E. coli was achieved using the pBAD vector system. The amplified gene fragment was cloned into the pBAD vector, which contains a galactose-inducible promoter. The recombinant plasmid was transformed into E. coli strain DH5α, which was grown in the presence of 0.2% galactose to induce expression of the gene. The induced culture was harvested and the recombinant C3 ADPRT was purified using affinity chromatography.

**Isolation of DNA from Botulinum:**

DNA was isolated from Botulinum using a standard phenol-chloroform extraction method. The DNA was then treated with RNase A to remove any RNA contamination and was precipitated with ethanol.

**Restriction endonucleases and ligases:**

Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, T4 DNA polymerase, and Klenow fragment of E. coli DNA polymerase I were used in the experiments. Competent E. coli cells were prepared by the method of Karasawa, Osaka, Japan.

**Oligonucleotide synthesis:**

Oligonucleotide primers used for PCR and DNA sequencing were synthesized on an Applied Biosystems model 3800 DNA synthesizer and purified with OligoSynthesis Purification Cartridges (Applied Biosystems).

**Experimental Procedures:**

Purified C3 ADP-ribosyltransferase was digested with lysyl endopeptidase and the digest was applied to reversed-phase HPLC as described under "Experimental Procedures". Isolated peptides were numbered R1 to R10.

**Fig. 1.** BPLC profile of lysyl endopeptidase-digest of C3 ADP-ribosyltransferase. Purified C3 ADP-ribosyltransferase was digested with lysyl endopeptidase and the digest was applied to reversed-phase HPLC as described under "Experimental Procedures". Isolated peptides are numbered R1 to R10.

**Fig. 2.** BPLC profile of V8 protease-digest of C3 ADP-ribosyltransferase. Purified C3 ADP-ribosyltransferase was digested with V8 protease and the digest was applied to reversed-phase HPLC as described under "Experimental Procedures". Isolated peptides are numbered V1 to V12.

**Fig. 3.** BPLC profile of endoproteinase Asp-N-digest of C3 ADP-ribosyltransferase. Purified C3 ADP-ribosyltransferase was digested with endoproteinase Asp-N and the digest was applied to reversed-phase HPLC as described under "Experimental Procedures". Isolated peptides are numbered D1 to D9.

**Fig. 4.** BPLC profile of trypsin-digest of C3 ADP-ribosyltransferase. Purified C3 ADP-ribosyltransferase was digested with trypsin and the digest was applied to reversed-phase HPLC as described under "Experimental Procedures". Isolated peptides are numbered T1 to T12.