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Hybrid proteins between *Pseudomonas* exotoxin A and poliovirus protease $2A^{pro}$

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Abstract Two hybrid proteins between *Pseudomonas aeruginosa* exotoxin A (PE) and poliovirus protease $2A^{pro}$ have been generated. One hybrid protein contains the poliovirus $2A^{pro}$ sequence replacing the region of PE corresponding to amino acids 413–607. The other hybrid contains in addition the transforming growth factor sequence. The two hybrid proteins were efficiently synthesized in *E. coli* cells using the inducible pET vectors. Both hybrid toxins cleaved p220 (eIF-4γ) when the recombinant plasmids were transfected in COS cells infected with recombinant vaccinia virus bearing the T7 RNA polymerase gene.

Key words: Poliovirus $2A^{pro}$; Initiation factor; *Pseudomonas* exotoxin A; Viral protease

1. Introduction

A variety of hybrid toxins and immunotoxins have been generated to selectively kill tumor cells and HIV-infected cells. In addition, several regions of these toxins have been replaced with other protein domains giving rise to hybrid proteins with novel functions [1,2]. Perhaps one of the best characterized toxin used in this type of studies is *Pseudomonas aeruginosa* exotoxin A (PE), a single-chain polypeptide that contains 613 amino acids. PE is arranged in several domains: domain Ia (aa 1–252) involved in binding the toxin to the receptor, domain II (253–364) translocates PE across the membrane, domain Ib (365–404) of unknown function, and domain III (405–613) endowed with catalytic activity, inactivates elongation factor 2 by ADP-rybosylation [3].

Domain I of PE has been replaced by other protein moieties, including growth factors and interleukins to target the toxic effects of PE to specific cell types [2,4–6]. Besides the enzymatic domain of PE, i.e. domain III, has been replaced with the enzyme barnase [7]. The resulting hybrid protein was shown to display RNAase activity [7]. We reasoned that replacement of PE domain III by the poliovirus protease $2A^{pro}$ would produce a hybrid protein able to cleave p220 (also known as eIF-4γ). This hybrid protein would be a very useful tool to analyze the function of this initiation factor in mammalian cells. In the present report we describe the construction of hybrid toxins between PE and poliovirus $2A^{pro}$. These hybrid proteins retain the $2A^{pro}$ activity and are able to cleave p220 when expressed in COS cells.

2. Materials and methods

2.1. Construction of expression plasmids

Vectors containing the $2A^{pro}$ sequence were constructed by standard cloning procedures [8]. Plasmid pVC47355 f(+)T was kindly provided by Dr. I. Pastan (Bethesda, MD) [7,9]. This plasmid contains the sequences encoding the chimeric toxin PE-TGFα under the control of a bacteriophage T7 promoter. Plasmid pPE was prepared from pVC47355 f(+)T by digestion with *Nde*I and self-ligating the 4.9 kb fragment.

Two oligonucleotides were designed to hybridize with regions 3386–3403 (primer 5'-2A) and 3832–3815 (primer 3'-2A) of poliovirus type 1 cDNA from vector pT7XLD, generously provided by Dr. E. Wimmer (Stony Brook, NY). The PCR-amplified product containing the sequence of poliovirus 2A was purified using the Gene Clean kit and was digested with *Sac*II. Plasmids pVC47355 f(+)T and pPE were double-digested with *Sac*II + *Stu*I, giving rise to 4.5 and 4.3 kb DNA fragments, respectively. These DNA fragments were ligated with the PCR-amplified fragment to obtain plasmids pVC-III + 2A and pPE-III + 2A, respectively, and the recombinants were screened by restriction digestion analyses. These plasmids were then used to transform BL21(DE3) *E. coli* cells [10]. The region amplified by polymerase chain reaction was sequenced by the dideoxy method (Sequenase; US Biochemical Corp.).

2.2. Growth and induction of recombinant bacteria

Single clones of BL21(DE3) cells, which inducibly express T7 RNA polymerase, containing the indicated plasmid, were grown overnight at 37°C in LB medium in the presence of 100 μg/ml ampicillin. Then the cells were diluted 100-fold in M9 medium [8] supplemented with 0.2% glucose and antibiotics or in LB medium supplemented with antibiotics. When the cultures reached $A_{660\,nm}$ of 0.4–0.6 they were induced by the addition of 1 mM IPTG. Rifampicin (Sigma) was used at 150 μg/ml to inhibit transcription by *E. coli* RNA polymerase.

2.3. Labelling bacterial proteins

For labelling the proteins synthesized, aliquots of cultures were collected and incubated with 5 μCi/ml [^{35}S]methionine (1.45 Ci/mmol; Amersham Corp.) for 15 min at 37°C. The labelled bacteria were pelleted for 1 min at 12,000 rpm in an Eppendorf microcentrifuge and dissolved in lysis buffer (160 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 100 mM dithiothreitol, and 0.033% Bromophenol blue). After SDS-PAGE, fluorography was carried out with 1 M salicylic acid. The gels were dried and exposed to X-AR films (Kodak) at −70°C.

2.4. COS cells and virus

COS cells were grown in tissue culture dishes (Nunc) and Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% newborn calf serum. The recombinant vaccinia virus bearing the T7 RNA polymerase (VT7) (kindly given by Dr. Moss, NIH, Bethesda) was grown in HeLa cells in DMEM with 2% newborn calf serum. Only the intracellular virus was collected after freezing and thawing.

2.5. Transfection of DNAs with VT7 expression system

For transfection experiments, COS cells were plated on 24-well dishes (Nunc) 24 h before infection with VT7 (m.o.i. 5). After 45 min of virus adsorption, a mixture of DNA (0.2 μg/well) and Lipofectin (2 μg/well) was added to cells as described by the manufacturer (Gibco, BRL). Cells were harvested at the times indicated in figure legends.

2.6. Immunoblot assays

Samples used for immunoblot analyses were dissolved in sample buffer; samples for analyses of p220 were harvested in sample buffer

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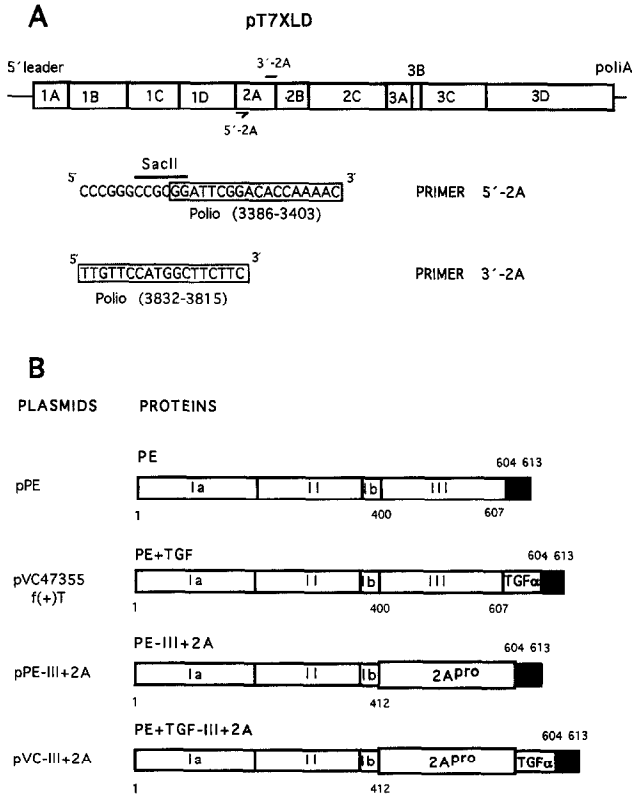
Fig. 1. (A) Primers used for polymerase chain reaction amplification. The region that hybridizes with poliovirus cDNA and the restriction enzyme sites are shown. In the upper part, the poliovirus genome has been represented showing the regions complementary to the primers. (B) Schematic representation of the hybrid proteins between *Pseudomonas* exotoxin A and poliovirus $2A^{pro}$. The filled box represents amino acids 604–613 of PE. →



with 1 mM PMSF (phenylmethyl-sulfonylfluoride) at the indicated times, to avoid degradation of p220. After boiling, the samples were electrophoresed in SDS-PAGE gels as indicated in each figure legend. The immunoblot assay was carried out as previously described [11]. Polyclonal p220 antibodies were obtained from rabbits immunized with synthetic peptides.

3. Results

The cloning and sequencing of the *Pseudomonas aeruginosa* gene encoding exotoxin A has allowed the construction of a variety of hybrid toxic proteins [2,4]. Fig. 1 schematically shows the hybrids obtained between PE and poliovirus $2A^{pro}$. Residues 413–607 from PE were replaced by the sequence encoding poliovirus $2A^{pro}$. This construct still retains the last amino acids (604–613) of PE, including the sequence REDLK which is important for the efficient delivery of the toxin to the cytosol [2]. The other construct contained, in addition, the sequences that encode for transforming growth factor (TGF). This is impor-

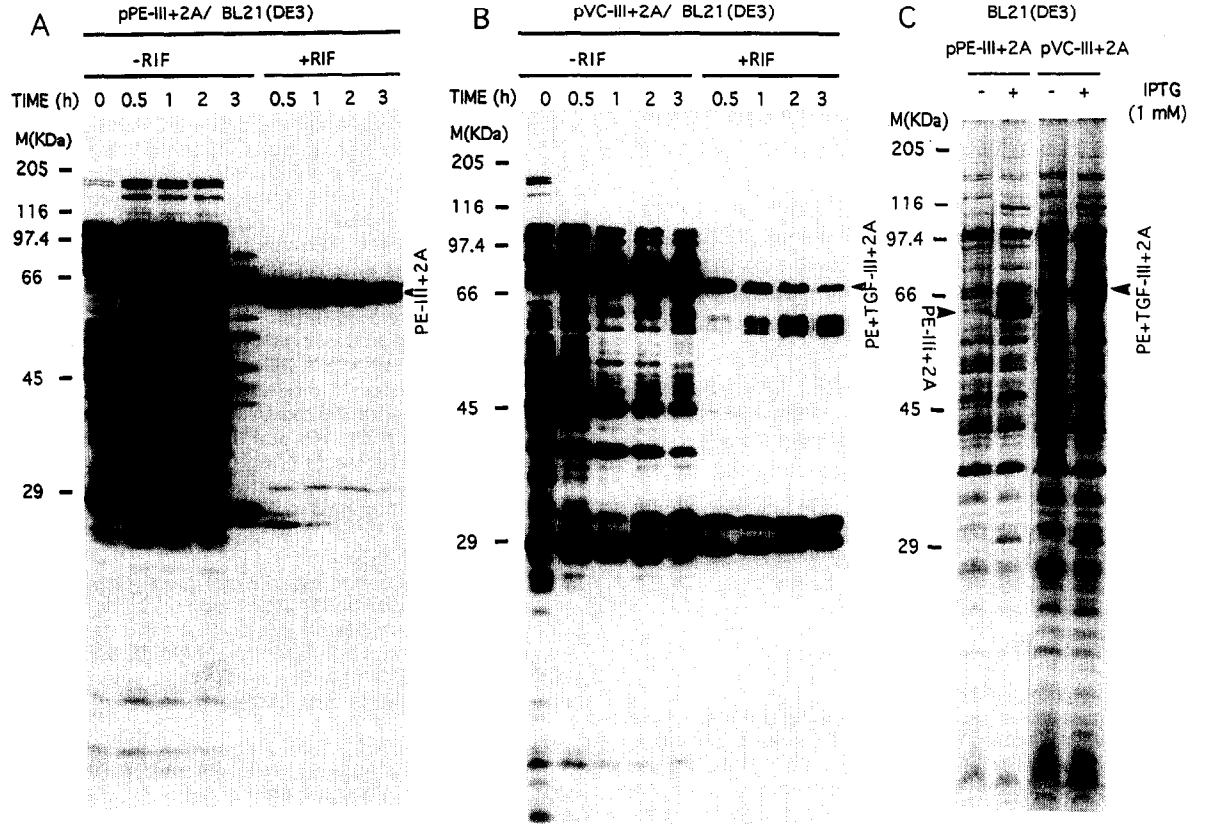


Fig. 2. Induction of hybrid proteins between *Pseudomonas* exotoxin A and poliovirus $2A^{pro}$ in *E. coli*. (A and B) BL21(DE3) *E. coli* cells bearing plasmids pPE-III + 2A or pVC-III + 2A, respectively, were grown and induced with 1 mM IPTG as described in section 2. At the indicated times post-induction, proteins were labelled with [^{35}S]methionine and analyzed by SDS-PAGE. When indicated, 150 μg/ml rifampicin was added to the cultures 20 min after addition of IPTG. (C) Coomassie blue staining of total protein extracts obtained from BL21(DE3) *E. coli* cells containing pPE-III + 2A or pVC-III + 2A induced with 1 mM IPTG (indicated as +) or not (−) at 3 h post-induction. The position of PE-III + 2A and PE + TGF-III + 2A proteins and molecular size markers are indicated.

tant to confer a wider range of cell types recognized by this hybrid toxin [2].

The hybrid genes were generated as described in section 2 and were placed under the control of a T7 promoter in pET vectors. Upon transformation of BL21 (DE3) *E. coli* cells, clones were selected and the synthesis of the hybrid toxins assayed. Fig. 2 shows that induction of these clones with IPTG gives rise to a prominent protein band that migrates with the expected size of the hybrid protein in each case. These bands are clearly visible by Coomassie blue staining (Fig. 2C). These induced proteins correspond to PE-III + 2A or PE + TGF-III + 2A. Pulse-labelling of these clones with [^{35}S]methionine upon induction with both IPTG and rifampicin shows more clearly the protein which is induced since rifampicin inhibits the

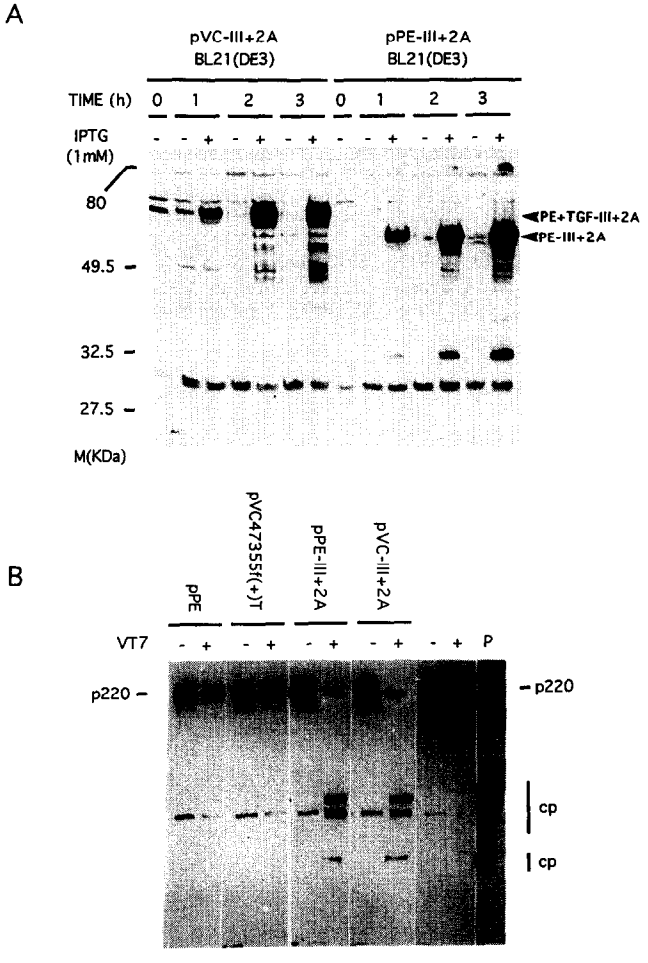


Fig. 3. (A) Immunoblot analyses of hybrid proteins between PE and poliovirus 2A^{pro}. BL21(DE3) *E. coli* cells containing pPE-III + 2A or pVC-III + 2A were grown and induced with 1 mM IPTG (indicated as +) or not (−). At the times indicated, cells were harvested and separated by 15% SDS-PAGE, transferred to nitrocellulose membranes and reacted with a specific anti-2A rabbit antiserum. The position of PE-III + 2A and PE + TGF-III + 2A proteins and molecular size markers are shown. (B) Cleavage of p220 in COS cells. COS cells grown in 24-well dishes were infected with recombinant vaccinia virus bearing the T7 RNA polymerase (m.o.i. 5 pfu/cell). After virus adsorption, cells were transfected with the indicated plasmids using lipofectin, as described in section 2. Samples were collected at 17 h.p.i. and immunoblot analyses of p220 were performed by 7.5% SDS-PAGE with rabbit polyclonal p220 antibody. P, poliovirus-infected HeLa cells; VT7, recombinant vaccinia virus containing the T7 RNA polymerase; Cp, cleavage products.

background of bacterial protein synthesis. In the case of the bacterial clone bearing the construct pVC-III- 2A there are several induced polypeptides, two of which had a lower molecular weight of around 29–31 kDa. We believe that one of these low MW components may correspond to β-galactosidase that is induced by IPTG treatment [12], while the other polypeptides may represent cleavage products.

In order to identify the nature of these polypeptides, they were transferred to a nitocellulose membrane and immunoreacted with specific 2A^{pro} antiserum. Clearly, there is only one major prominent polypeptide reacting against this antiserum in the clone bearing the PE + TGF-III + 2A construction (Fig. 3A). This polypeptide migrates with an apparent MW of 69 kDa, which corresponds to the expected size of the hybrid protein. On the other hand, two major proteins are apparent in the clone bearing the construction PE-III + 2A; the major one corresponds in size to the hybrid toxin whereas we do not know the nature of the lower one. There are several possibilities to account for the appearance of this low MW component that contains 2A^{pro} sequences. One is that it is generated by cleavage from the major product. Another possibility is that incorrect initiation of translation takes place in phase with the 2A^{pro} sequence giving rise to a lower MW protein. We favor the first possibility, because poliovirus proteases are active in *cis* and *trans* even as precursor polypeptides. Nevertheless, the main conclusion from these results is that these bacterial clones synthesize a major protein that reacts with anti-2A^{pro} antiserum, indicating that 2A^{pro} sequences are present in the hybrid protein.

Finally, to analyze if these hybrids between PE and poliovirus 2A^{pro} are active in p220 cleavage, several approaches were followed. The first one consisted of the partial purification of the hybrid proteins in order to test their effects on culture cells. Partially purified preparations were toxic for mouse cells, but only after prolonged incubation (about 2 days). Analysis of p220 cleavage at this time did not reveal clear results because of the dead cells (results not shown). The other approach used was to directly express these constructs in mammalian cells. Since the hybrid proteins were under the control of a T7 promoter in pET vectors, they were directly transfected in COS cells previously infected with recombinant vaccinia virus carrying the T7 RNA polymerase gene. Analysis of p220 after 17 h post-infection clearly shows that this factor becomes cleaved when the cells were transfected with pPE-III + 2A of pVC-III + 2A, but not with control plasmids pPE or pVC47355 f(+)T (Fig. 3B). The specificity of this cleavage is demonstrated because p220 is degraded only if cells transfected with the hybrid constructs are infected by recombinant vaccinia virus.

4. Discussion

The mechanisms by which picornaviruses arrest cellular protein synthesis after infection is a subject of intense research [13–15]. Despite the efforts of a number of laboratories, there is not as yet a definitive model to account for all the existing experimental evidence on the shut-off of host translation by picornaviruses. Suggestions that poliovirus blocks host translation by proteolytic cleavage of a p220 polypeptide (eIF-4γ), a component of initiation factor eIF-4F [16], have not been corroborated by more recent experimental findings [17–19]. This lack of knowledge comes, at least in part, from the fact that very

little is known about the effects that the expression of individual poliovirus genes have in mammalian cells. One approach that can be used in the case of poliovirus proteins endowed with enzymatic capacity, such as the two proteases $2A^{pro}$ and $3C^{pro}$, should be to introduce these proteins directly into cells, in order to analyze their potential detrimental effects on host metabolism. As an initial step in this direction, we have synthesized hybrid proteins between PE and $2A^{pro}$. These hybrids still retain the $2A^{pro}$ activity as measured by their capacity to cleave p220, despite the fact that $2A^{pro}$ is flanked by other protein sequences in both hybrids. Further efforts in this direction would concentrate on the production and purification of one of these hybrid proteins in large amounts so as to continue the analyses of its effects on culture cells.

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