Journal of Virology, May 1996, p. 3319–3324
0022-538X/96/$04.00+0
Copyright © 1996, American Society for Microbiology
Vol. 70, No. 5

Hybrid Proteins between *Pseudomonas aeruginosa* Exotoxin A and Poliovirus 2A^{pro} Cleave p220 in HeLa Cells

ISABEL NOVOA,[1] MATT COTTEN,[2] AND LUIS CARRASCO[1]*

Centro de Biología Molecular, UAM-Consejo Superior de Investigaciones Científicas, Universidad Autónoma de Madrid, Canto Blanco, 28049 Madrid, Spain,[1] and Research Institute of Molecular Pathology, 1030 Vienna, Austria[2]

Received 11 September 1995/Accepted 12 January 1996

Cleavage of p220, a component of the initiation factor eIF-4F, has been correlated with the inhibition of host translation during poliovirus infection. To obtain p220 cleavage in the absence of any other poliovirus gene products, hybrid proteins containing *Pseudomonas aeruginosa* exotoxin A and poliovirus protease 2A^{pro} have been constructed. The addition of the hybrid molecules to cultured cells did not lead to substantial p220 cleavage. However, the simultaneous presence of the hybrid toxin with replicationally inactive chicken adenovirus particles results in efficient cleavage of p220 in the intact cells. Under these conditions, cellular translation continues unabated for several hours, arguing against a direct requirement for intact p220 in each round of the initiation of translation of cellular mRNAs.

Hybrid proteins consisting of toxins and proteins that selectively bind to target cells are becoming promising tools to specifically kill tumor and human immunodeficiency virus-infected cells. The domain of the toxin responsible for binding to the cell surface can be replaced by other molecules that confer different receptor specificity (4, 16, 28, 30). A variety of bacterial and plant toxins have been employed in this type of study. Perhaps one of the most frequently used and best-known toxins in this regard is *Pseudomonas aeruginosa* exotoxin A (PE), a protein of 613 amino acids that is secreted into the medium by the bacterium (11, 29, 45). PE, like many other protein toxins, is composed of several domains: domain I is involved in the attachment of the toxin to cellular receptors (19), domain II translocates the protein across the membrane (20, 21), and domain III is endowed with enzymatic activity, i.e., the ADP-ribosylation of elongation factor 2 (EF-2) (17, 39, 45). We reasoned that the replacement of domain III of PE by poliovirus protease 2A^{pro} would produce a hybrid toxin with protease activity. One activity of 2A^{pro} is to cleave a cellular polypeptide, p220, that forms part of the translation initiation factor eIF-4F. The hybrid toxin consisting of PE and 2A^{pro} would be useful to analyze the effects of p220 cleavage on cellular translation in the absence of any other poliovirus polypeptides.

Deletion of domain III of PE abrogates the ADP-ribosylation activity of the toxin (28). Thus, a hybrid toxin containing the first 412 amino acids of PE fused to barnase is devoid of ADP-ribosylation activity but retains the RNase activity of the barnase molecule (32, 33). Therefore, we decided to make hybrid proteins containing the first 412 amino acids of PE directly fused to poliovirus protease 2A^{pro} followed by the last 10 residues of PE (residues 604 to 613), which are necessary for efficient transport and toxin delivery to the cytosol (38). In addition, another hybrid toxin that contains the transforming growth factor α (TGF-α) sequence placed between 2A^{pro} and PE (residues 604 to 613) was generated (Fig. 1A). The presence of TGF-α should confer a wide range of cells for toxin binding, because this toxin will be able to bind not only to cells bearing the PE receptor but also to cells containing the TGF receptor (29). To this end, construction of the plasmids encoding the different fusion proteins was carried out by standard molecular cloning procedures (36). Plasmids pMal-PE, pMal-PE-TGF, pMal-PE-III+2A, and pMal-PE+TGF-III+2A were constructed by digestion with *Hin*dIII plasmids pPE, pVC47355f(+)T, pPE-III+2A, and pVC-III+2A (26), respectively. Afterwards, 5′ extensions were removed with mung bean nuclease. Subsequent digestions with *Eco*RI yielded 1,859-, 2,018-, 1,711-, and 1,870-bp fragments, respectively. These DNA fragments were ligated to the pMal-c2 vector (New England BioLabs) digested with *Xmn*I-*Eco*RI and used to transform *Escherichia coli* DH5α cells. The recombinants were screened by restriction digestion analysis. To facilitate the purification of these hybrid proteins, they were fused to the maltose-binding protein (MBP). Expression of both hybrid proteins fused to MBP is very efficient in *E. coli* cells on induction with IPTG (isopropyl-β-D-galactopyranoside) (Fig. 1B). The migration of the induced proteins corresponded to the expected sizes of the fusion proteins: 106 kDa for MBP-PE-III+2A and 112 kDa for MBP-PE+TGF-III+2A. To purify these proteins, bacteria were induced for 2 h, and the supernatants of ruptured cells were purified by affinity chromatography as described previously (34) (Fig. 1B). Cleavage of these hybrid proteins was achieved by incubation with factor Xa to yield MBP and the corresponding hybrid protein, either PE-III+2A or PE+TGF-III+2A (Fig. 1B). We found that the uncleaved protein showed 2A^{pro} activity similar to the cleaved toxins without MBP (see below). Therefore, the fusion protein containing MBP was used in subsequent experiments.

To assess the proteolytic activities of both hybrid proteins, the cleavage of two different substrates was analyzed. In the first assay, the mRNA encoding poliovirus ΔVP3VP1Δ2A was obtained and translated in rabbit reticulocyte lysates (Fig. 2A). Translation of this mRNA generates a protein that contains the poliovirus structural proteins ΔVP3VP1 followed by truncated 2A^{pro} inactive in cleaving the ΔVP3VP1Δ2A. Thus, an intact precursor, ΔVP3VP1Δ2A^{pro}, that can be cleaved in *trans* by active 2A^{pro} is synthesized (24). The addition of either hybrid protein MBP-PE-III+2A or MBP-PE+TGF-III+2A generates ΔVP3VP1 and Δ2A (Fig. 2A), indicating that the 2A^{pro} present in these proteins is able to recognize and cleave this substrate in *trans*. This result is particularly interesting because it shows not only that 2A^{pro} is active when it contains a foreign sequence at its amino terminus (MBP-PE-III+2A)

* Corresponding author. Fax: 34-1-397-47-99.

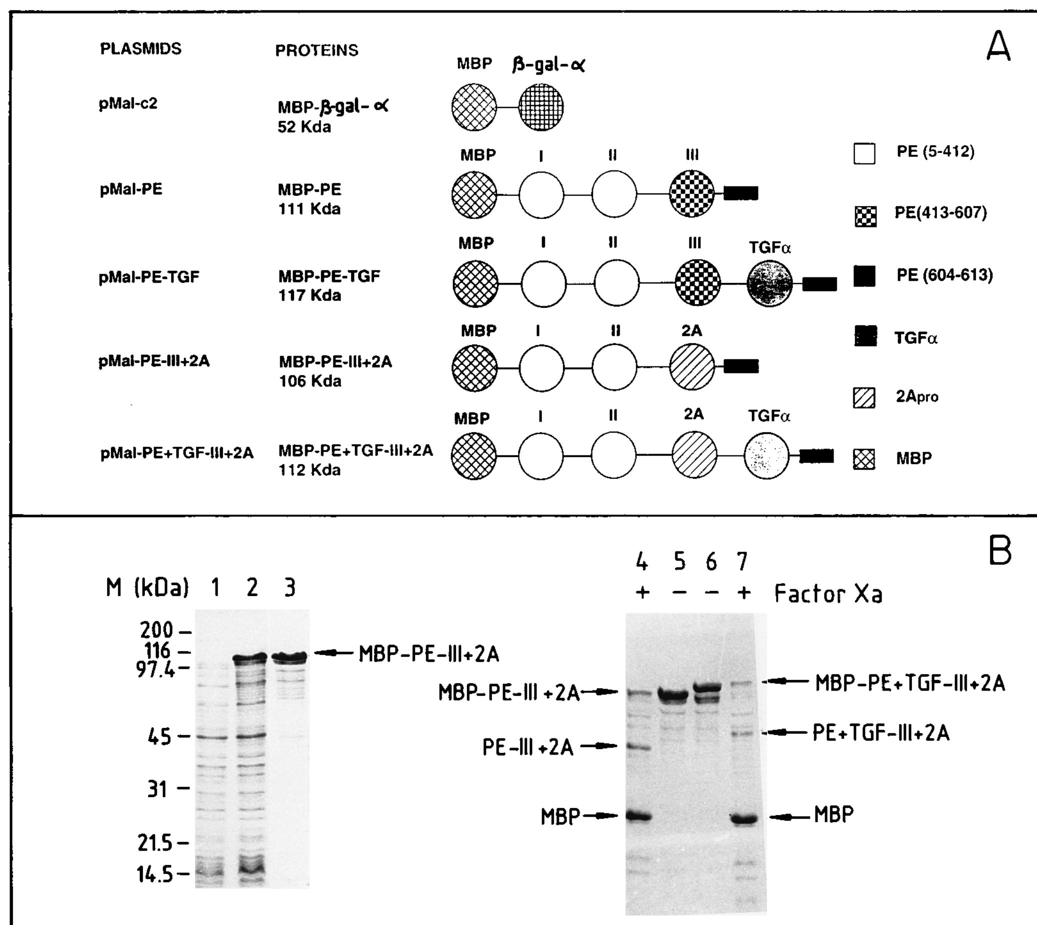

FIG. 1. (A) Schematic representation of proteins used in this study; (B) expression and purification of MBP-PE-III+2A. Lanes: 1, proteins from uninduced *E. coli* cells; 2, proteins from cells induced with 1 mM IPTG; 3, protein fractions eluted from the amylose resin column; 4 to 7, assay of MBP-PE-III+2A (lanes 4 and 5) and MBP-PE+TGF-III+2A (lanes 6 and 7). Purified proteins were incubated in the presence (lanes 4 and 7) or absence (lanes 5 and 6) of 5% (wt/wt) factor Xa for 22 h. Samples were separated by sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis (SDS–15% PAGE) and stained with Coomassie blue. The positions of MBP-PE-III+2A, MBP-PE+TGF-III+2A, PE-III+2A, PE+TGF-III+2A, MBP, and molecular mass markers (M) are indicated.

but also that the activity of $2A^{pro}$ is retained when the protein is flanked by sequences unrelated to poliovirus at both ends of the molecule.

More important for the type of experiments planned in this study with intact cells was to assay the activity of these hybrid proteins in p220 cleavage in cell-free systems. Incubation of rabbit reticulocyte lysates with these hybrids generates the cleavage products of p220 (Fig. 2B). The antibodies used (raised against human p220 peptides) recognize only one fragment of the rabbit p220 molecule that corresponds to the carboxy end of p220 (1). The amino-terminal products of p220 cleavage from rabbit lysates are not recognized by our antibodies, because there are more differences in the amino-terminal region than in the carboxy-terminal region of the p220 peptides from different species (2). Notably, MBP-PE-III+2A was more active than MBP-PE+TGF-III+2A in the cleavage of both substrates. At present we cannot conclude that this inefficient cleavage by MBP-PE+TGF-III+2A is due to the presence of additional sequences at the carboxy terminus of poliovirus 2A or to the presence of TGF itself, which partially blocks 2A activity. Nevertheless, these studies indicated that $2A^{pro}$ activity was present in both hybrid molecules as shown by cleavage of ΔVP3VP1Δ2A and rabbit p220.

Our aim with the construction of these hybrid toxins was to generate new tools to cleave p220 in intact cells in the absence of poliovirus infections in order to analyze the effects of p220 (and thus eIF-4F) inactivation on protein synthesis. Therefore, the purified hybrid proteins were added to the culture medium of HeLa or L929 cells. Cleavage of p220 was not observed on incubation of the hybrid proteins with cultured cells (Fig. 2C). Occasionally there was partial cleavage of p220 (around 10 to 20%) but only after prolonged incubation (48 h). Since no differences were found between the different cell types employed, we continued our work with HeLa cells, since this cell line is used in most studies on poliovirus-induced arrest of protein synthesis (5). Although disappointing, these results do not differ much from the known features of PE or PE-barnase entry. Thus, only a low percentage (about 10%) of PE is cleaved during entry, and only a fraction of the cleaved PE enters the cytosol (25). It has been calculated for other toxins that less than 1% of the bound toxin appears in the cytoplasm (27, 37).

We have found in the past that this inefficient toxin delivery is drastically enhanced by animal virus particles (6, 13). In fact, the entry of intact PE is augmented 1,000- to 10,000-fold by simultaneous incubation with adenovirus particles (14). Recently, chicken adenovirus particles (CELO virus) unable to replicate in mammalian cells have been successfully employed

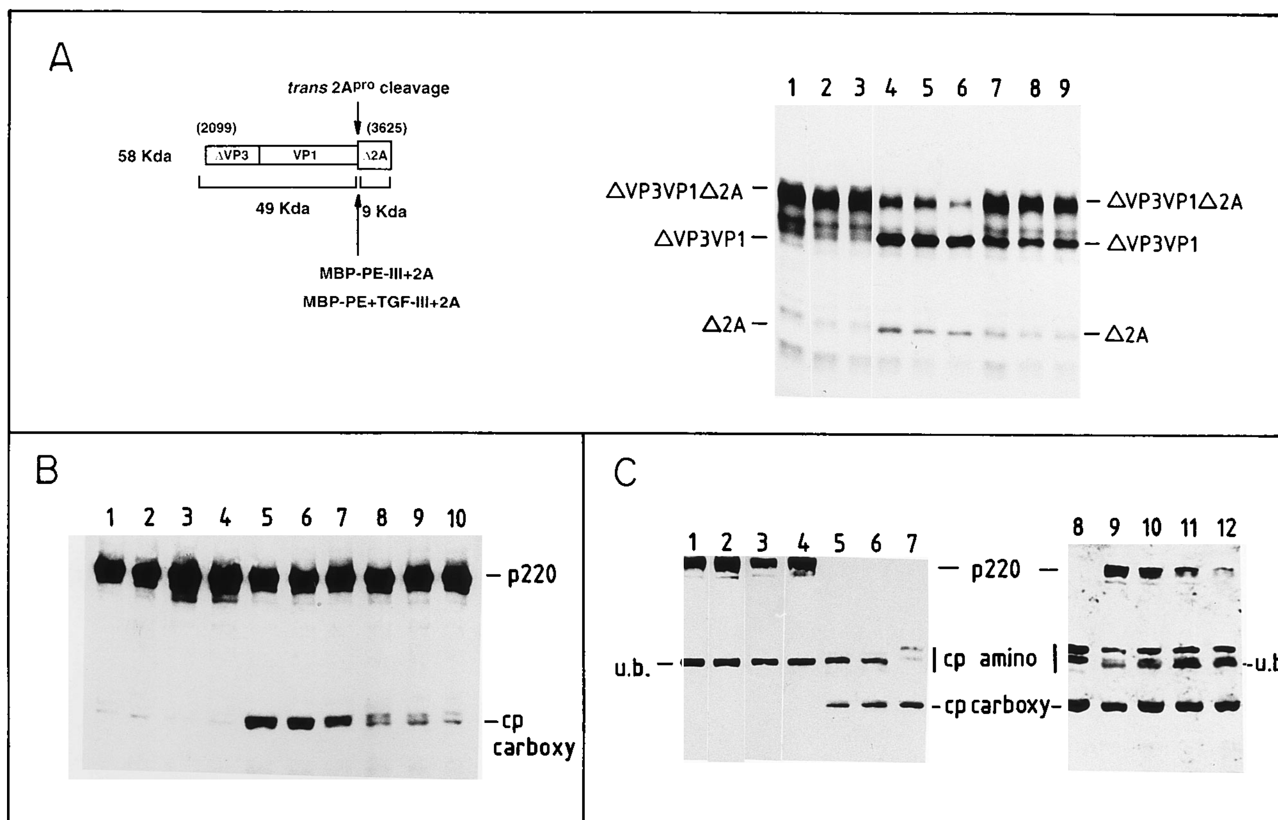

FIG. 2. Activity of MBP-PE-III+2A and MBP-PE+TGF-III+2A in vitro and in intact HeLa cells. (A) A schematic representation of the products encoded by RNA transcripts of Pf1MI-linearized plasmid pT7.1D2AB(ΔVP3VP1Δ2A) is shown on the left; on the right, the results of an in vitro 2A^{pro} cleavage assay are shown. ΔVP3VP1Δ2A synthesized in rabbit reticulocyte lysates (lane 1), as described previously (24), was incubated for 2 h at 30°C with 4 μg of MBP-PE (lane 2); with 4 μg of MBP-PE-TGF (lane 3); with 5, 2, and 1 μg of MBP-PE-III+2A (lanes 4 to 6, respectively); or with 5, 2, and 1 μg of MBP-PE+TGF-III+2A (lanes 7 to 9, respectively). The positions of ΔVP3VP1Δ2A, ΔVP3VP1, and Δ2A are indicated. (B) p220 cleavage assay of rabbit reticulocyte lysates. Rabbit reticulocyte lysates (Promega) were incubated for 2.5 h at 30°C with buffer (lane 1); with 5, 2.5, and 1 μg of MBP-β-gal-α (lanes 2 to 4, respectively); with 5, 2.5, and 1 μg of MBP-PE-III+2A (lanes 5 to 7, respectively); or with 5, 2.5, and 1 μg of MBP-PE+TGF-III+2A (lanes 8 to 10, respectively). Proteins were separated by SDS–7.5% PAGE and analyzed by Western blot (immunoblot) of anti-p220 polyclonal antibodies (1). Intact p220 and the carboxy-terminal fragment of p220 (cp carboxy) are indicated. (C) Cleavage of p220 in HeLa cells. (Left) HeLa cells grown in 24-well dishes were incubated at 37°C for 18 h without (lane 1) or with 80 μg of purified MBP-PE+TGF-III+2A (lane 2), 100 μg of purified MBP-PE-III+2A (lane 3), 10 μl of CELO virus (10^{12} virus particles per ml) (7) (lane 4), 100 μg of MBP-PE-III+2A and 10 μl CELO virus (lane 5), 100 μg of MBP-PE+TGF-III+2A and 10 μl CELO virus (lane 6), or poliovirus (lane 7). (Right) HeLa cells grown in 24-well dishes were incubated with 10 μl of CELO virus (10^{12} virus particles per ml) in the presence of increasing amounts of purified MBP-PE-III+2A. Lanes: 8, poliovirus-infected cells; 9 to 12, 30, 50, 80, and 100 μg of MBP-PE-III+2A and CELO virus, respectively. Proteins were separated by SDS–7.5% PAGE and analyzed by Western blot with anti-p220. Intact p220 and the amino-terminal (cp amino) and carboxy-terminal (cp carboxy) fragments of p220 are indicated. ub, unspecific band.

to transform these cells with plasmid DNA (7). Therefore, we tested the effects of CELO virus on the entry of the hybrid toxins, as determined by the extent of p220 cleavage. CELO virus cannot replicate in mammalian cells (7). The results obtained for p220 cleavage in cultured HeLa cells by the two hybrid toxins drastically changed when CELO virus was added. After 18 h of incubation with 100 μg of each toxin in the presence of CELO virus, the cleavage of p220 was very efficient, while no cleavage was observed with CELO virus alone (Fig. 2C, lanes 1 to 7). p220 cleavage under these conditions is similar to a poliovirus infection. Concentrations of the hybrid toxins lower than 100 μg per well (500 μg/ml) result in partial cleavage of p220 (Fig. 2C, lanes 8 to 12). These results suggest that the toxins bound to the cells or internalized in endosomes are efficiently delivered to the cytosol by permeabilization of the cells with CELO virus. Next, we compared the efficiency of p220 cleavage by MBP-PE-III+2A with or without Xa treatment and found no differences, suggesting that the presence of MBP in the hybrid toxin does not affect its entry or 2A^{pro} activity (data not shown). The kinetics of p220 cleavage under these conditions show that significant cleavage is already apparent at 8 to 10 h, and virtually no intact p220 is detected by 15 to 17 h (results not shown). The conclusion from these results is that conditions for very efficient p220 degradation can be found in intact cells in the absence of poliovirus infection. We do not know if cleavage products obtained after p220 digestion with MBP-PE-III+2A are exactly the same as those obtained with genuine poliovirus 2A. However, they comigrate with the p220 cleavage products obtained from poliovirus-infected cells.

The findings described above raise the possibility of assaying protein synthesis in HeLa cells containing primarily proteolytically cleaved p220. These experiments were carried out by pulse-labeling HeLa cells with [^{35}S]methionine as described previously (18). Figure 3A shows that HeLa cells containing extensively degraded p220 after 17 h of treatment efficiently synthesize proteins, suggesting that the integrity of p220 is not required to initiate the translation of these cellular mRNAs, at least those already engaged in protein synthesis. Moreover, 4 or even 8 h later, i.e., 21 or 25 h after treatment, respectively, p220 still remains cleaved, while cellular mRNA translation continues unabated. Since p220 is in excess relative to eIF-4F

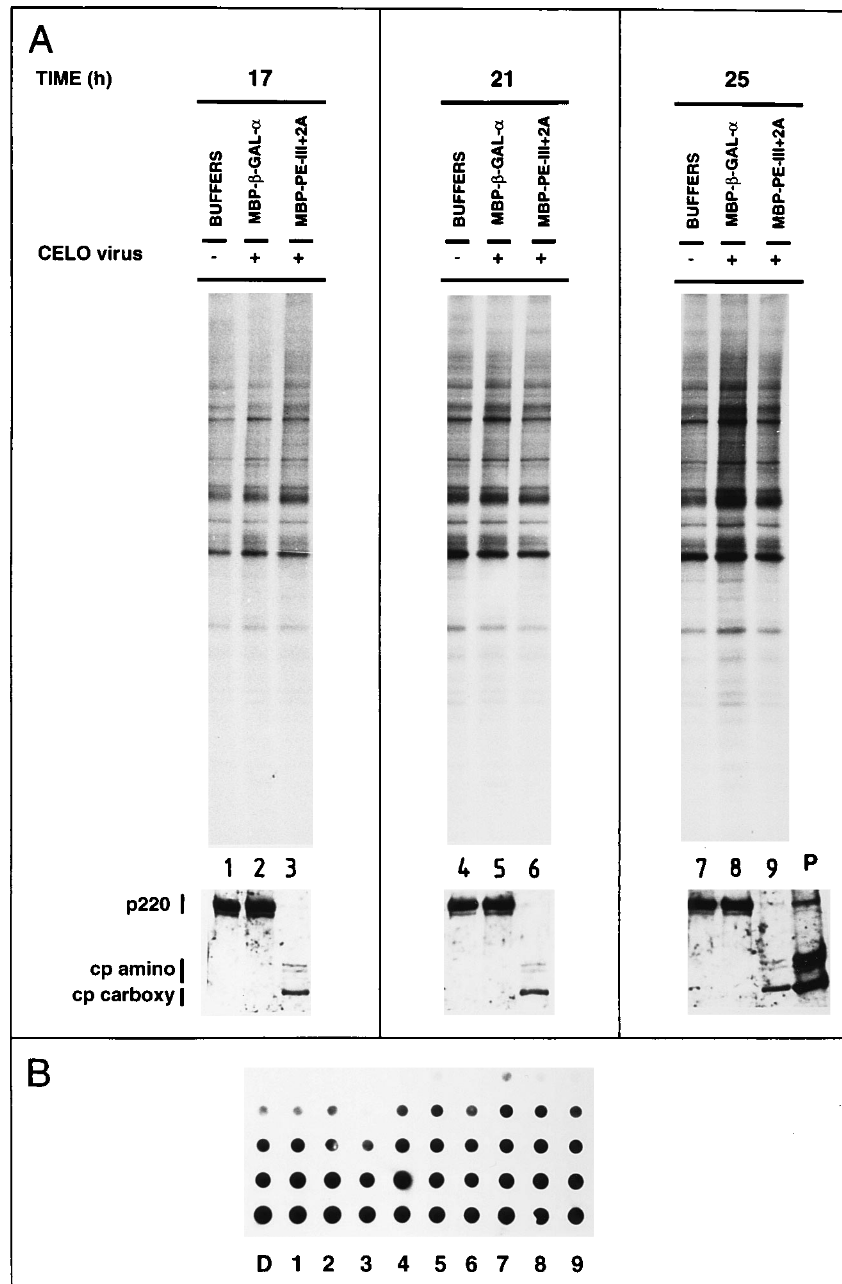

FIG. 3. Protein synthesis and p220 cleavage in HeLa cells incubated with MBP-PE-III+2A and CELO virus. HeLa cells grown in 35-well dishes were incubated with buffer only (lanes 1, 4, and 7), with 400 μg of MBP-β-gal-α and 40 μl of CELO virus (lanes 2, 5, and 8), or with 400 μg of MBP-PE-III+2A and 40 μl of CELO virus (10^{12} virus particles per ml) (lanes 3, 6, and 9). At 17 h (lanes 1 to 3), 21 h (lanes 4 to 6), and 25 h (lanes 7 to 9), cells were labeled with [^{35}S]methionine (20 μCi/ml) for 1 h. Cell extracts were prepared, and the protein content was determined. (A) (Upper panel). Total protein synthesis analyzed by SDS–15% PAGE; (lower panel). Western blot of the same samples with anti-p220 antibodies (1). P, poliovirus-infected HeLa cells (control). (B) RNA extracted from these cells was hybridized with biotinylated riboprobe corresponding to α-actin (human) (15) by dot blot (36). The α-actin-biotinylated riboprobe was synthesized from a Bluescript SK(−)–α-actin plasmid with T7 RNA polymerase (35). Intact p220 and the amino-terminal (cp amino) and carboxy-terminal (cp carboxy) fragments of p220 are indicated. D, DNA of SK(−)–α-actin.

(two molecules of p220 per molecule of eIF-4F) (12), it is possible that some uncleaved p220 present in hybrid toxin-treated cells is still sufficient to support translation in these cells. As a control, the mRNAs present in these cells were extracted and hybridized with a probe against actin mRNA (Fig. 3B). The results indicate that untreated cells and cells treated with the hybrid toxin contain similar levels of the mRNA encoding actin. Therefore, p220 cleavage alone does not lead to an immediate arrest of ongoing cellular translation, as occurs during the infection of HeLa cells by poliovirus.

The use of toxins to target other proteins or peptides in cells has been successful, although efficient internalization has not been achieved (4, 16, 43). The results of this study indicate that the use of a truncated version of PE to introduce poliovirus protease $2A^{pro}$ is by itself not very effective. However, the combination of this approach with the permeabilizing capacity

of animal virus particles (13), particularly adenoviruses (8, 9, 14), greatly improves the delivery of hybrid toxin to the cytoplasm. The use of this combined approach introduces the hybrid toxin containing PE and $2A^{pro}$ into most cells in a few hours, as judged by the cleavage of p220.

The major conclusion from these results is that cellular translation takes place when p220 has been substantially degraded. Thus, the present results show that ongoing cellular protein synthesis continues for hours in the absence of detectable p220. Under the conditions studied, reinitiation of cellular mRNA translation takes place, since the translation of an average-size mRNA occurs in 5 to 10 min. Several possibilities can be put forward to explain these findings: (i) some remaining uncleaved p220 is still sufficient to support the initiation of translation, (ii) the p220 cleavage products are still active in translation, and (iii) p220 participates in only the first initiation event, when the mRNA reaches the translational machinery. Further work with the system described herein could help to clarify which of these possibilities is operating.

Two additional questions arise from these findings. Does p220 cleavage play any physiological role during the poliovirus life cycle? What are the poliovirus protein and the mechanism used to block host translation if p220 cleavage is not sufficient? With regard to the first question, two opposite views can be considered: (i) p220 is cleaved fortuitously as other cellular proteins become proteolytically degraded during poliovirus infection (42), and (ii) p220 cleavage plays a part during poliovirus growth in cultured cells or at the organism level. At present we favor the second view for several reasons. First, cleavage of p220 is conserved not only by the three poliovirus serotypes but also by other picornaviruses, such as rhinoviruses and aphtoviruses (22, 40). If p220 is required at some step of cellular gene expression, poliovirus will disarm the cell to respond to its infection. This function may not be as important during poliovirus infection of fibroblasts in culture but may be physiologically relevant when poliovirus infects other cells of the organism, including immune cells.

The use of poliovirus protease $2A^{pro}$ in the hybrid proteins examined in this work was intended to analyze the effects of p220 cleavage on protein synthesis in the absence of other poliovirus proteins. This objective has been achieved. Efficient p220 cleavage is attained in cultured cells, and these cells continue to synthesize proteins for hours. Previous attempts by other investigators were directed at examining the impact of $2A^{pro}$ on reporter gene expression but not on cellular translation itself (10, 41, 44). Certainly, the individual expression of $2A^{pro}$ was detrimental for reporter gene expression (10, 41). Only one study addressed the question of the step blocked by $2A^{pro}$, reaching the conclusion that the major blockade for chloramphenicol acetyltransferase expression occurred at the transcriptional level (10). Contrary to previous views, the cleavage of p220 may not be responsible for the inhibition of host translation by poliovirus infection. In fact, we and others have provided evidence that there is no correlation between the timing or the extent of p220 cleavage and the decrease of protein synthesis during poliovirus infection (3, 23, 31). Therefore, the possibility that a poliovirus gene product different from $2A^{pro}$ is directly responsible for the blockade of host translation remains open.

The expert technical assistance of M. A. Sanz is acknowledged.

DGICYT project no. PB94-0148 and the institutional grant to the CBM of Fundación Ramón Areces are acknowledged for their financial support. I.N. is a holder of a Gobierno Vasco fellowship.

REFERENCES

1. **Aldabe, R., E. Feduchi, I. Novoa, and L. Carrasco.** 1995. Efficient cleavage of p220 by poliovirus $2A^{pro}$ expression in mammalian cells: effects on vaccina virus. Biochem. Biophys. Res. Commun. **215:**928–936.
2. **Allen, M. L., A. M. Metz, R. T. Timmer, R. E. Rhoads, and K. S. Browning.** 1992. Isolation and sequence of the cDNAs encoding the subunits of the isozyme form of wheat protein synthesis initiation factor 4F. J. Biol. Chem. **267:**23232–23236.
3. **Bonneau, A. M., and N. Sonenberg.** 1987. Proteolysis of the p220 component of the cap-binding protein complex is not sufficient for complete inhibition of host cell protein synthesis after poliovirus infection. J. Virol. **61:**986–991.
4. **Brinkmann, U., and I. Pastan.** 1994. Immunotoxins against cancer. Biochim. Biophys. Acta Rev. Cancer **1198:**27–45.
5. **Carrasco, L., and J. L. Castrillo.** 1987. The regulation of translation in picornavirus-infected cells, p. 115–146. *In* L. Carrasco (ed.), Mechanisms of viral toxicity in animal cells. CRC Press, Boca Raton, Fla.
6. **Carrasco, L., M. J. Otero, and J. L. Castrillo.** 1989. Modification of membrane permeability by animal viruses. Pharmacol. Ther. **40:**171–212.
7. **Cotten, M., E. Wagner, K. Zatloukal, and M. L. Birnstiel.** 1993. Chicken adenovirus (CELO virus) particles augment receptor-mediated DNA delivery to mammalian cells and yield exceptional levels of stable transformants. J. Virol. **67:**3777–3785.
8. **Cotten, M., E. Wagner, K. Zatloukal, S. Phillips, D. T. Curiel, and M. L. Birnstiel.** 1992. High-efficiency receptor-mediated delivery of small and large (48 kilobase) gene constructs using the endosome-disruption activity of defective or chemically inactivated adenovirus particles. Proc. Natl. Acad. Sci. USA **89:**6094–6098.
9. **Curiel, D. T., S. Agarwal, E. Wagner, and M. Cotten.** 1991. Adenovirus enhancement of transferrin-polylysine-mediated gene delivery. Proc. Natl. Acad. Sci. USA **88:**8850–8854.
10. **Davies, M. V., J. Pelletier, K. Meerovitch, N. Sonenberg, and R. J. Kaufman.** 1991. The effect of poliovirus proteinase 2Apro on cellular metabolism. J. Biol. Chem. **266:**14714–14720.
11. **Douglas, C. M., C. Guidi-Rontani, and R. J. Collier.** 1987. Exotoxin A of *Pseudomonas aeruginosa*: active, cloned toxin is secreted into the periplasmic space of *Escherichia coli*. J. Bacteriol. **169:**4962–4966.
12. **Duncan, R., S. C. Milburn, and J. W. Hershey.** 1987. Regulated phosphorylation and low abundance of HeLa cell initiation factor eIF-4F suggest a role in translational control. Heat shock effects on eIF-4F. J. Biol. Chem. **262:**380–388.
13. **Fernández-Puentes, C., and L. Carrasco.** 1980. Viral infection permeabilizes mammalian cells to protein toxins. Cell **20:**769–775.
14. **FitzGerald, D. J., R. Padmanabhan, I. Pastan, and M. C. Willingham.** 1983. Adenovirus-induced release of epidermal growth factor and pseudomonas toxin into the cytosol of KB cells during receptor-mediated endocytosis. Cell **32:**607–617.
15. **Hanauer, A., M. Levin, R. Heilig, D. Daegelen, A. Kahn, and J. L. Mandel.** 1983. Isolation and characterization of cDNA clones for human skeletal muscle a-actin. Nucleic Acids Res. **11:**3503–3516.
16. **Houston, L. L.** 1993. Targeted delivery of toxins and enzymes by antibodies and growth factors. Curr. Opin. Biotechnol. **4:**739–744.
17. **Hwang, J., D. J. FitzGerald, S. Adhya, and I. Pastan.** 1987. Functional domains of Pseudomonas exotoxin identified by deletion analysis of the gene expressed in E. coli. Cell **48:**129–136.
18. **Irurzun, A., S. Sánchez-Palomino, I. Novoa, and L. Carrasco.** 1995. Monensin and nigericin prevent the inhibition of host translation by poliovirus, without affecting p220 cleavage. J. Virol. **69:**7453–7460.
19. **Jinno, Y., V. K. Chaudhary, T. Kondo, S. Adhya, D. J. FitzGerald, and I. Pastan.** 1988. Mutational analysis of domain I of *Pseudomonas* exotoxin. J. Biol. Chem. **263:**13203–13207.
20. **Jinno, Y., M. Ogata, V. K. Chaudhary, M. C. Willingham, S. Adhya, D. FitzGerald, and I. Pastan.** 1989. Domain II mutants of *Pseudomonas* exotoxin deficient in translocation. J. Biol. Chem. **264:**15953–15959.
21. **Kasturi, S., A. Kihara, D. FitzGerald, and I. Pastan.** 1992. Alanine scanning mutagenesis identifies surface amino acids on domain II of *Pseudomonas* exotoxin required for cytotoxicity, proper folding, and secretion into periplasm. J. Biol. Chem. **267:**23427–23433.
22. **Kirchweger, R., E. Ziegler, B. J. Lamphear, D. Waters, H.-D. Liebig, W. Sommergruber, F. Sobrino, C. Hohenadl, D. Blaas, R. E. Rhoads, and T. Skern.** 1994. Foot-and-mouth disease virus leader proteinase: purification of the Lb form and determination of its cleavage site on eIF-4γ. J. Virol. **68:**5677–5684.
23. **Lloyd, R. E., and M. Bovee.** 1993. Persistent infection of human erythroblastoid cells by poliovirus. Virology **194:**200–209.
24. **Martínez-Abarca, F., M. A. Alonso, and L. Carrasco.** 1993. High level expression in *Escherichia coli* cells and purification of poliovirus protein $2A^{pro}$. J. Gen. Virol. **74:**2645–2652.
25. **Montecucco, C., E. Papini, and G. Schiavo.** 1994. Bacterial protein toxins penetrate cells via a four-step mechanism. FEBS Lett. **346:**92–98.
26. **Novoa, I., E. Feduchi, and L. Carrasco.** 1994. Hybrid proteins between *Pseudomonas* exotoxin A and poliovirus 2Apro. FEBS Lett. **355:**45–48.
27. **Papini, E., R. Rappuoli, M. Murgia, and C. Montecucco.** 1993. Cell pene-

tration of diphtheria toxin. Reduction of the interchain disulfide bridge is the rate-limiting step of translocation in the cytosol. J. Biol. Chem. **268:**1567–1574.
28. **Pastan, I., V. Chaudhary, and D. J. FitzGerald.** 1992. Recombinant toxins as novel therapeutic agents. Annu. Rev. Biochem. **61:**331–354.
29. **Pastan, I., and D. FitzGerald.** 1989. Pseudomonas exotoxin: chimeric toxins. J. Biol. Chem. **264:**15157–15160.
30. **Pastan, I., and D. FitzGerald.** 1991. Recombinant toxins for cancer treatment. Science **254:**1173–1177.
31. **Perez, L., and L. Carrasco.** 1992. Lack of direct correlation between p220 cleavage and the shut-off of host translation after poliovirus infection. Virology **189:**178–186.
32. **Prior, T. I., D. J. FitzGerald, and I. Pastan.** 1991. Barnase toxin: a new chimeric toxin composed of pseudomonas exotoxin A and barnase. Cell **64:**1017–1023.
33. **Prior, T. I., D. J. FitzGerald, and I. Pastan.** 1992. Translocation mediated by domain II of *Pseudomonas* exotoxin A: transport of barnase into the cytosol. Biochemistry **31:**3555–3559.
34. **Rodríguez, P. L., and L. Carrasco.** 1993. Poliovirus protein 2C has ATPase and GTPase activities. J. Biol. Chem. **268:**8105–8110.
35. **Rodríguez, P. L., and L. Carrasco.** 1994. Nonradioactive northwestern analysis using biotinylated riboprobes. BioTechniques **17:**702–705.
36. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
37. **Sandvig, K., O. Garred, P. K. Holm, and B. van Deurs.** 1993. Endocytosis and intracellular transport of protein toxins. Biochem. Soc. Trans. **21:**707–711.
38. **Seetharam, S., V. K. Chaudhary, D. FitzGerald, and I. Pastan.** 1991. Increased cytotoxic activity of *Pseudomonas* exotoxin and two chimeric toxins ending in KDEL. J. Biol. Chem. **266:**17376–17381.
39. **Siegall, C. B., V. K. Chaudhary, D. J. FitzGerald, and I. Pastan.** 1989. Functional analysis of domains II, Ib and III of *Pseudomonas* exotoxin. J. Biol. Chem. **264:**14256–14261.
40. **Sommergruber, W., H. Ahorn, H. Klump, J. Seipelt, A. Zoephel, F. Fessl, E. Krystek, D. Blaas, E. Kuechler, H.-D. Liebig, and T. Skern.** 1994. 2A proteinases of coxsackie- and rhinovirus cleave peptides derived from eIF-4gamma via a common recognition motif. Virology **198:**741–745.
41. **Sun, X. H., and D. Baltimore.** 1989. Human immunodeficiency virus tat-activated expression of poliovirus protein 2A inhibits mRNA translation. Proc. Natl. Acad. Sci. USA **86:**2143–2146.
42. **Urzáinqui, A., and L. Carrasco.** 1989. Degradation of cellular proteins during poliovirus infection: studies by two-dimensional gel electrophoresis. J. Virol. **63:**4729–4735.
43. **Van Oosterhout, Y. V. J. M., F. W. M. B. Preijers, H. M. C. Wessels, and T. De Witte.** 1992. Cytotoxicity of CD3-ricin A chain immunotoxins in relation to cellular uptake and degradation kinetics. Cancer Res. **52:**5921–5925.
44. **Whetter, L. E., S. P. Day, O. Elroy-Stein, E. A. Brown, and S. M. Lemon.** 1994. Low efficiency of the 5′ nontranslated region of hepatitis A virus RNA in directing cap-independent translation in permissive monkey kidney cells. J. Virol. **68:**5253–5263.
45. **Wilson, B. A., and R. J. Collier.** 1992. Diphtheria toxin and *Pseudomonas aeruginosa* exotoxin A: active-site structure and enzymic mechanism. Curr. Top. Microbiol. Immunol. **175:**27–41.