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The Amino-Terminal One-Third of the Influenza Virus PA Protein Is Responsible for the Induction of Proteolysis

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We have previously described the fact that the individual expression of influenza virus PA protein induced a generalized proteolysis (J. J. Sanz-Ezquerro, S. de la Luna, J. Ortin, and A. Nieto, J. Virol. 69:2420–2426, 1995). In this study, we have further characterized this effect by mapping the regions of PA protein required and have found by deletion analysis that the first 247 amino acids are sufficient to bring about this activity. PA mutants that were able to decrease the accumulation levels of coexpressed proteins also presented lower steady-state levels due to a reduction in their half-lives. Furthermore, the PA wild type produced a decrease in the stationary levels of different PA versions, indicating that is itself a target for its induced proteolytic process. All of the PA proteins that induced proteolysis presented nuclear localization, being the sequences responsible for nuclear transport located inside the first 247 amino acids of the molecule. To distinguish between the regions involved in nuclear localization and those involved in induction of proteolysis, we fused the nuclear localization signal of the simian virus 40 T antigen to the carboxy terminus of the cytosolic versions of PA. None of the cytosolic PA versions affected in the first 247-amino-acid part of PA, which were now located in the nucleus, were able to induce proteolysis, suggesting that conservation of a particular conformation in this region of the molecule is required for the effect observed. The fact that all of the PA proteins able to induce proteolysis presented nuclear localization, together with the observation that this activity is shared by influenza virus PA proteins from two different type A viruses, suggests a physiological role for this PA protein activity in viral infection.

The genome of influenza virus consists of eight single-stranded RNA segments of negative polarity, each one in a ribonucleoprotein complex with viral nucleoprotein (NP) and the three subunits of the polymerase (PB1, PB2, and PA) (18). After the infection of susceptible cells, the ribonucleoproteins are transported to the nucleus, where transcription of the genome occurs (13). Transcription initiation is carried out by a cap-snatching mechanism (17), and polyadenylation of the mRNAs takes place with a five- to seven-U stretch close to the 5′ end of the negative-strand template used as a signal (21, 28). Replication of the viral RNAs is carried out in two steps: (i) synthesis of positive-strand replicative intermediates that are exact copies of the virion RNAs (cRNAs) (12) and (ii) use of these molecules as templates to synthesize new copies of negative-stranded viral RNAs (17, 18). RNA replication also occurs in the nucleus of the infected cell (15, 20, 31) and requires viral protein synthesis, at least of NP (31).

The individual roles of the different polymerase subunits in these processes have been only partially assigned. The PB1 subunit contains conserved protein motifs present in other RNA virus polymerases (27) that appear to be essential for polymerase activity (4). This subunit is probably responsible for elongation of the nascent chain (6). The PB2 subunit has been shown to interact with cap 1 structures (5, 33) and is possibly involved in the recruitment of capped cellular RNAs (6). Antibodies specific for PB2 abolish the endonucleolytic activity of the entire polymerase complex that is required for the endonucleolytic cleavage of the host cellular mRNA precursors, suggesting that this subunit is responsible for this activity (11, 19). Little is known about PA function in the expression of the genome. Its presence is an absolute requirement for the expression of viral RNAs in systems reconstituted in vivo (7, 14, 23). In infected cells, although no specific role for PA has been described, the observed phenotype of temperature-sensitive mutants in the PA gene suggests that this subunit is mostly involved in viral RNA synthesis (22).

As we have previously described, when PA protein is individually expressed, it brings about the induction of generalized proteolysis that reduces the steady-state levels of coexpressed proteins (30). This effect could explain the failure to establish mammalian cells stably expressing this protein (32). We have also observed that nuclear localization of PA protein closely correlates with chromatin condensation and aberrant nuclear morphology (25). In this study, we decided to go further into the characterization of the induction of proteolysis exerted by PA by studying which regions of PA are necessary to produce the effect. We found that the amino-terminal one-third of the protein is sufficient to induce the proteolysis and that there is a correlation between nuclear localization of the protein and induction of proteolysis, suggesting that nuclear transport of PA could be a prerequisite for proteolysis.

MATERIALS AND METHODS

Cells, viruses, and plasmids. The COS-1 cell line (9) was obtained from Y. Gluzman, and the HeLa cell line was purchased from the American Type Culture Collection. Cell cultures were grown in Dulbecco's modified Eagle medium containing 5% fetal bovine serum. Vaccinia virus VTF7-3 is a recombinant virus that expresses the phage T7 RNA polymerase (8).

All plasmids used in this study contained a T7 RNA polymerase promoter upstream of the cloned gene to drive transcription by this enzyme. pGEM3 recombinant plasmids encoding the influenza virus PA, PB2, and NS1 proteins from strain A/Victoria/75 have been described elsewhere (23). Plasmid pGPA PR8, encoding PA protein from the A/PR/8/34 strain, was constructed by insertion of the relevant *Eco*RI fragment from plasmid PAR303 (34) into the pGEM3

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plasmid. The His-PA protein is a fusion protein of PA with a tag of six histidine residues in the N terminus. It is encoded by plasmid pRSETPA, which contains the PA cDNA from the A/Victoria/75 strain cloned into the *Bam*HI site of the pRSETA plasmid (10). Plasmid pT7 EMC-CAT, encoding the chloramphenicol acetyltransferase (CAT) protein, has been described elsewhere (30).

Construction of mutants. Most of the pGEM3 plasmids expressing different PA mutants were generated by digestion of recombinant pGPA at different restriction sites in the PA gene and the vector's polylinker. The terminal deletion mutants pGPA/St, pGPA/Av, pGPA/Bg, pGPA/B, and pGPA/H were constructed by digestion with *Sty*I, *Ava*I, *Bgl*II, *Bam*HI, and *Hin*dIII treatment, respectively, followed by a filling-in reaction with Klenow enzyme and autoligation. An incorrect filling-in reaction after *Bgl*II treatment in the construction of mutant pGPA/Bg generated an insertion of a serine at position 509 of the PA amino acid sequence, (plasmid pGPAS509). Mutants pGPA/K and pGPA/D contain deletions of nucleotides 1 to 279 and 1 to 1486, respectively, and were generated by Transformer site-directed mutagenesis (Clontech) with the *trans*-oligonucleotide *Ssp*I-*Eco*RV used as described by the supplier as the selection oligonucleotide and with the oligonucleotides 5′ GTTTACTACTGTCCAGGC CATCCCGGGGAATTCCGG 3′ for the pGPA/K mutant and 5′ GTCGGCC TTTGTGGCCATCCCGGGGAATTCCGG 3′ for the pGPA/D mutant used as the mutagenic primers. The PA mutants with internal deletions were generated as follows. pGPAΔBS and pGPAΔBA were generated by digestion of pGPAwt DNA with *Bgl*II-*Sty*I and *Bam*HI-*Afl*II respectively, filling-in reaction, and autoligation. pGPAΔSB was generated by subcloning of the *Asp* 718-*Sca*I fragment from pGPA, in pGPA digested with *Bam*HI, filled in with Klenow polymerase, and digested with *Asp* 718. pGPAΔHS was generated by insertion into the pGEM3 plasmid cut with *Asp* 718 and *Sal*I enzymes of two fragments of pGPAwt DNA, one generated by *Hin*dIII and a filling-in reaction followed by *Asp* 718 digestion and the other generated by *Sca*I and *Sal*I restriction reactions. pGPAΔSH was generated by insertion of a fragment of pGPA generated by *Hin*dIII restriction followed by mung bean nuclease treatment and *Sal*I digestion into pGPA digested with *Stu*I and *Sal*I endonucleases. An incorrect reaction with mung bean nuclease generated mutant pGPA/S, which expresses only the first 186 amino-terminal amino acids of PA sequence. An incorrect filling-in reaction of the *Bam*HI site in the generation of mutant pGPAΔSB produced the terminal deletion mutant pGPA/Sc, which expresses the first 342 N-terminal amino acids of PA sequence. Deletion mutant pGPAΔ3 was generated by subcloning of the *Ava*I fragment from pSEPA (24) into the pGPA plasmid cut with the *Ava*I enzyme. Point mutants pGPA151 and pGPA162 were produced with the Transformer site-directed mutagenesis kit from Clontech, with a degenerated oligonucleotide with the sequence 5′ TTCA/GCTGGGGAGGAAATGGCCGACA AAGGCCGACTACA/GCTCTT 3′ used as the mutagenic primer, which produced a change of alanine for threonine at positions 151 and 162 of the amino acid sequence. For the generation of single-amino-acid insertion mutants, an adaptation of the procedure of Barany (2) was used. Plasmid pGPA was digested with limiting amounts of the restriction endonuclease *Dde*I for 15 min at 37°C; the amount of enzyme able to produce a partial digestion with a sizable fraction of linear plasmid DNA was determined after a dose-effect experiment. With this limiting amount of enzyme, we used an amount of ethidium bromide that ensures that only one cut is introduced per DNA molecule (26). The pGPA DNA linearized under these conditions was isolated from an agarose gel, filled in with Klenow polymerase, and recircularized. By this method, mutants pGPAI672 and pGPAI550 were generated, which contain an insertion of an isoleucine at the indicated positions. Occasionally, the ligation was not accurate and a nucleotide was deleted, leading to a frameshift. This was the case for mutant pGPA/Dd, which encodes the first 100 amino acids of PA protein followed by 10 extra nonspecific amino acids. Plasmid pARPA154 has been previously described (30), and plasmid pG3PA154 contains the sequence of this point PA mutant (24) cloned into *Eco*RI-*Xba*I sites of the pGEM3 plasmid.

For the generation of mutants carrying the nuclear localization signal from the simian virus 40 (SV40) large T antigen (16), the Transformer site-directed mutagenesis kit (Clontech) was used, with pGPAwt plasmid as the template. The selection oligonucleotide used was the *trans*-oligonucleotide *Ssp*I-*Eco*RV described by the supplier, and the mutagenic primer used was an oligonucleotide with the sequence 5′ GCATTGCCACAACTACACCTTCCTCTTCTTCTTGG GCCTTAATGCATGTGTTAG 3′ that introduced the T antigen nuclear localization signal (NLS) coding region inserted in frame at the carboxy end of the PA sequence. Replacement in this new construct (pGPA-T), of *Pvu*II-*Sty*I fragments from either pGPAΔ3, pGPAΔSH, or pGPA154, yielded the new plasmids pGPAΔ3-T, pGPAΔSH-T, and pGPA154-T, which express these PA mutants with the T antigen NLS at the carboxy terminus. All mutant plasmids were sequenced by the dideoxy method (29) with ad hoc oligonucleotide primers.

Transfection. Subconfluent monolayers of cells were infected with VTF7-3 virus at a multiplicity of infection of 5 to 10 PFU per cell. After 1 h of adsorption at 37°C, cells were transfected with the indicated mixtures of plasmids by the liposome-mediated method with cationic liposomes. The total amount of transfected DNA per dish was kept constant, by adjustment if necessary with pGEM3 plasmid to avoid differences in transfection efficiency. Cells were incubated at 37°C in serum-free Dulbecco's modified Eagle medium until harvested (usually 16 h posttransfection). Different plasmid DNA preparations were used, and experiments were repeated at least three times; the figures show results from representative experiments.

Western blotting. Western blots (immunoblots) were done as previously described (30). The following primary antibodies were used: for PB2 protein, PARB2 8N, a rabbit antiserum that was prepared by immunizing animals with a carboxyl-terminal-truncated form of PB2 (1/100 dilution); for PA protein, a mixture of monoclonal antibodies (MAbs) 9, 11, 12, and 14 (1/40 dilution from culture supernatant) (3); for NS1 protein, a rabbit anti-NS1 serum prepared by hyperimmunization with purified protein (1/600); and for CAT enzyme, a rabbit serum raised to be specific for the protein expressed in *Escherichia coli* (5Prime-3Prime, Inc.) at a 1/50 dilution.

Pulse-chase experiments. Pulse-chase and immunoprecipitation experiments were carried out as previously described (30) with MAb 12 (3) for PA immunoprecipitation.

Immunofluorescence. Subconfluent monolayers of HeLa cells were infected with VTF7-3 and transfected with PA expression plasmids as indicated in the figures. At 6 h posttransfection, the cells were fixed with methanol at −20°C and stored in phosphate-buffered saline (PBS) buffer at 4°C. Cells were saturated with 2% bovine serum albumin (BSA) in PBS and incubated for 1 h at room temperature with a PA-specific mixture of MAbs diluted in 0.1% BSA in PBS. After being washed with PBS, cells were stained for 1 h at room temperature with a 1/1,000 dilution of goat anti-mouse fluorescein isothiocyanate-labeled antibody (Southern Biotechnology) in PBS–0.1% BSA. Finally, the preparations were washed with PBS, mounted in Mowiol (Aldrich), and photographed with a Zeiss fluorescence microscope.

RESULTS AND DISCUSSION

PA protein is a target for its induced proteolytic process. During our previous studies, we observed that the expression of the PA polymerase subunit from the A/Victoria/3/75 influenza virus strain induced the proteolysis of coexpressed proteins (30). This unexpected behavior could be the consequence of a specific peculiarity of PA of this influenza virus A strain or could represent a more general feature of the PA subunits from different type A influenza viruses. To answer this question, we studied the accumulation levels of the influenza virus proteins PB2 and NS1, as well as CAT, upon cotransfection of each of the recombinant plasmids coding for these proteins with pGEM3 plasmids encoding the PA subunit from either of the influenza virus strains A/Victoria/3/75 and A/PR/8/34. COS-1 cell cultures were first infected with a recombinant vaccinia virus expressing the T7 RNA polymerase and then transfected with the appropriate mixtures of plasmids. The accumulation of each reporter protein was determined by Western blotting with specific sera. Similar reductions of the steady-state levels of PB2, NS1, and CAT proteins were observed when any of these proteins were coexpressed with the PA subunit from either the A/Victoria/3/75 or A/PR/8/34 strain, but not when the previously reported inactive point mutant PA154 (30) was used as the control (data not shown). The expression of the different PA proteins used in these experiments was analyzed by immunoblotting, showing that both PA proteins were expressed at similar levels that were lower than that obtained from the PA154 mutant (data not shown). This result is in agreement with our previous observations suggesting that PA proteins able to reduce the accumulation of coexpressed proteins were also able to decrease their own accumulation. These data indicate that the effect on triggering proteolysis is shared by PA proteins from different human influenza A viruses, in particular A/Victoria/3/75 and A/PR/8/34 strains, which are among the most distant ones within the PA proteins, it being possible that this conserved biological activity has a physiological role for virus infection.

Having taken into account the observations presented above, we decided to identify the regions of PA protein able to induce activation of the proteolytic process. We had previously described the construction of a collection of PA deletion mutants, both terminal and internal, that were used for the identification of the nuclear localization signal of PA (24). In the present studies, we used all of these mutants and some new deletion and point mutants whose construction is described in

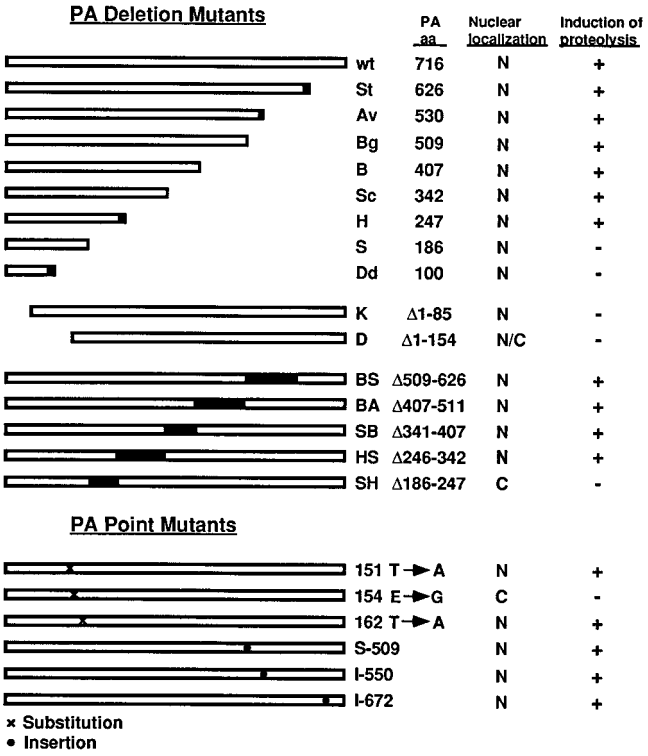

FIG. 1. PA mutants used in this study. Numbers to the right correspond to the amino acids (aa) of PA protein that are expressed by each construct; solid boxes at the end of carboxy-terminal-deleted mutants are extra amino acids, nonrelated to PA, caused by the frameshift. N, nuclear; C, cytosolic; N/C, nuclear and cytosolic (see text for details on intracellular localization phenotypes).

Materials and Methods. A diagram of all mutants used is shown in Fig. 1, together with their phenotype with regard to nuclear localization. In our previous report (24), we observed that wild-type PA could be detected in the nucleus or in the cytosol of the expressing cell in a variable proportion; for that reason, we defined as the nuclear (N) phenotype the pattern showing PA protein located exclusively in the nucleus in at least a portion of the expressing cells. In contrast, we defined the cytosolic (C) phenotype as the pattern in which the protein was exclusively located in the cytoplasm in every expressing cell. Three mutants, PA/Sc, PA/H, and PA/S, presented a pattern of cellular distribution which is almost exclusively nuclear. Subcellular localization of mutant PA/Dd was difficult to ascertain because of the small size of the encoded protein that can probably diffuse across the nuclear envelope. As we have described previously (24), mutant PA/D has a nuclear and cytosolic distribution in every expressing cell. The pGEM3 recombinant plasmids encoding the mutant PA genes were used to transfect COS-1 cells previously infected with VTF7-3 recombinant virus, and the accumulation of each mutant PA protein was determined by Western blotting. The accumulation of the PA mutant proteins is shown in Fig. 2A. Every recombinant plasmid gave raise to a protein which was recognized by a mixture of specific MAbs and whose size was in agreement with that expected according to the different PA constructs. The results presented in Fig. 2A indicate that the steady-state levels of the various PA mutants are very different. Some deletion mutants, like PA/Bg and PAΔHS, show an accumulation indistinguishable from that of wild-type PA. Other PA mutants, like PA/S and PAΔSH, showed consistently higher levels of accumulation than the wild-type protein. Two mutants, PA/H and PA/Sc (arrows), which encode the first 247 and first 342 amino acids of the PA molecule, respectively, showed particularly low steady-state levels. It should be noticed that these are the mutants that presented a pattern of cellular distribution almost exclusively nuclear. In contrast, the

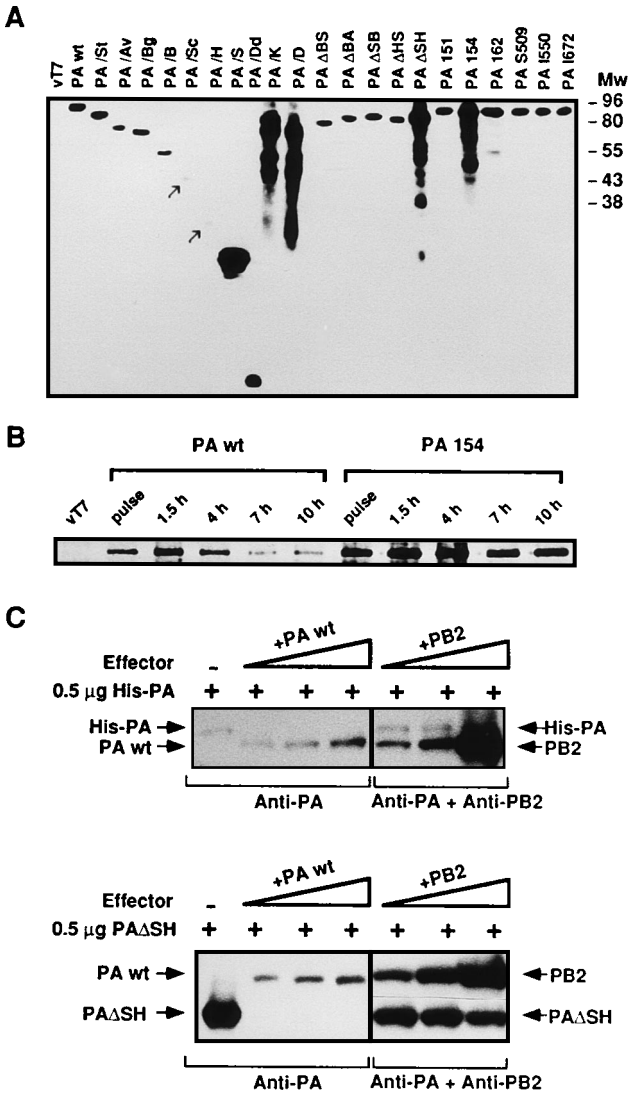

FIG. 2. PA protein is a target for its induced proteolytic process. (A) PA mutants present different accumulation levels. COS-1 cells were infected with VTF7-3 and transfected with 0.5 μg of each of the pGPA plasmids encoding the different PA mutants. Equal amounts of total cell extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 12% polyacrylamide gel and Western blotting with a mixture of PA-specific MAbs. (B) Half-life of PA wild-type (wt) protein is shorter than that of PA 154. Cells infected with VTF7-3 and transfected with 0.12 μg of pGPA wild-type or pARPA154 plasmid were labeled at 16 h posttransfection with a mixture of [^{35}S]Met and [^{35}S]Cys (700 μCi/ml) for 15 min. Cells were lysed in radioimmunoprecipitation assay buffer immediately (pulses) or after incubation for the indicated chase times. Cell extracts were used for immunoprecipitation of PA protein with a specific MAb. After SDS-PAGE, the gel was dried and exposed. (C) PA protein is a direct target for the degradation effect. COS-1 cells were infected with VTF7-3 and transfected with 0.5 μg of either the PRSETPA (upper panel) or the pGPAΔSH (lower panel) plasmid and no pGPAwt (lane −) or increasing amounts (0.25, 0.5, and 2 μg) of either pGPAwt or pGPB2. The total amount of DNA transfected in each case was kept constant (2.5 μg), being supplemented when necessary with pGEM3. Total cell extracts were prepared at 24 h posttransfection and analyzed by Western blotting with either PA-specific MAbs (anti-PA) or a PB2-specific antiserum (anti-PB2). Mw, molecular weight.

following terminal deletion mutant, PA/B, which encodes the first 407 amino acids of PA protein, presents accumulation levels in the same range as that of wild-type PA. For a better comparison of the PA accumulation levels, we used the same amount of cell extracts and the same exposition time for the chemiluminiscent detection of the Western blot signals. For that reason, the signal obtained with some mutants (PA/K, PA/D, PAΔSH, and PA154) is saturated; when shorter times were used, it could be observed that the main band that accumulated corresponded to the entire protein, although smaller products were also observed. These results for PA protein accumulation may represent the capacity of the different PA mutants to induce or not induce the extensive proteolysis caused by PA's expression if PA itself could be used as the target for proteolysis.

The decrease in protein accumulation induced by PA protein expression is the consequence of a reduction in the half-life of the coexpressed proteins (30). Thus, we checked whether the observed differences in the steady-state levels of PA mutant proteins could be due to variations in the half-life of the encoded proteins. To that aim, pulse-chase experiments were carried out in which wild-type PA and the previously reported mutant PA154 inactive in proteolysis induction, which greatly differ in their degree of accumulation, were expressed in COS-1 cells via the VTF7-3 system. Cells were either pulsed with [^{35}S]Met-[^{35}S]Cys or pulsed and chased with an excess of unlabeled amino acids. The amount of labeled protein was determined by autoradiography after immunoprecipitation, and the results are shown in Fig. 2B. Although the rate of synthesis of wild-type PA is slower than that of mutant PA154, the most obvious difference to be observed refers to the half-lives of both proteins. Quantitative data of this experiment showed that wild-type protein is reduced two to three times in its half-life compared with the mutant protein. We interpret the decrease in the synthesis of wild-type PA to be the result of the observed reduction in the accumulation of the T7 RNA polymerase in the presence of wild-type protein (data not shown). Similar results were obtained when we analyzed different mutants, in which a correlation between high accumulation levels and longer half-lives or lower steady-state levels with shorter half-lives was observed.

In order to further characterize the effect of PA upon its own molecule, we used wild-type PA as an effector over two different versions of PA used as reporters. We then transfected COS-1 cells previously infected with VTF7-3 with 0.5 μg of pRSETPA plasmid, which encodes a fusion protein of PA with a tag of 35 amino acids in the N terminus of the molecule, or with 0.5 μg of plasmid pGPAΔSH encoding the deletion mutant PAΔSH, in either the absence or the presence of increasing amounts of wild-type pGPA plasmid or plasmid encoding PB2 protein as the control. The results are presented in Fig. 2C, in which it is shown that wild-type PA protein is able to induce the degradation of its own molecule. These results indicate that whatever the mechanism responsible for the PA induced-proteolysis may be, PA protein itself can be used as a target.

Mapping of the PA protein regions involved in the induction of proteolysis. We next analyzed the ability to induce proteolysis of the different PA mutants by using as reporters two viral proteins, PB2 and NS1, as well as CAT. Cultures of COS-1 cells, previously infected with VTF7-3 recombinant virus, were cotransfected with either pGPB2, pGNS1, or pT7 EMC-CAT plasmid and each of the PA mutant plasmids. The amounts of accumulated PB2, NS1, and CAT proteins were determined by Western blotting as described in Materials and Methods. The expression of the different PA constructs in the cotransfected cells was monitored by immunoblotting, and in every case, the level of accumulation of the different mutants was similar to that presented in Fig. 2A (data not shown). The upper part of Fig. 3 shows the PB2, NS1, and CAT-specific bands obtained by immunoblotting of a typical experiment. After quantitation by scanning densitometry of three independent experiments, the results presented in the lower part of Fig. 3 were obtained. It could be observed that PAs with carboxy-terminal deletions up to amino acid 247 showed a reduction in the steady-state levels of the reporter proteins similar to that obtained with the wild-type PA (mutants St, Av, Bg, B, Sc, and H). We also observed that the amino-terminal deletion had a very strong effect on proteolytic activity, as indicated by mutants without the first 85 and 154 amino acids (mutants K and D) which lose this property. An internal deletion mutant lacking amino acids 186 to 247 (PAΔSH) also showed loss of activity. Taken together, the phenotypes of the mutants analyzed lead to the following conclusions: (i) the first 247 amino acids of the protein are sufficient to bring about this biological activity (mutant PA/H) and (ii) the region comprising the first 85 amino acids and that located between positions 186 and 247 of the PA subunit contain sequences that are necessary to induce proteolysis of the cotransfected protein. In other words, we can conclude that the amino-terminal portion of the PA molecule is responsible for the extensive proteolytic activity shown by this protein, either upon the coexpressed proteins or upon its own molecule.

Is the presence of PA protein in the nucleus necessary for the induction of proteolysis? Every one of the PA genes able to induce proteolysis described above encodes proteins that localize into the nucleus when expressed individually in mammalian cells. As we had previously described, two regions of the PA protein are responsible for its translocation to the nucleus: sequence positions 124 to 139 and 186 to 247, as delimited by mutants PAΔ3 and PAΔSH, respectively (24). The first region includes a nucleoplasmin-like NLS, while the second does not show any similarity to previously described NLSs (24). Since there are mutants that are targeted to the nucleus and that have lost the capacity to induce proteolysis (mutants PA/K and PA/D), we can conclude that location in the nucleus is not the only requirement for PA protein to be active. However, the question about the necessity of nuclear localization of PA for cellular proteolytic activity remains. If PA's presence in the nucleus is a prerequisite, we should expect that some potentially active mutants would lose their biological activity upon localization in the cytosol. To answer this question, we tested if the previously described PAΔ3 cytosolic mutant lacking the nucleoplasmin-like NLS (24) was able or unable to induce proteolysis. This PA mutant has the two regions of PA that we have mapped so far as required for the induction, but these sequences will be expressed in the context of a cytosolic protein. Cotransfection experiments were performed with the pGPA or pGPAΔ3 plasmid plus either the pGPB2, pGNS1, or pT7 EMC-CAT plasmid. Figure 4A shows the steady-state levels of wild-type PA and PAΔ3 mutant proteins plus those of the pGPAΔSH protein used as the control, while the accumulation levels of PB2, NS1, and CAT proteins obtained in cotransfected cells are shown in Fig. 4B. These results indicate that mutant PAΔ3 is accumulated to high levels in the transfected cells and that it is unable to reduce the steady-state levels of the PB2, NS1, and CAT proteins in coexpression experiments, in contrast with the behavior of wild-type PA. This result reinforces the strict correlation found between nuclear localization and the capacity to induce proteolysis, but we cannot eliminate the possibility that deletion of a short stretch of the protein, such as that containing the

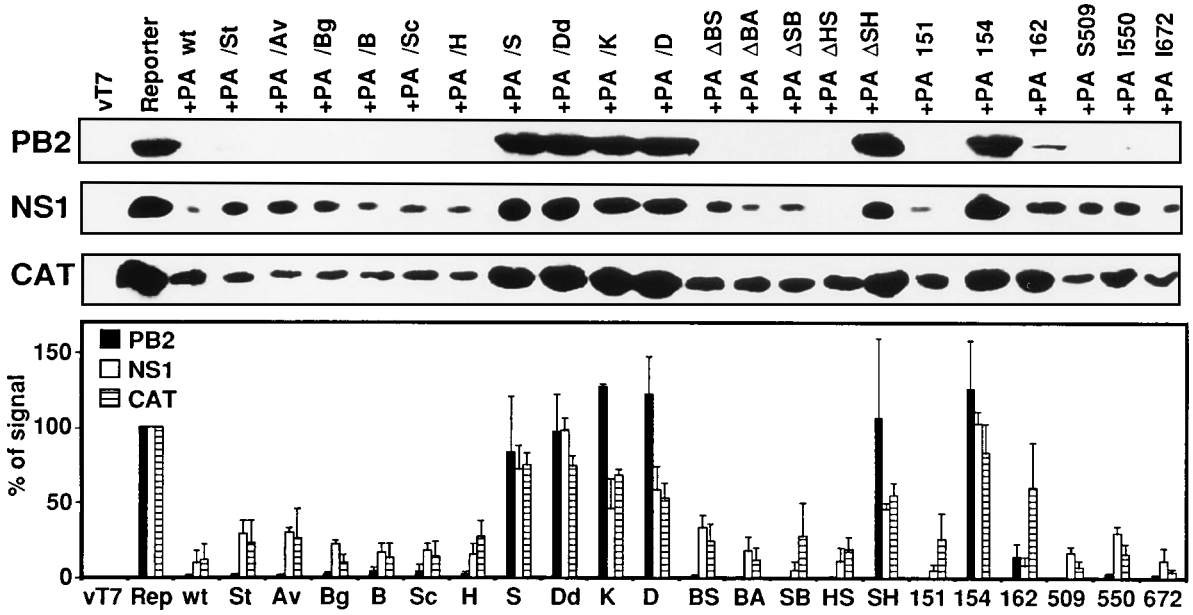

FIG. 3. Effect of the different PA mutants on the accumulation of reporter proteins. Cells were infected with VTF7-3 and transfected with 0.25 μg of each reporter plasmid (pGPB2 [PB2], pGNS1 [NS1], or pT7EMCCAT [CAT]) together with 0.5 μg of the corresponding pGPA plasmid, as indicated in the top line. Total cell extracts were prepared and analyzed by Western blotting for the accumulation of each reporter protein. Results from a representative experiment are shown. The lower histogram shows the quantitation by densitometry of the enhanced chemiluminescence signals of Western blots from three different experiments. The results are presented as the percentage of remaining signal, taking as 100% the percentage obtained when the reporter proteins were singly expressed. Standard deviation bars are given.

nucleoplasmin-like NLS of 15 amino acids, or even point mutations, such as that affecting amino acid 154, could produce a conformational change that would eliminate PA-induced proteolysis. To distinguish whether regions involved in nuclear localization of PA were functional only in transport or were also involved in the induction of proteolysis, we fused the nuclear localization signal of the SV40 T antigen (16) to the carboxy-terminal part of the wild-type PA or the cytosolic PA mutants. The localization of the different PA constructs carrying the nuclear localization signal of T antigen was tested in HeLa cells upon infection with VTF7-3 and transfection with plasmids expressing the new versions of the PA proteins. The cellular distribution behavior of PA protein in this system is similar to that described for SV40-PA recombinants in COS-1 cells (24). The results presented in Fig. 5A show that the NLS from T antigen was able to confer nuclear localization to PA mutant proteins with a previous cytosolic phenotype, although nuclear localization was never complete. When we analyzed the ability to induce proteolysis of these heterologous constructs, with PB2 protein as the reporter, we observed that T antigen-tagged wild-type PA was active but the T antigen-tagged PA mutants were not (Fig. 5B). These results indicate that the regions involved in nuclear transport of PA are also involved in the induction of proteolysis. A scheme summarizing the data obtained from the mutational analysis and showing the regions of PA involved in the nuclear transport of the molecule and in the induction of proteolysis is shown in Fig. 6.

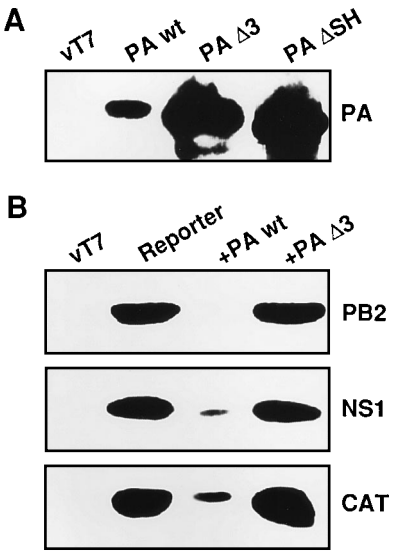

FIG. 4. Cytosolic PA protein containing regions involved in proteolysis induction is not able to decrease the accumulation of reporter proteins. (A) Expression of PAΔ3 protein. COS-1 cells were infected with VTF7-3 and transfected with 0.5 μg of pGPA plasmids as indicated. Total cell extracts were used for Western blotting with PA-specific antibodies. (B) Effect of expression of PAΔ3 protein on the accumulation of reporter proteins. Cells infected with VTF7-3 were transfected with 0.25 μg of each reporter plasmid (pGPB2 [PB2], pGNS1 [NS1], or pT7EMCCAT [CAT]) together with 0.5 μg of pGEM3 (Reporter) or 0.5 μg of pGPA plasmids as indicated. Cell extracts were analyzed by Western blotting with specific antisera as indicated to the right. vT7, nontransfected cells.

Concluding remarks. The generality of the PA-induced proteolytic effect has been studied by looking at the phenotypes of two different PA genes derived from type A viruses. Since both genes were active and are among the most distant ones within the PA proteins from the human viruses, the induction of proteolysis seems to be a general characteristic of PA proteins from type A strains. To gain insight into the general proteolysis induced by PA protein expression, we have carried out an extensive deletion mapping study. The sequences responsible for PA-induced protein degradation are located in the amino-terminal one-third of the protein, as indicated by the negative phenotype of deletions affecting amino acids 1 to 85, 124 to

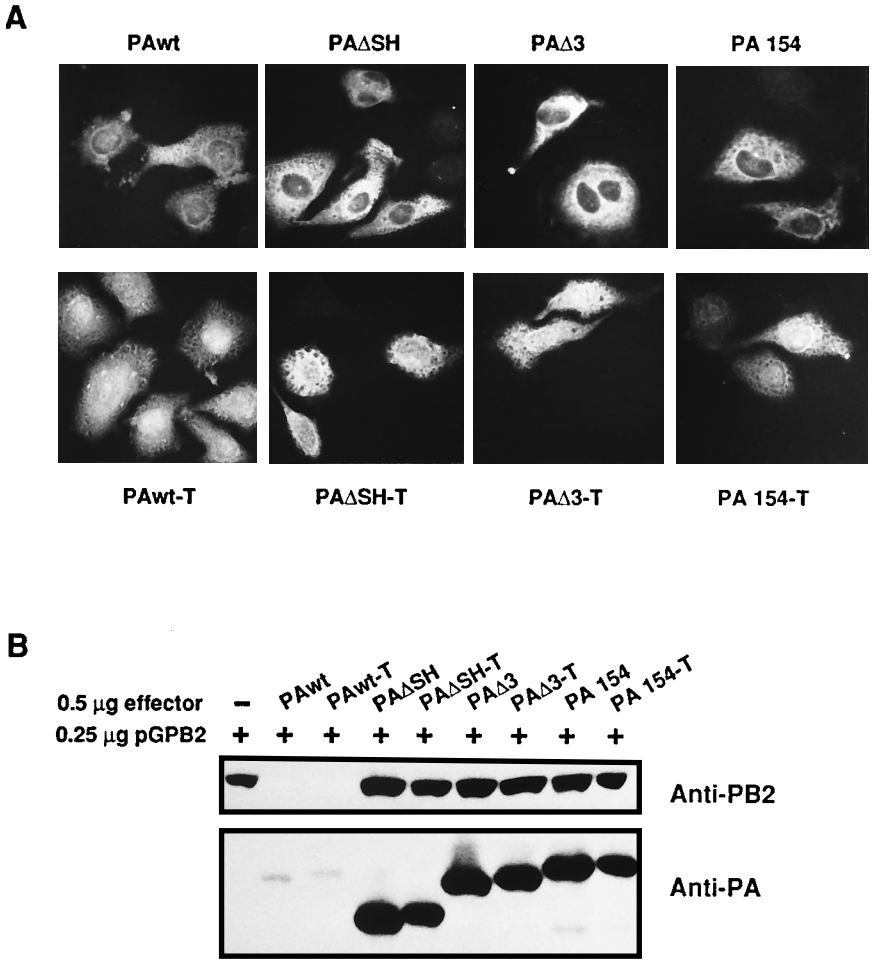

FIG. 5. Cytosolic mutants of PA protein are not active in proteolysis induction after relocalization into the nucleus. (A) Subcellular localization of PA mutants containing the nuclear localization signal from SV40 T antigen (PAs-T). Subconfluent monolayers of HeLa cells were infected with VTF7-3 and transfected with either 0.3 μg (pGPA [PAwt] and pGPA-T [PAwt-T]) or 0.1 μg (pGPAΔ3 [PAΔ3], pGPAΔ3-T [PAΔ3-T], pGPAΔSH [PAΔSH], pGPAΔSH-T [PAΔSH-T], pGPA154 [PA 154], and pGPA154-T [PA 154-T] of PA expression plasmids together with pGEM3 as the carrier (4 μg of total DNA per 35-mm-diameter dish). At 6 h posttransfection, the cells were fixed with methanol at −20°C and subjected to indirect immunofluorescence with PA-specific MAbs as described in Materials and Methods. (B) Effect of the expression of PA mutants containing the T antigen NLS on the accumulation of PB2. COS-1 cells were infected with VTF7-3 and transfected with 0.25 μg of pGPB2 plasmid together with no pGPA (lane −) or 0.5 μg of pGPA plasmids as indicated. Total cell extracts were analyzed by Western blotting with PB2-specific (upper panel) or PA-specific (lower panel) antibodies.

139, and 186 to 247 and the activity of a protein including the first 247 amino acids of the PA sequence. We have carried out an extensive search in the data bank, looking for homologies with the first 247 residues of the PA protein, and no significant homologies could be found with any previously sequenced protein, including cellular and viral proteases.

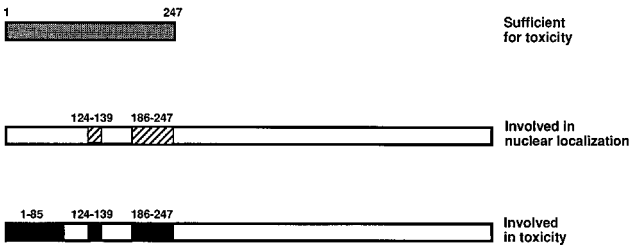

FIG. 6. Sequences in PA protein involved in nuclear localization and in induction of proteolysis map to the amino-terminal one-third of the protein. Note the overlap between nuclear localization signals and regions involved in the proteolytic activity in the PA protein. This scheme summarizes data presented in this report as well as previous results (24).

As described above, influenza virus PA protein has the ability to induce its own degradation. At present, we cannot distinguish between the induction of cellular proteases after PA expression and the presence in PA protein of an intrinsic proteolytic activity. However, if that were the case, it would represent a protease with no sequence homologies to those previously described. In this context, it should be emphasized that PA toxicity takes place not only in eukaryotic cells but also in bacteria. We have repeatedly looked for PA expression in bacteria, and although PA protein in synthesized, it is unable to accumulate (data not shown). We have also carried out preliminary experiments with PA expression in *Saccharomyces cerevisiae* and have observed that PA-expressing plasmids isolated from *S. cerevisiae* undergo a reorganization with deletion of the first 450 nucleotides of PA (the first 150 amino acids), suggesting that reorganization of the plasmid is taking place in order to avoid PA toxicity.

All active PA proteins are nuclear proteins, and it should be noted that the two PA mutants that show the lowest level of accumulation are mutants that have a stronger, almost exclusive nuclear localization, supporting the notion that a nuclear signal is necessary to trigger the proteolysis and/or that the susceptibility to PA degradation is highest in this subcellular compartment.

In influenza virus-infected cells, it can be observed that, although at late times PA protein is located in the nucleus, early in infection it is still in the cytosol (1, 25). This is in contrast to the other core influenza viral proteins, PB1, PB2, and NP, that are located in the nucleus as soon as they can be detected (1, 25). This delay in nuclear targeting suggests that the transport of the PA subunit to the nucleus may be regulated during infection. This fact, together with the apparent requirement for nuclear localization to induce protein degradation and the observation that this characteristic is shared by influenza virus PA proteins from type A viruses, suggests a physiological role for this PA activity during influenza virus infection.

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