Mutational analysis of the influenza virus A/Victoria/3/75 PA protein: studies of interaction with PB1 protein and identification of a dominant negative mutant

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The RNA polymerase activity and PB1 binding of influenza virus PA mutants were studied using an in vivo-reconstituted polymerase assay and a two hybrid system. Deletions covering the whole PA protein abolished polymerase activity, but the deletion of the 154 N-terminal amino acids allowed PB1 binding, indicating that the PA protein N terminus is not absolutely required for this interaction. Further internal or C-terminal deletions abolished PB1 interaction, suggesting that most of the protein is involved in this association. As a novel finding we showed that a single amino acid insertion mutant, PAI672, was responsible for a temperature-sensitive phenotype. Mutant PAS509, which had a serine insertion at position 509, bound to PB1 like wild-type PA but did not show any polymerase activity. Over-expression of PAS509 interfered with the polymerase activity of wild-type PA, identifying PAS509 as a dominant negative mutant.

The influenza virus RNA polymerase is composed of three polymerase proteins, PB1, PB2 and PA, and catalyses two distinct types of RNA synthesis; (i) synthesis of mRNA (transcription) and (ii) amplification of the vRNA (replication). For mRNA synthesis, 5’-capped, host cell-derived RNA fragments are used as primers and polyadenylation occurs at a signal located 17–22 nucleotides before the 5’ end of the template. Replication occurs without primer and the vRNA template is copied to a full-length positive-stranded RNA (cRNA), which serves as template for vRNA synthesis. It has been shown that free nucleoprotein (NP) might be a control element for anti-termination, but little is known about the mechanism of the transcription–replication switch and the detailed role of the components of the RNA polymerase (Krug et al., 1989). Comparative sequence analysis and experimental data suggest that PB1 is the polymerase itself (Biswas & Nayak, 1994; Poch et al., 1990). PB2 binds CAP1 structures and is probably responsible for the binding of the vRNA promoter (Fodor et al., 1993; Ulmanen et al., 1981) and the endonucleolytic cleavage of the host cell primers (Licheng et al., 1995). The function of PA is unknown. It is essential for the activity of in vivo-reconstituted polymerase (reviewed in Mena et al., 1995) and genetic evidence suggests a role for PA in replication rather than in transcription (reviewed in Mahy, 1983). PA protein induces a general proteolysis of co-expressed proteins (Sanz-Ezquerro et al., 1995), but it is not clear if this property is essential for polymerase activity. The region of PA responsible for the induction of proteolysis maps to the N-terminal third of PA, as determined by mutational analysis (Sanz-Ezquerro et al., 1996). The expression of the P proteins in Xenopus oocytes showed that complex formation occurs in the absence of virus RNA. The co-immunoprecipitation of pairs of P proteins indicated that PB2 and PA can interact independently with PB1, yet cannot form a complex directly with each other (Digard et al., 1989).

In this report we describe the use of two in vivo-reconstitution assays to analyse the RNA polymerase activity and the PB1 binding of a large set of PA mutants. Polymerase deficient, but interacting mutants have been characterized as dominant negative and therefore identify regions of the molecule relevant for its biochemical activity. Fig. 1 shows the most relevant mutants used in this study. C-terminal, N-terminal and internal deletions covered the entire PA protein sequence. In addition, three point mutants in the N terminus (positions 151, 154 and 162) and three insertion mutants in the C-terminal third of PA (PAI550, PAI672 and PASS09) were analysed. Mutant PASS09 contained a serine insertion after position 509 just downstream of a conserved sequence with homology to a nucleotide-binding motif (position 502–509). The construction of all mutants has been described previously (Sanz-Ezquerro et al., 1996). The subcellular localization and expression of the individual mutants used in this study have been shown previously (Sanz-Ezquerro et al., 1996).
Fig. 1. PA mutants: PB1 association and polymerase activities. The last PA-encoded amino acid of each C-terminal deletion mutant is indicated. Black boxes indicate a frameshift and a short stretch of unspecific sequence until the next stop codon is reached. For the N-terminal and internal mutants the size and location of the deletion is indicated. The insertion mutants are named with the inserted amino acid and the position upstream of the insertion. The PB1–PA interaction was measured using the two hybrid system as described in the text. The PB1 association of the interacting PA mutants is indicated as the percentage luciferase activity of that achieved by the wt PA co-PA fusion protein. The interaction assay of the non-interacting mutants was repeated at least twice and the values for the interacting mutants represent the average of six independent experiments. The polymerase activity of the individual PA mutants was determined by reconstitution of the RNA polymerase in vivo as described. The activity of mutant 1550 is indicated as the percentage CAT activity of that achieved by the wt PA protein. The polymerase assay was repeated two to three times with each mutant.

Firstly, we tested whether the PA mutants were able to reconstitute in vivo an active polymerase complex. The expression of the three P proteins and NP using the vaccinia virus–T7 RNA polymerase expression system allowed the expression of an influenza virus-like vRNA containing the CAT gene flanked by the non-coding regions of the NS gene (Piccone et al., 1993) as previously described (Mena et al., 1994). In addition to the mutants shown in Fig. 1, three mutants with smaller deletions in the C-terminal half of PA (PAΔ463-511, PAΔ407-511 and PAΔ407-425) and one
expression of wt PA allowed CAT expression (data not shown).

We considered it of interest to examine if any of the mutations introduced a temperature-sensitive (ts) phenotype. Thus, the RNA polymerase activity was reconstituted at 37 °C or 33 °C as described (Mena et al., 1994). Mutant PA154 and all deletion mutants remained inactive at 33 °C and mutants PA151 and PA162 showed only slightly increased activity at 33 °C compared to 37 °C (data not shown). Nevertheless, interesting results were obtained with the insertion mutants (Fig. 2). Mutant PA1672, completely inactive at 37 °C, reached 3.4% of wt activity at 33 °C, a level significantly higher than background (0.3%). Mutant PA1550 was also about twofold more active at the permissive temperature. Similar insertion mutants with a ts phenotype for RNA polymerase activity have been observed for the PB2 gene (Perales et al., 1996). Interestingly, a ts mutant (s263) of fowl plague virus carries a missense mutation (Ala to Val change) at position 671 of PA (Herget & Scholtissek, 1993). Insertion mutants, in contrast to missense mutations that are in general found to be responsible for ts mutations, could be genetically quite stable since reversion would require the deletion of exactly three nucleotides. Nevertheless mutations in PA or other virus genes might suppress the ts phenotype (Mandler et al., 1991). Influenza viruses with ts insertion mutations could be rescued using described procedures (for review see García-Sastre & Palese, 1993) to generate recombinants with reduced pathogenicity and enhanced genetic stability.

For the mutants that were negative for CAT activity it could not be distinguished if the active site of PA, complex formation or just the general folding of PA was affected by the mutation. Thus, we further studied the PA–PB1 association of the mutants using a GAL4/VP16-based two hybrid system developed for the transfection of animal cells (Sadowski et al., 1988 and references therein). Plasmids were constructed expressing the GAL4 DNA-binding domain fused to the N terminus of PB1, and the VP16 activation domain fused to the N terminus of PA. For the construction of GAL4 fusions the multiple cloning site (MCS) of pSG424 (Sadowski & Patshne, 1989) was replaced by the MCS of pTM1 (Elroy-Stein et al., 1989). PCR fragments of PB1 and PA cDNAs of strain A/Victoria/75 with a SmaI restriction site upstream of the ATG and a XbaI site downstream of the stop codon were transferred to the modified pSG424 using the introduced SmaI and XbaI sites. The VP16–PAwt, –PAAD and –PAAK fusion plasmids were constructed by transferring the PA genes of pSG424PA, pGEM3PAAD or pGEM3PAAK into the EcoRI–XbaI site of pAASVVP16 (Aso et al., 1992) whose polylinker had been previously replaced by the MCS of pGEM3 (EcoRI–HindIII). All other pVPPA mutant plasmids were constructed by replacing the EcoRI–XbaI fragment of pVPPA with the corresponding fragment from the pGEM3PA mutant plasmid. The expression of the VPPA fusion proteins was confirmed by Western blot analysis using GAL4–VP16- and additional deletion mutant (PAΔSA) in the N-terminal half (A186–280) of PA were analysed (data not shown). The polymerase activities of the PA mutants are summarized in Fig. 1. None of these deletion mutants was active in the polymerase assay. This result is not surprising since these drastic mutations might not only affect the structure, the intracellular localization or the active site of PA but also complex formation and thus indirectly the activity of the other polymerase subunits. The point mutant PA154, the only cytoplasmic mutant beside PAASA and PAASH, was inactive, whereas the nuclear mutants (PA151, PA162) with mutations in the same region (Sanz-Ezquerro et al., 1996) remained active (data not shown). Of the three insertion mutants only PAI550 showed activity, albeit to a reduced level (20%). The expression of the individual PA mutants was analysed by Western blot using a part of the same size and the majority of the mutants was expressed to similar or higher levels (PA, PAASA, PAASH, PA154) than the wild-type (wt) control (data not shown). Only the mutants PASc and PAH1 were reproducibly accumulated at levels lower than wt PA and other mutants. However, the low expression levels of these mutants cannot be the reason for the negative result in polymerase activity, since much lower, almost undetectable

\[d_{\text{AC-CM}} \quad A_{\text{C-CM}} \quad C_{\text{M}}\]

Fig. 2. CAT activity and its competition by PA mutants. CAT activity of wt PA and other mutants. However, the low expression levels of these mutants cannot be the reason for the negative result in polymerase activity, since much lower, almost undetectable
PA-specific antisera (data not shown). All VPPA mutant proteins were of the expected size and accumulated to similar levels as wt VPPA, except for the two shortest mutants expressing C-terminal portions of PA (VPPAh, VPPAS), which were five- to 10-fold less abundant than wt VPPA. The reporter plasmid pGL-G5 (constructed and provided by P. Stäheli, Department of Virology, University of Freiburg, Germany) contains five GAL4 DNA-binding sites upstream of an E1B promoter and the luciferase gene.

The association of PA and PB1 in vivo was measured by transfection of subconfluent monolayers of COS-1 cells (p35 dishes) with 1 μg of pGL-G5, 2.5 μg of pVPPA (or mutant pVPPA) and 250 or 500 ng of pSG424PB1, using cationic liposomes for transfection. Cells were harvested 48 h post-transfection and assayed for luciferase activity (Brasier et al., 1989). The transactivation mediated by this association of VPPA and GAL4–PB1 resulted in a high level of luciferase expression, reaching about 10% of that achieved with GAL4–VP16 by transfection of 1 μg of pSGVP690 (Sadkowski & Patshne, 1989; data not shown). When either pSG424PB1 or pVPPA were transfected together with plasmids expressing either the unfused VP16 activation domain or the unfused GAL4 binding domain, only basal level of luciferase activity (1–3% of wt activity) could be detected. Thus, neither VPPA nor GAL4–PB1 had transactivation ability by itself.

The interaction of the PA mutants with PB1 is shown in Fig. 1. Deletion of the 154 N-terminal amino acids (mutant VPPAΔD) barely affected the association with PB1, indicating that the N terminus is not absolutely required for such interaction. Since none of the 12 C-terminal and internal PA deletion mutants showed binding to PB1 it might be concluded that the entire C-terminal three quarters of PA are involved in this association. PB1 may be bound at several points and/or by a highly structure-dependent element formed by discontinuous regions of PA. The structure is indeed important since a deletion of the 154 N-terminal amino acids did not affect binding to PB1, but a deletion of only 85 amino acids (mutant VPPAΔK) or the amino acid substitution at position 154 (mutant VPPA154) were inhibitory. Thus, the sequence located between position 85 and 154 does not contribute to interaction but can inhibit the binding mediated by other sequences. The binding capacity of other mutants might be inhibited similarly. The insertion mutants VPPAS159 and VPPAI672 bound to PB1 with similar affinity to VPPAwt, and VPPAI550 showed about 50% of the binding activity of VPPAwt.

In the experiments presented so far, we identified three mutants (PAAD, PSS159 and PAI672) which were negative in polymerase activity, but interacted with PB1 to a similar extent as wt PA. We were interested to know whether these mutants could enter the polymerase complex and thus inhibit its activity in a dominant negative manner. Therefore, we performed in vivo competition experiments at 37 °C (Fig. 3). A low amount of wt pGEM3PA (5 ng per dish) was transfected together with 100 ng of pGEM3PB2 and pGEM3PB1 for the reconstitution of the polymerase complex. In spite of this, the system was saturated with PA protein, since transfection of an additional 100 ng of pGEM3PAwt did not increase CAT activity (Fig. 3). The decrease in CAT activity observed after over-expression of wt PA or the non-interacting mutant PAABA (Fig. 3) can be explained by the proteolytic activity induced by PA protein, which reduced the concentration of the co-expressed proteins (PB1, PB2, NP and CAT; Sanz-Ezquerro et al., 1995). The over-expression of PSS159 (Fig. 3) reduced CAT expression more than 100-fold compared to competition with wt PA or PAABA proteins, indicating that PSS159 competes for complex formation. It has been shown that the proteolytic activities induced by PSS159, wt PA and PAABA are identical (Sanz-Ezquerro et al., 1996) and hence cannot be responsible for this result. Thus, mutation S159 did not alter the structure but might have affected an active site of PA. In this mutant a serine is inserted just downstream of a conserved putative nucleotide-binding motif (G$_{302}$FIKGC$_{307}$R$_{308}$S$_{309}$), which closely resembles the classic GXXGKT/S nucleotide-binding motif, and suggests that the function of PA requires the binding of nucleotides. Despite their strong interaction with PB1 in the two hybrid system, PAAD and PAI672 could not compete efficiently under these conditions (data not shown), indicating that their binding properties are not complete in terms of complex formation.

In conclusion, two in vivo-reconstituted systems were used to screen a series of PA mutants for their polymerase activity.
and complex formation capacities. The inability to identify a small region of PA able to interact with PBI suggests that several distant positions of its amino acid sequence might fold together to form conformation-dependent interaction domains. Several mutations in the C-terminal third of the protein affected its polymerase activity without dramatically altering the PBl-binding properties. In particular, the insertion of a serine downstream of a putative nucleotide-binding domain generated a dominant negative mutant, identifying this region as a potential active site of PA. Further experiments will show if this dominant negative mutant is able to interfere with RNA replication in influenza virus-infected cells.

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