Disruption of the Viral Polymerase Complex Assembly as a Novel Approach to Attenuate Influenza A Virus*

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To develop a novel attenuation strategy applicable to all influenza A viruses, we targeted the highly conserved protein-protein interaction of the viral polymerase subunits PA and PB1. We postulated that impaired binding between PA and PB1 would negatively affect trimeric polymerase complex formation, leading to reduced viral replication efficiency in vivo. As proof of concept, we introduced single or multiple amino acid substitutions into the protein-protein-binding domains of either PB1 or PA, or both, to decrease binding affinity and polymerase activity substantially. As expected, upon generation of recombinant influenza A viruses (SC35M strain) containing these mutations, many pseudo-revertants appeared that partially restored PA-PB1 binding and polymerase activity. These polymerase assembly mutants displayed drastic attenuation in cell culture and mice. The attenuation of the polymerase assembly mutants was maintained in IFNα/β receptor knock-out mice. As exemplified using a H5N1 polymerase assembly mutant, this attenuation strategy can be also applied to other highly pathogenic influenza A virus strains. Thus, we provide proof of principle that targeted mutation of the highly conserved interaction domains of PA and PB1 represents a novel strategy to attenuate influenza A viruses.

Infection with influenza viruses annually claims 250,000–500,000 lives worldwide (1). As exemplified by the 1918 influenza pandemic, which resulted in more than 50 million deaths (2), global spread of a pandemic influenza virus strain can lead to high morbidity and mortality. Besides inactivated vaccines, a live attenuated influenza virus vaccine (FluMist) (3, 4) is currently used for vaccination. Importantly, the degree of attenuation is critical for the safety of the live vaccines, but it also affects their efficacy. For safety reasons, children below the age of two and immunocompromised persons are excluded from vaccination with these live vaccines (3). Furthermore, the live vaccine used in the 2007–2008 season was 50% less efficacious than the inactivated vaccine that was used (5). In the currently used influenza A virus (FluA)3 live vaccine, the degree of attenuation is fixed because a specific master strain is used (3). Thus, to vary the degree of attenuation and to identify new sites that contribute to the safety of live vaccines, new attenuation approaches applicable for all FluA strains are desirable.

A promising target for attenuation of all known FluA strains is the trimeric polymerase complex. Its assembly from the subunits PA, PB1, and PB2 is crucial for polymerase activity and thus virus replication (6–8). PB1 represents the central scaffold protein that binds to PA and PB2 (6, 9–11). Several recently published crystal structures have defined the highly conserved protein-protein-binding domains for PA-PB1 and PA-PB1-PB2 (11–13). Alteration of these conserved residues abrogates subunit interactions accompanied by restricted assembly of polymerase heterotrimers, resulting in decreased polymerase activities (6, 14).

We therefore hypothesized that inefficient polymerase assembly would lead to impaired viral growth and thus attenuation. We identified mutations in the PA-binding domain of PB1 (PB1L8N), in the PB1-binding domain of PA (PAW706E), or in both domains simultaneously (PB1L8N/PAW706E), all of which reduced PA-PB1 binding affinities, leading to impaired polymerase assembly and activity. We observed a correlation between reduction in polymerase activity and increased attenuation in cell culture and mice. Importantly, the attenuated phenotype of the polymerase assembly mutants was maintained in the IFNα/β receptor knock-out mice. Thus, targeting of the highly conserved protein-protein interaction domains of PA and PB1 represents a novel strategy to attenuate influenza A viruses.

EXPERIMENTAL PROCEDURES

Generation of Recombinant Influenza A Viruses—Generation of the recombinant viruses A/SC35M (H7N7) (15) and A/Thailand/KAN-1/2004 (H5N1) (16) was performed as described (14, 16) in 6-well tissue plates with 105 293T cells per well using the eight pHW2000 plasmids (300 ng of each) expressing the individual segments and the four pCAGGS plasmids (150 ng of each) coding for PA, PB1, PB2, and NP. After

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3 The abbreviations used are: FluA, influenza A virus; MDCK, Madin-Darby canine kidney; IFNAR, IFNα receptor; aa, amino acid(s); p.i., post infection.
24 h, released virus was used to infect MDCK cells. Sequences of viral genomes were determined from particles released in the supernatant of the MDCK cells 48 h post infection. Rescued viruses were plaque-purified, and MDCK cells were infected for propagation of virus stocks.

**Virus Infections and Growth Assays**—MDCK cells or human lung cells (A549) were infected at a multiplicity of infection of 0.001 for 24–36 h. Virus titers in the cell supernatants were determined at the indicated time points by plaque assay and expressed as plaque-forming units (pfu)/ml. For passaging the mutant viruses 10 times in cell culture, MDCK cells were infected with $\sim 10^5$ pfu corresponding to a multiplicity of infection of 0.001 for 24–36 h.

**Mouse Experiments**—BALB/c mice were obtained from JANVIER SAS (Strasbourg, France). IFNα receptor knock-out (IFNAR$^{-/-}$) mice (17) backcrossed 12 generations to C57BL/6 were generated by J. Sprent and M. Rubinstein (The Scripps Research Institute, La Jolla, CA) and kindly provided by A. Diefenbach (Institute of Medical Microbiology and Hygiene (IMMH), Freiburg, Germany). 6–8-week-old animals were used for all infection experiments, which were performed in accordance with the guidelines of the local animal care committee. Animals were euthanized if severe symptoms developed or body weight loss approached 25% of the initial value. Lung homogenates were prepared using the FastPrep24 system (MP Biomedicals). For passaging the mutant viruses 10 times in cell culture, MDCK cells were infected with $\sim 10^5$ pfu corresponding to a multiplicity of infection of 0.001 for 24–36 h.

**Molecular Modeling**—Based on the structural model of PB1 (aa 1–15) bound to PA (Protein Data Bank (PDB) 2znl), a homology model was derived for SC35M using Prime (Schroedinger Suite 9.1.107) on an Intel 6600 Core2Duo processor machine (3 GB of RAM) running openSUSE 11.2. To accurately reflect conformational shifts in PA and PB1 induced by the V121 exchange, the side chains of amino acids forming the proximate shell around this residue were refined using Prime Refinement with default settings. Thereby, a main shift of the side chain of Met-595 is observed, which is displaced by the bulker substituent of V121.

**Plasmid Constructions**—The pHW2000 plasmid system (18) was used for influenza A virus rescue. To obtain rescue plasmids containing the mutant PB1 or PA genes, site-directed mutagenesis was performed. To generate the pCAGGS expression plasmids encoding PA or PB1 mutants, parts of the corresponding open reading frames were PCR-amplified, digested with AgeI and NotI (PB1) or Cfr9I and Bsp119I (PA), and subsequently cloned into pCAGGS-SC35M-PB1-HA and pCAGGS-SC35M-PA-HA.

**Co-immunoprecipitation and Immunoblot Analysis**—293T cells were transfected with the indicated plasmids in 6-well plates using METAFECTENE (Biontex, Martinsried, Germany). Cells were incubated 24 h post transfection with lysis buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 0.5 mM EDTA, 0.5% Nonidet P-40, 1% protease inhibitor mix G, (Serva, Heidelberg, Germany), 1 mM DTT) for 15 min on ice. After centrifugation by 13,000 rpm at 4 °C, supernatant was incubated with HA- or FLAG M2-specific antibodies (Sigma) coupled to agarose beads, respectively, for 1 h at 4 °C. After three washes with 1 ml of washing buffer (lysis buffer without protease inhibitor mix), bound material was eluted under denaturing conditions, separated on SDS-PAGE gels, and transferred to PVDF membranes. Viral polymerase subunits were detected with antibodies directed against the HA (Covance, Berkeley, CA) or His (Qiagen) or FLAG tag (Sigma).

**Enzyme-linked Immunosorbent Assay (ELISA)**—ELISA was essentially performed as described previously (19) using biotinylated PB1$_{1–15}$ peptides bound to streptavidin-coated microwell plates and cell extract containing HA-tagged PA WT or mutant proteins.

**Cells**—293T, A549, and MDCK cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, and 1% penicillin/streptomycin. All cells were maintained at 37 °C and 5% CO$_2$.

**Peptides**—Peptides were synthesized as described in Ref. 19 using a Pioneer automatic peptide synthesizer (Applied Biosystems, Foster City, CA).

**Reconstitution of the Influenza Virus Polymerase Activity**—293T cells were transiently transfected with a plasmid mixture containing the influenza A virus-derived PB1, PB2, PA, and NP expression plasmids and a polymerase I-driven plasmid transcribing an influenza A virus-like RNA coding for the reporter protein firefly luciferase to monitor viral polymerase activity (14). The transfection mixture also contained a plasmid constitutively expressing Renilla luciferase, which served to normalize variation in transfection efficiency. The reporter activity was determined 24 h post transfection and normalized using the Dual-Glu® luciferase assay system (Promega).

**Statistical Analysis**—With the exception of the mouse experiments, error bars represent S.E. from at least three independent experiments. For the animal experiments, the indicated number of mice was used for calculating S.E.

**Ethics Statement**—All animal experiments were performed in compliance with the German animal protection law (Tierschutzgesetz). The mice were housed and handled in accordance with good animal practice as defined by the Federation of Laboratory Animal Science Associations (FELASA) and the national animal welfare body GV-SOLAS. The animal welfare committees of the University of Freiburg (Regierungspräsidium Freiburg) approved all animal experiments.

**RESULTS**

**Mutation at the PA-PB1-binding Interface Leads to Escape Mutants with Impaired Viral Replication**—To identify mutations in PA or PB1 that attenuate FluA to a desirable level, we devised an experimental procedure to force spontaneous escape mutants (Fig. 1A). The first step is the generation of PA or PB1 mutant proteins with substantially impaired polymerase activity and subunit interaction. In a second step, viruses expressing these mutant proteins are created by reverse genetics techniques. Taking into account the rapid mutability of FluA, we expected, dependent on the severity of the introduced mutations, the following possible outcomes: (i) mainte-
Polymerase Assembly Mutants

A. Generation of PA/PB1 mutants with low or no polymerase activity → Rescue of the PA/PB1 mutant viruses → No rescue → Maintenance of inserted mutation → Attenuation → Viable → Escape mutants with recovered polymerase activity → No attenuation

B. PB1 variants

| PB1 variant | PB1<sub>1-15</sub> |
|-------------|---------------------|
| WT          | MDVNPPLLFLKIPQA     |
| 8D          | MDVNPITDFLKIPQA     |
| 11D         | MDVNPITLFLDIPQA     |
| 13D         | MDVNPITLFLKDIPQA    |
| 4D,5D       | MDVDDTLFLKIPQA      |
| 6D,7D       | MDVNPDDFLKIPQA      |
| 8D,9D       | MDVNPITDLDLIPQA     |
| 10D,11D     | MDVNPITLFDIPQA      |
| 4D,8D,11D   | MDVDPITLFDLIPQA     |
| 4D,5D,10D,11D | MDVDPITLFDLIPQA   |
| 6D,7D,8D,9D | MDVNPDDDLKIPQA      |

C. Relative activity (%)

D. PB1<sub>6D</sub><sub>7D</sub>, PB1<sub>8D</sub>, PB1<sub>8D9D</sub>, PB1<sub>10D11D</sub>

E. Virus titer (log<sub>10</sub> pfu/ml)

Post infection
nance of the inserted mutations, resulting in attenuated viruses; (ii) maintenance of the inserted mutations, leading to non-viable viruses; or (iii) introduction of compensatory mutations. These mutations could restore wild-type (WT) viability or display an attenuated phenotype (Fig. 1A).

To efficiently disrupt the highly hydrophobic binding interface between PA and PB1 (12, 13), we replaced either single or multiple aa in the PA-binding domain of PB1 by the charged aa aspartic acid (Fig. 1B) and determined viral polymerase activity in human 293T cells (Fig. 1C). Single substitutions by Asp outside (P13D) or at the border (K11D) of the PA-binding core region (12, 13, 19) resulted in a moderately decreased or unchanged polymerase activity (Fig. 1C). In contrast, replacement of the leucine at position 8 (L8D), which lies within the core-binding 310 helix and is known to be essential for placement of the leucine at position 8 (L8D), which lies within PB1L10D,K11D, respectively (Table 1, Fig. 1D–E). Double substitutions within or at the core region (12, 13) resulted in a moderately decreased or unchanged polymerase activity (Fig. 1C). Single substitutions by Asp outside (P13D) or at the border (K11D) of the PA-binding core region (12, 13, 19) resulted in a moderately decreased or unchanged polymerase activity (Fig. 1C). In contrast, replacement of the leucine at position 8 (L8D), which lies within the core-binding 310 helix, is known to be essential for binding to PA (9, 19), abrogated polymerase activity to a level below 1% of WT activity (Fig. 1C). Double substitutions at other positions in the N terminus of PB1 (PB11–15) reduced the polymerase activity below 3% of WT activity (Fig. 1C).

Next, rescue experiments were performed to generate recombinant SC35M viruses encoding for the above mentioned PB1 mutants. To prevent simple reversion to the WT sequence, 2–3 nucleotide exchanges were performed to mutate the individual aa positions in PB1 (supplemental Fig. S2). As expected, we could generate the mutant virus coding for PB1L10D,K11D without compensatory mutations (Table 1) and impaired growth (supplemental Fig. S1A). However, besides the PB1L10D,K11D mutant, we were also able to generate recombinant SC35M mutated to PB1T6D,L7D, PB1L8D, PB1L8D,F9D, and PB1L10D,K11D respectively (Table 1, Fig. 1D). However, the majority of these viruses represented pseudo-revertants, which we defined as viruses containing a non-introduced, non-WT mutation at the site of the mutated residue, which partially or fully restores the WT phenotype. Specifically, during the rescue of SC35M coding for PB1T6D,L7D, the pseudo-revertant PB1T6D,D7Y emerged frequently (Fig. 1D), whereas rescue of the PB1L10D,K11D mutant virus produced two viruses coding for PB1L10D,K11D and PB1L10D,D11V (Fig. 1D, Table 1). Importantly, several independent attempts to rescue mutant viruses coding for PB1L8D resulted exclusively in virus mutants harboring changes to PB1D8A, PB1D8V, or PB1D8N (Fig. 1D, Table 1). PB1D8V or PB1D8A mutant viruses showed only slightly reduced viral growth (Fig. 1E, data not shown), whereas the mutant virus coding for PB1D8N displayed a pronounced attenuation at 12 h p.i. (Fig. 1E). No additional mutations were found in the polymerase genes of this virus (Table 1, supplemental Fig. S2A). Independent attempts to generate SC35M coding for PB1L8D,F9D yielded three virus mutants: PB1L8D,F9D, PB1L8D,D9F, and PB1L8D,D9F (Fig. 1D). Because PB1D8F represents the wild-type aa at this position, the PB1L8D,D9F and PB1L8D,D9F mutants were phenotypically indistinguishable from the PB1D8F or PB1L8D,D9F mutants, respectively. However, the PB1L8D,D9F mutant represents a recombination event that resulted in restoration of the PB1 open reading frame downstream of the mutated PB1 site (supplemental Fig. S3). As shown in Fig. 1E, the PB1L8D,D9F mutant showed no attenuation in cell culture (Fig. 1E).

Pseudo-reversions in PB1 Partially Restore Polymerase Activity and Trimeric Polymerase Complex Assembly—We reasoned that the pseudo-reversions PB1D8N and PB1D8V would...
rescue polymerase activity through restoration of PA-PB1 binding and thus polymerase complex assembly. Indeed, 60% of WT polymerase activity was achieved with PB1\text{D8N}, whereas PB1\text{D8V} even exceeded WT activity (Fig. 2A). Using an ELISA-based binding assay (19) and peptides corresponding to the 15 N-terminal aa of PB1 (pPB1\text{N}), we determined
that PA did not bind to pPB1L8D, whereas binding was observed to both pPB1DRN and pPB1DRV, although with significantly lower affinity than pPB1WT (Fig. 2B). To test whether the pseudo-reversion affects the formation of PA–PB1 dimers, we performed co-immunoprecipitation studies. Although PB1DRN weakly interacted with PA, no interaction was detected between PB1L8D and PA (Fig. 2C). In contrast, complex formation between PA and PB1DRV was comparable with that of PA and WT PB1 (Fig. 2C). When co-immunoprecipitation was performed with all three polymerase subunits, both PB1DRN and PB1DRV supported trimeric polymerase complex assembly, whereas PB1L8D did not (Fig. 2D). Consistent with earlier observations (14), lack of trimeric polymerase assembly was accompanied by reduced levels of PB2 (Fig. 2D, cell extract). We speculate that the reduced levels of PB2 are the result of an enhanced degradation of the unbound PB2. To better understand the reason for the diminished binding, we modeled the PA-binding domain of PB1L8D, PB1DRV, and PB1DRN into the PA–PB1 crystal structure. Within the hydrophobic groove of PA (12, 20), Leu-8 plays a key role in protein binding affinity by mediating contacts to Ile-621, Val-636, and Leu-640 (Fig. 2E). Most strikingly, mutation to L8D causes total affinity loss by repulsion of the charged amino acid from this non-polar region (Fig. 2F). L8N still places hydrophilic chemical functionalities unfavorably in this binding groove but may establish a hydrogen-bond interaction with Pro-620 that likely increases the binding affinity (Fig. 2G). In contrast, the D8V mutation apparently causes a reduction of the hydrophobic interaction area due to its smaller dimensions when compared with 8L (Fig. 2H), resulting in weaker binding but not in a complete loss of binding affinity.

Molecular Modeling Reveals Attenuating Mutations in the PA-Binding Site of PA—To test whether mutations in the PA-binding domain of PA have the potential to cause attenuation of influenza virus, we mutated the highly conserved tryptophan at position 706 (Fig. 3A) to either glutamine (PAW706E) or arginine (PAW706R). Molecular modeling suggests that the negatively charged carboxylate moiety of W706E will attract Lys-643 of PA to form a salt bridge (Fig. 3B). This mutation abrogated binding of PA to PB1 in a biochemical pulldown assay (12). We also expected that the mutation W706R would abrogate the interaction with PB1 because W706R positions a positively charged group in the close vicinity of Lys-643 of PA, resulting in the destabilization of the PA protein conformation (Fig. 3C). Both mutants, PAW706E and PAW706R, failed to bind to peptides comprising the N-terminal 15 aa of WT PB1 (Fig. 3D). When compared with WT PA, the polymerase activity was reduced by ~90% for PAW706E and ~98% for PAW706R (Fig. 3E). SC35M coding for PAW706E could be generated by reverse genetics without further compensatory mutations in PA (Fig. 3F) or both PB1 and PB2 (Table 1). As expected, this mutant virus was significantly attenuated by 2.5 log10 24 h.p.i. in MDCK (Fig. 3G) and A549 cells (supplemental Fig. S1B). Attempts to rescue SC35M-PAW706R revealed a pseudo-reversion (PAW706G) (Fig. 3F), which was barely impaired in viral growth in MDCK cells (Fig. 3G) and attenuated by 2 log10 24 h.p.i. in A549 cells (supplemental Fig. S1B). To test the feasibility of generating viruses harboring mutations in both PB1 and PA, we tried to rescue a virus coding for both PAW706E and PB1DRN. In one rescue attempt, we obtained a virus mutant harboring a reversion to WT PB1 and no changes in PA (Table 1). In a second independent rescue attempt, we obtained a virus mutant (SC35M-PAW706E PB1L8I) harboring a mutation in PB1 to N8I without changes in PA (Fig. 3H), which displayed considerably impaired growth in MDCK (Fig. 3I) and A549 cells (supplemental Fig. S1C) with 3–4 log10 titer reduction 24 h.p.i., corresponding to a small plaque phenotype of SC35M-PAW706E PB1L8I when compared with WT virus (supplemental Fig. S4). Thus, virus mutants with aa changes in both PB1 and PA can be generated and show impaired growth properties.

Polymerase Assembly Mutants Are Attenuated in Mice—To evaluate the degree of attenuation of our mutant viruses in vivo, we infected BALB/c mice with SC35M mutants. Following intranasal infection, we monitored for weight loss and survival and determined viral lung titers. Although WT-infected mice succumbed to death following infection with 10⁵ pfu, all mice infected with 10⁴ pfu of SC35M-PB1DRN survived despite an initial weight loss (Fig. 4A). Consistent with the lack of attenuation in cell culture, infection with SC35M-PB1DRV was as pathogenic as WT SC35M (supplemental Fig. 5). Mice infected with 10⁵ or 10⁴ pfu of SC35M-PAW706E (Fig. 4B) or SC35M-PAW706E PB1L8I (Fig. 4C) survived with marginal reduction of body weight, whereas at 10⁵ pfu, 25 and 37.5% of the animals survived infection with SC35M-PAW706E and SC35M-PAW706E PB1L8I, respectively. The degree of attenuation observed with the polymerase assembly mutant viruses correlates with decreased viral lung titers (Fig. 4D).

To demonstrate that our findings apply to other influenza A viruses, we introduced the W706E mutation in PA of the highly pathogenic H5N1 virus A/Thailand/(KAN-1)/2004 (clade 1) (21). When compared with infection with WT KAN-1, this mutant virus (KAN-1-PAW706E) was attenuated by 4 log10 24 h.p.i. in MDCK cells (supplemental Fig. 1D) and showed an increase in LD50 of ~10,000-fold (Fig. 4E, data not shown) as well as significantly lower lung titers (5 log10) in BALB/c mice (Fig. 4F).

To determine the safety of our mutant viruses, we infected IFNAR−/− mice, which are highly susceptible to virus infection (17, 22, 23), with up to 10⁹ pfu of the attenuated strain SC35M-PAW706E or 10 pfu of WT virus. All SC35M-PAW706E-infected mice survived, whereas all animals infected with WT virus succumbed to infection (Fig. 4G). At an infection dose of 10⁴ pfu, 66% of the SC35M-PAW706E-infected mice survived. As expected, the viral lung titer of mice infected with 10⁴ pfu SC35M-PAW706E for 48 h was at least 1000-fold reduced when compared with WT-infected mice (Fig. 4H).

Polymerase Assembly Mutants Remain Attenuated after Passing in Cell Culture and Mice—To determine whether the polymerase assembly mutants can easily revert to WT, we performed three serial lung passages of SC35M-PB1DRN, SC35M-PAW706E, and SC35M-PAW706E PB1L8I in BALB/c mice. No changes were observed in PA, whereas mutations to either V92M or V92L outside the PA-binding domain (12, 13,
FIGURE 3. Mutations in the PB1-binding site of PA affect viral growth properties. A–C, Trp-706 contributes to PA-PB1 binding by formation of surface contacts with PB1 Asn-4 and PB1 Pro-5 to PA. A, a model of these contacts as in Fig. 2H. The surface of PA around Trp-706 is shown in mesh style, and the surface of PB1 is shown in opaque style, respectively. B, the negatively charged carboxylate moiety introduced by the PA W706E mutation may attract PA Lys-643 to form a salt bridge (wild-type model in gray; conformation predicted for the mutant in green). C, the positive charge introduced by the W706R mutation is likely to have a repulsive effect due to the positive charge of Lys-643. D, determination of the PA binding affinities of the PA mutants by ELISA using cell extracts containing mutant PA-HA. OD, optical density; 706E, W706E; 706R, W706R. E, determination of the FluA polymerase activity in the presence of the indicated PA mutants. PA−, omission of PA. F, rescue of SC35M mutants coding for PA mutants. The arrow indicates the site of polymorphism. 706G, W706G. G, viral growth of PA mutant viruses in MDCK cells. H, rescue of a SC35M mutant with mutations in PA and PB1. 8N, D8N; 8I, L8I. I, viral growth curve of SC35M-PAW706EPB1L8I in MDCK cells.
19) appeared in PB1 of SC35M-PAW706E and SC35M-PAW706EPB1L8I, respectively (Fig. 5, A and B). Infection of IFNAR0/0 mice with 100 pfu of SC35M-PAW706E passaged three times in mice resulted only in a transient weight loss (Fig. 5C), demonstrating that this virus was still strongly attenuated. Furthermore, serially passaging SC35M-PAW706E and SC35M-PAW706EPB1L8I 10 times in MDCK cells also led to a mutation to PB1V92M (Fig. 6, A and B), as well as PAT639A. Importantly, growth of both passaged viruses remained impaired in MDCK cells (Fig. 6C). Together, these results indicate that the polymerase assembly mutants can acquire mutations in the viral genome during the passages in mice or cell culture but that these mutations do not reverse their attenuated phenotype. Therefore, the polymerase assembly mutants are phenotypically stable.

**DISCUSSION**

The objective of this study was to generate attenuated influenza A viruses based on defects in the assembly of the polymerase complex. Accordingly, we reasoned that alteration of key amino acids involved in the subunit interactions would force the virus to escape by acquisition of new mutations, some of which would fail to restore efficient polymerase complex assembly and activity. Indeed, the majority of the rescued mutant viruses represented pseudo-revertants with various degrees of attenuation. Only a few intentionally introduced mutations (e.g., PB1L10D,K11D and PAW706E) were tolerated and, as predicted, recombinant viruses expressing these proteins were attenuated. However, the occurrence of compensatory mutations at other aa positions in

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**FIGURE 4.** Polymerase assembly mutants are attenuated in mice. A–C, survival (right) and weight loss (left) of 6–8-week-old female BALB/c mice (n = 6/group) after intranasal infection with the indicated doses of SC35M mutant viruses encoding PB1D8N (PB18N) (A), PAW706E (PA706E) (B), or both PAW706E and PB1L8I (PB18I) (C). WT, wild-type virus SC35M. D, BALB/c mice (n = 5) were infected with 10^7 pfu of the indicated virus mutants. 48 h p.i., lung titers were determined by plaque assay. E, weight loss and survival of 6–8-week-old female BALB/c mice (n = 10/group) after intranasal infection with the indicated doses of KAN-1 or KAN-1-PB1L8I. F, lung titers in mice (n = 5) infected with 10^3 pfu of either KAN-1 or KAN-1-PB1L8I at 48 h p.i. G, weight loss and survival of 8–10-week-old IFNAR0/0 mice (n = 7/group) infected with the indicated doses of WT virus or SC35M-PAW706E. H, lung titer in IFNAR0/0 mice (n = 3) infected with 10 pfu of either WT or SC35M-PAW706E at 48 h p.i.

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**FIGURE 5.** Characterization of polymerase assembly mutants after serial passages in mice. A, mutations in PB1 and PA observed after serial passages of the indicated virus mutants in BALB/c mice. mP1–3, mouse passage 1–3; mouse passage 1–3; mouse passage 1–3. B, electropherogram of PB1 and PA sequences after serial passages of the indicated viruses in BALB/c. The arrows indicate the site of mutation or polymorphism. SC35M-PB18N, SC35M-PB1D8N, SC35M-PAW706E. C, survival and weight loss of 8–10-week-old IFNAR0/0 mice (n = 6) infected with the SC35M-PAW706E passaged three times in mice (PA706E (mP3)).

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**FIGURE 6.** Characterization of polymerase assembly mutants after serial passages in cell culture. A, mutations in PB1 and PA observed after serial passages of the indicated virus mutants in MDCK cells. cP5 and cP10, cell passage 5 and 10. B, electropherogram of PB1 and PA sequences after serial passages of the indicated viruses in MDCK cells. The arrows indicate the site of mutation or polymorphism. SC35M-PAW706E, SC35M-PAW706EPB18I, SC35M-PAW706EPB1L8I. C, viral growth of the indicated mutant viruses in MDCK cells.
PA or PB1 was not observed. This strongly suggests that only few aa exchanges are tolerated within these binding domains without loss of affinity. The lack of compensatory mutations especially in the relatively small core PA-binding domain might be caused by the involvement of several aa of the 310 helix in mediating contact to PA (12, 13, 19).

The amino acids within the PA-binding domain of PB1 (e.g. Leu-8) and the PB1-binding domain of PA (e.g. Trp-706), which are essential for polymerase subunit interaction, are highly conserved and identical among all known influenza A virus strains (12–14, 24). We therefore infer that targeting these key residues results in attenuation of virtually all influenza A virus strains. As exemplified by two unrelated viruses, the highly pathogenic H5N1 strain KAN-1 and the mouse-adapted strain SC35M (H7N7) mutation in PA (W706E) resulted in severe attenuation of both viruses in mice. However, the degree of attenuation is higher for the H5N1 mutant virus. This might reflect differences in intrinsic PA/PB1 binding affinities between these virus strains, as described recently for fluA and influenza B viruses (14).

We also observed that targeting different key residues within the PA- or PB1-binding domains could vary the degree of attenuation because the mutation L8N in PB1 resulted in a less attenuated phenotype of SC35M in cell culture and mouse models when compared with the W706E mutation in PA or a combination of both mutations. The possibility to fine-tune the individual degree of attenuation by defined substitutions in PA or PB1 represents a unique tool to avoid over-attenuation. The repertoire of suitable mutations could possibly be extended to the similarly conserved PB2-PB1-binding site (11).

The polymerase assembly mutant viruses are also drastically attenuated in mice with immune system defects, as demonstrated in IFNAR0/0 mice, which are highly susceptible to virus infection (17, 22, 23). We attribute this observation to the host-independent polymerase assembly defect of these viruses, which forces attenuation under all conditions. Our findings suggest that live vaccines harboring these mutations should possess a good safety profile in patients with impaired antiviral defense mechanisms.

Several reasons might account for the genetic stability of the polymerase assembly mutants in mice and cell culture. The observed reduction in growth kinetics of these mutants further decreases the population size and thus the likelihood that viable escape mutants will emerge. Furthermore, the PB1/PA-binding interface is highly conserved and cannot tolerate many aa changes without reducing the binding affinity (12–14, 24). Thus, we speculate that point mutations, which cause reversions to wild-type polymerase activity, are unlikely.

Live attenuated viruses confer several advantages as vaccines over traditionally used inactivated viruses, including induction of cellular as well as humoral immunity, broader protection among influenza virus subtypes, and better protection in children (3). However, the currently used cold-adapted influenza vaccine (FluMist) is not approved for children under the age of two or immunocompromised patients (3). Our attenuation strategy might contribute to a better safety profile for these viruses, as demonstrated by infection of IFNAR0/0 mice. Furthermore, the cold-adapted master strain A/AnnArbor/6/60 H2N2 contains a fixed degree of attenuation (3), whereas we have developed a strategy to easily identify new mutations, which allow attenuation to be fine-tuned.

Overall, generation of polymerase assembly mutants can be applied to all influenza A viruses, resulting in adjusted degrees of attenuation. Thus, this novel approach may also contribute to the development of phenotypically stable live vaccines against seasonal and pandemic influenza. Furthermore, this attenuation strategy could also be used to make successful reassortment between attenuated live vaccines and circulating influenza A viruses less probable. Such reassortment events are of great concern because the resulting viruses might have an increased pathogenicity. By targeting a protein-protein interaction domain encoded on two segments, exchange of only one segment should maintain attenuation. However, our mutations could be combined with several other attenuation strategies, which have been described (3, 25–28).

We provide proof of principle that disruption of a protein-protein interaction site essential for polymerase complex formation results in an attenuation of influenza viruses. Thus, targeting the interaction domains of viral proteins that are essential for productive infection may represent a universal approach that could be applied to many other viruses.

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