Immunization of pigs with an attenuated pseudorabies virus recombinant expressing the haemagglutinin of pandemic swine origin H1N1 influenza A virus

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Received 11 September 2013
Accepted 15 January 2014

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Pigs can be severely harmed by influenza, and represent important reservoir hosts, in which new human pathogens such as the recent pandemic swine-origin H1N1 influenza A virus can arise by mutation and reassortment of genome segments. To obtain novel, safe influenza vaccines for pigs, and to investigate the antigen-specific immune response, we modified an established live-virus vaccine against Aujeszky’s disease of swine, pseudorabies virus (PrV) strain Bartha (PrV-Ba), to serve as vector for the expression of haemagglutinin (HA) of swine-origin H1N1 virus. To facilitate transgene insertion, the genome of PrV-Ba was cloned as a bacterial artificial chromosome. HA expression occurred under control of the human or murine cytomegalovirus immediate early promoters (P-HCMV, P-MCMV), but could be substantially enhanced by synthetic introns and adaptation of the codon usage to that of PrV. However, despite abundant expression, the heterologous glycoprotein was not detectably incorporated into mature PrV particles. Replication of HA-expressing PrV in cell culture was only slightly affected compared to that of the parental virus strain. A single immunization of pigs with the PrV vector expressing the codon-optimized HA gene under control of P-MCMV induced high levels of HA-specific antibodies. The vaccinated animals were protected from clinical signs after challenge with a related swine-origin H1N1 influenza A virus, and challenge virus shedding was significantly reduced.

INTRODUCTION

The 2009 pandemic H1N1 swine origin influenza viruses (SoIVs) belong to the genus Influenzavirus A within the family Orthomyxoviridae (King et al., 2012). Influenza A viruses possess segmented, negative-strand RNA genomes, and are highly variable with respect to sequence and antigenic properties of their envelope glycoproteins haemagglutinin (HA) and neuraminidase (NA). They cause respiratory and general diseases in many mammalian and bird species, but aquatic birds are generally considered as natural hosts in which infections are mostly asymptomatic (Palese & Shaw, 2007). However, pigs also represent important reservoir hosts harbouring subtypes H1N1, H3N2 and H1N2 (Van Reeth, 2007). Because pigs are susceptible to both avian and mammalian influenza viruses, they may function as mixing vessels for the development of new human pathogens by reassortment of genome segments from different viruses (Webster et al., 1993). Obviously, the reassortments and mutations resulting in the recent pandemic H1N1 SoIV also occurred in swine before the virus became efficiently transmissible to and between humans (Garten et al., 2009; Furuse et al., 2010; Smith et al., 2009).

To prevent further development and spread of zoonotic viruses in the pig population, swine influenza should be controlled more thoroughly. Due to the mostly moderate course of disease and the considerable costs of inactivated vaccines, pigs are still not generally immunized. Moreover, the available porcine vaccines containing mixtures of inactivated H1N1, H1N2 and H3N2 subtype influenza viruses do not reliably confer protection, in particular if the challenge virus HA is not closely related to that contained in the vaccine (Ma & Richt, 2010; Van Reeth, 2007). Thus, besides the humoral immune response induced by inactivated vaccines, cell-mediated and mucosal responses might be required for establishment of a solid and broad immunity. To overcome these problems, live-attenuated vaccines possessing attenuating mutations, e.g. in non-structural proteins, the viral RNA polymerase or the HA cleavage site, have been developed (Babiuk et al., 2011; Pena et al., 2011; Vincent et al., 2011).
et al., 2012). Although the protective efficacy of these vaccine candidates is promising, they bear risks caused by the high mutation and recombination potential of influenza viruses.

Therefore, vectored live-virus vaccines containing only single or few influenza virus genes might be safer alternatives. Furthermore, vectored vaccines facilitate epidemiological surveillance, since they support serological differentiation of naturally infected from vaccinated animals (DIVA) (van Oirschot, 1999) by testing for the presence or absence of serum antibodies against influenza virus antigens which are not contained in the vaccine. Vectored vaccines based on attenuated fowlpox virus, Newcastle disease virus or avian herpesviruses, and usually expressing HA, are efficacious against highly pathogenic avian influenza viruses (HPAIVs) (Li et al., 2011; Pavlova et al., 2009; Taylor et al., 1988; Veits et al., 2006) and are already used in practice. Accordingly, a live attenuated porcine virus might be a promising vector for vaccines against swine influenza.

Pseudorabies virus (PrV) or Swine herpesvirus 1 (SuHV-1) is a member of the subfamily Alphaherpesvirinae of the family Herpesviridae within the order Herpesvirales (King et al., 2012). Like influenza viruses, herpesviruses produce enveloped particles containing viral glycoproteins, but possess dsDNA genomes. PrV causes Aujeszky's disease, which leads to abortions in sows and mortality of piglets. Furthermore, PrV causes fatal infections in many other mammalian species excluding higher primates and humans. For control and eventual eradication, attenuated PrV live-virus vaccines with defined gene deletions have been developed and used to eliminate PrV infections in domestic pigs in parts of Europe and North America (Mettenleiter, 2000; Pomeranz et al., 2005).

The use of attenuated PrV strains as viral vectors for the expression of antigens of other viral and non-viral pathogens has been reported repeatedly, and the induction of both humoral and cell-mediated immune responses against the foreign proteins, as well as protective efficacy have been demonstrated (Jiang et al., 2007; Qian et al., 2004; Sedegah et al., 1992; Thomsen et al., 1987b; van Zijl et al., 1991; Wei et al., 2010). The antigens previously expressed in PrV vectors also include H3 HA of a swine influenza virus, but protective efficacy of the respective virus recombinant has so far only been shown in a mouse model (Tian et al., 2006). Another animal alphaherpesvirus, equine herpesvirus 1 (EHV-1), has been used as a viral vector for the expression of the HA gene of pandemic H1N1 SoIV and shown to protect mice, and partially also pigs against challenge infections (Said et al., 2011, 2013).

We attempted to generate vectored influenza vaccines specifically targeted to immunization of pigs. To this end, we used the established PrV vaccine strain Bartha (PrV-Ba) (Bartha, 1961), which is fully sequenced and stably attenuated by multiple mutations, including a large deletion encompassing the genes encoding three membrane proteins, of which the highly immunogenic glycoprotein E (gE) is suitable for DIVA diagnostics (Lomniczi et al., 1987; Szpara et al., 2011; van Oirschot, 1999). To facilitate the use of PrV-Ba as a vector, its genome was cloned as a bacterial artificial chromosome (BAC) (Fig. 1a). The HA transgene was obtained from pandemic H1N1 SoIV A/Regensburg/D6/2009 (Lange et al., 2009). To enhance its expression, different promoters, intron insertions and codon-optimization were evaluated. The in vitro replication properties of the obtained PrV recombinants were analysed. To assess protective efficacy, pigs were infected with HA-expressing PrV, and the induction of HA-specific antibodies, together with protection against challenge infection with the related pandemic H1N1 SoIV A/California/7/09 (Garten et al., 2009), were investigated. In particular, the effect of vaccination on influenza virus shedding was examined, since it correlates with the risk of transmission to other animals or humans.

RESULTS

Optimization of transient HA expression

The reverse-transcribed ORF encoding the HA of pandemic SoIV A/Regensburg/D6/2009 was first cloned into a eukaryotic expression vector under control of the human cytomegalovirus immediate-early promoter (P-HCMV) (Fig. 1b). However, transient HA expression from pcDNA-sirH1 appeared very weak in indirect immunofluorescence (IF) tests of transfected rabbit kidney (RK13) cells (not shown), and was undetectable in Western blot analyses (Fig. 2) using a monospecific antiserum (rabbit α-GST-H1). To enhance protein expression, a synthetic intron (IVS) was inserted within the 5′-non-translated part of the transcription unit (Fig. 1b), and the promoter was replaced by the homologous immediate early promoter of murine cytomegalovirus (P-MCMV). Both modifications led to a successive increase of HA expression, and proteins with an apparent molecular mass of approximately 75 kDa and also smaller precursors or degradation products became clearly visible in Western blot analyses of transfected cell lysates (Fig. 2). In addition, a synthetic HA gene (synH1) was cloned in which codon usage had been adapted to that of the prospected viral vector PrV without affecting the encoded amino acid sequence. Interestingly, this modification again significantly enhanced HA expression in transfected mammalian cells (Fig. 2).

Generation of HA-expressing PrV mutants

To investigate the effects of promoter and codon usage on HA expression in a PrV vector, four different virus recombinants were generated containing the authentic sirH1 ORF, or the synthetic synH1 ORF of SoIV A/Regensburg/D6/09 under control of P-HCMV or P-MCMV, respectively. The 5′-IVS was present in all constructs. The nonessential gG gene of PrV (Thomsen et al., 1987a) was chosen as the insertion site and provided the polyadenylation signal

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required for foreign gene expression (Fig. 1a). The viral vector was based on PrV-Ba which had been cloned as a BAC harbouring the F-plasmid replicon, and an EGFP expression cassette within the gG gene (Fig. 1a). This insertion contained two EcoRI restriction sites which were unique in the virus genome. Therefore, EcoRI digestion of pPrV-BaDgGG DNA prior to cotransfection of RK13 cells together with HA transfer plasmids derived from pUC-BaKJPHI or pUC-BaKJPMI promoted formation of the desired non-fluorescent virus mutants PrV-BaHI-sirH1, PrV-BaMI-sirH1, PrV-BaHI-synH1 and PrV-BaMI-synH1 by homologous recombination (Fig. 1a). Absence of gG expression in cells infected with pPrV-BaDgGG and derived virus recombinants was confirmed by Western blot analyses (Fig. 3a, lower panel).

To evaluate HA expression, RK13 cells infected with the PrV recombinants were analysed by Western blotting and IIF tests (Fig. 3). An antiserum from an H1N1 SoIV-infected pig showed specific IIF reactions with cells infected with either of the virus mutants, but not with PrV-Ba-infected or uninfected cells (Fig. 3b). Obviously, the PrV-adapted synH1 gene was much more abundantly expressed than the authentic sirH1 gene, and P-MCMV induced moderately enhanced expression compared to P-HCMV. In Western blot analyses using the α-GST-H1 serum, the sirH1 products were detectable only after overexposure, whereas the synH1 products appeared as abundant proteins of approximately 75 kDa (Fig. 3a, upper panel). In contrast, uncleaved (gBa) and furin-cleaved (gBb, gBc) forms of PrV-gB (Whealy et al., 1990) were detected in all
infected cells at comparable amounts (Fig. 3a, middle panel). Since PrV-BaMI-synH1 containing the codon-optimized HA ORF under control of P-MCMV exhibited the most abundant expression, this mutant was used in all subsequent experiments.

The PrV-expressed HA showed a similar molecular mass as the uncleaved precursor HA$_0$ in SoIV virions (Fig. 3a, upper panel). However, the approximately 60 kDa N-terminal cleavage product HA$_1$, which was predominant in influenza virus particles, was not detectable in PrV-infected RK13 cells, indicating that the required trypsin-like proteases are not expressed in these cells. The C-terminal HA fragment HA$_2$ starting at amino acid 345 was not detectable by the antiserum, because it was absent from the bacterial fusion protein used for rabbit immunization (Fig. 1b).

**Influenza virus HA is not incorporated into PrV virions**

Since HA is a major envelope glycoprotein of influenza virus particles, it was investigated whether it is also incorporated into PrV virions. Interestingly, the PrV-expressed HA was undetectable in Western blot analyses of purified virions of PrV-BaMI-synH1 with the z-GST-H1 serum, although HA was abundantly expressed in RK13

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**Fig. 2.** Transient HA expression. RK13 cells were transfected with the indicated HA expression plasmids and lysed after 48 h. Cells infected with PrV-BaMI-synH1 were used as positive controls. Proteins were separated by SDS-PAGE. Western blot reactions of the rabbit-α-GST-H1 serum are shown. Molecular mass markers are indicated.

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**Fig. 3.** HA expression of PrV recombinants. (a) For Western blotting cells were lysed 20 h after infection (m.o.i. of 2) with the indicated viruses, and proteins were separated by SDS-PAGE together with virion proteins of H1N1 SoIV A/Regensburg/D6/09. Identical blots were probed with z-GST-H1, z-PrV-gB or z-PrV-gG sera. Molecular mass markers and locations of specifically detected proteins are indicated. (b) For IIF tests RK13 cells were fixed 2 days after PrV-infection (m.o.i. of <0.001). HA expression was detected using an H1N1 SoIV-specific swine serum and fluorescein-conjugated secondary antibodies.
cells infected with the same virus (Fig. 4 upper panel). In contrast, the major PrV envelope protein gB was enriched in virions of PrV-BaMI-synH1 and PrV-Ba (Fig. 4, second panel). Purity of the analysed virions was demonstrated by the absence of viral membrane protein pUL34 (Fig. 4, lower panel), which is found in primary enveloped, but not in mature PrV particles (Klupp et al., 2000). Immunoelectron microscopy confirmed that the influenza virus protein is not detectably incorporated into virion envelopes of the PrV recombinant (not shown).

**In vitro replication of HA-expressing PrV**

Previous studies have shown that deletion of gG does not affect PrV-replication in cell culture (Thomsen et al., 1987a). To investigate whether insertion or expression of the HA transgene at this locus has an effect on replication properties, one-step growth kinetics and plaque sizes of PrV-BaMI-synH1 and parental PrV-Ba were compared in RK13 cells (Fig. 5). Replication of PrV-BaMI-synH1 was slightly delayed, but led to similar final virus titres as PrV-Ba (Fig. 5a). Consistently, cell-to-cell spread of the HA-expressing mutant was also slightly affected, leading to a reduction of plaque diameters by approximately 20% compared with plaques of PrV-Ba (Fig. 5b). Similar results were obtained with different plaque isolates of PrV-BaMI-synH1, indicating that abundant HA expression might interfere with expression of PrV genes required for replication. Nevertheless, the moderate defects should not complicate production of a respective live-virus vaccine.

**Immunization of pigs with PrV-BaMI-synH1 induces HA-specific antibodies**

To test the suitability of PrV-BaMI-synH1 as live-virus vaccine against pandemic H1N1 SoIV, 7-week-old piglets were infected intranasally with this virus recombinant, whereas control animals were immunized with PrV-Ba or not vaccinated. After 2 weeks, haemagglutination inhibition (HI) assays demonstrated HA-specific serum antibodies at titres of between 40 and 640 HI units in all animals vaccinated with PrV-BaMI-synH1, and one piglet developed detectable antibodies as early as 1 week after immunization (Fig. 6). As expected, none of the control animals showed positive reactions. However, a challenge with pandemic H1N1 SoIV A/California/7/09 performed 3 weeks after PrV-infection induced similar levels of HA-specific serum antibodies in both vaccinated groups, whereas naïve pigs developed lower antibody titres after primary SoIV infection (Fig. 6). Thus, vaccination with PrV-Ba may have caused an unspecific stimulation of the immune system. Remarkably, the maximum HI titres observed 2 weeks after challenge were only slightly higher than those achieved by vaccination with PrV-BaMI-synH1.

**Vaccination with HA-expressing PrV protects pigs and reduces influenza challenge virus shedding**

After vaccination with PrV-BaMI-synH1 or PrV-Ba, and after challenge infection with SoIV A/California/7/09 3 weeks later, the piglets were investigated daily for clinical signs. Significantly increased body temperatures were not observed throughout the entire trial, and no indications of disease were found after primary infection with the PrV vaccines. From days 2 to 7 after influenza virus infection, the naïve controls and the animals previously immunized with PrV-Ba developed moderate clinical symptoms such as epistaxis and sneezing, and single pigs showed swollen eyes or conjunctivitis. The pigs vaccinated with PrV-BaMI-synH1 developed only minimal respiratory symptoms (sporadic sneezing).

To investigate the effect of immunization on influenza virus shedding, nasal swabs of all animals were collected before and until day 10 after challenge infection. Viral RNA was isolated and analysed by real-time reverse transcriptase (RT)-PCR. As indicated by higher cycle threshold ($C_t$)
values, the viral loads of animals vaccinated with PrV-BaMI-synH1 were significantly lower than those of the control piglets (Fig. 7a). Whereas nasal swabs of all control animals contained SoIV RNA until day 6 after challenge, only four of the PrV BaMI-synH1 vaccinated pigs were positive until day 4, and subsequently the proportion of positive animals decreased more rapidly than in the control groups (Fig. 7a). These results were confirmed by titration of the shed SoIV on Madin–Darby canine kidney (MDCK) cells (Fig. 7b), although sensitivity of this method was slightly lower than that of PCR.

Two weeks after challenge, gross pathology and histopathologic analysis revealed no lesions of the lung or of the upper respiratory tract, either in vaccinated or in control animals (results not shown).

**DISCUSSION**

Swine influenza is a relevant respiratory disease of pigs causing considerable economic losses. Nevertheless, no stringent vaccination programmes have been developed to date. However, the recent human pandemic caused by H1N1 SoIV highlighted again that swine are an important reservoir host which can function as a mixing vessel for evolution of new zoonotic influenza viruses (Garten et al., 2009). Since improved influenza vaccines for swine are required, we selected an attenuated PrV live vaccine as porcine virus vector for the expression of influenza virus proteins.

PrV-Ba and similar vaccine strains have been successfully used to control and eliminate Aujeszky’s disease in domestic pigs in several countries (Mettenleiter, 2000; Pomeranz et al., 2005). Nevertheless, PrV is still prevalent in wild boar (Müller et al., 2011) and an important pathogen of domestic pigs in major parts of the world. Therefore, PrV vectors could be used as bivalent vaccines to protect swine against Aujeszky’s disease, and influenza for example, without raising labour requirements and costs. The suitability of PrV as a viral vector for foreign proteins has been shown previously; in one study PrV has already been used for expression of the HA of an H3N2 swine influenza virus (Tian et al., 2006). Whereas in this study investigation of protective efficacy was restricted to mice, we now demonstrate for the first time efficacy of a PrV-based vector vaccine against swine influenza in the natural host of both pathogens.

Since HA is known as the most immunogenic protein of influenza A viruses (Palese & Shaw, 2007), we attempted to express the HA of the pandemic H1N1 SoIV Regensburg/D6/09 in the PrV vector. Unfortunately, the authentic reverse-transcribed HA gene was barely expressed from plasmid constructs containing the strong HCMV immediate-early promoter-enhancer complex and a proper polyadenylation signal. This correlated with the described
inefficient expression of the authentic HA gene of another H1N1 SoIV isolate from plasmids designed for DNA vaccination (Tenbusch et al., 2010). Similar to expression of the HA gene of an H5N1 HPAIV (Pavlova et al., 2009), H1 expression was enhanced by insertion of a synthetic intron into the 5′-non-translated part of the transcription unit, confirming that mRNA splicing can influence transcription efficiency, stability and nuclear export of transcripts, and subsequent translation rates (Le Hir et al., 2003). An additional, although moderate, increase in HA expression could be achieved by substitution of the HCMV promoter by the homologous promoter of MCMV. However, after insertion of the corresponding expression cassettes into the PrV genome, only minor amounts of HA were detected in infected cells. To improve this inefficient expression, codon usage was adapted in a synthetic HA gene (synH1) to that of PrV which is characterized by a strong preference of third position C or G nucleotides (Klupp et al., 2004). After insertion into the PrV genome synH1 proved to be expressed at substantially higher levels than the authentic gene, which might be explained, for example, by increased resistance of the mRNA against degradation by the herpesvirus host-shutoff RNase (Shu et al., 2013). However, enhanced HA expression was also observed in cells transfected with synH1 expression plasmids, indicating virus-independent effects such as reduced competition with cellular transcripts for certain tRNAs. Furthermore, since influenza virus mRNAs are

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**Fig. 7.** Viral load in nasal swabs. Naïve pigs and animals immunized with the indicated PrV mutants were challenged with H1N1 SoIV A/California/7/09. (a) Total RNA from nasal swabs collected before (0) and 1–10 days after challenge was analysed by real-time RT-PCR for the influenza virus M gene. (b) Infectious virus titres in the swabs samples were determined on MDCK cells. Mean C\textsubscript{T} and TCID\textsubscript{50} values, standard deviations, together with the numbers of virus shedding animals are given. Asterisks indicate statistically significant differences between vaccinated and control animals (*P<0.05, **P<0.01, ***P<0.001).
transcribed by viral enzymes (Palese & Shaw, 2007), it is also conceivable that the authentic HA gene contains yet unidentified sequence elements interfering with expression by the cellular transcription machinery (which is also utilized by herpesviruses), and that these elements were incidentally removed by codon optimization.

Despite abundant expression of the codon-optimized HA in cells infected with the respective PrV mutants, it was not detectable in purified virions. Thus, although HA, like most envelope proteins of PrV, represents a type I integral membrane glycoprotein, it is obviously not targeted to or retained at the site of final herpesvirus envelopment, which is at Golgi-derived vesicles in the cytosol (Mettenleiter, 2002). In contrast, influenza virus particles are considered to bud at the plasma membrane (Palese & Shaw, 2007), and therefore their envelope proteins should accumulate at the surface of infected cells. In accord with our present findings, the H5 type HA, and the N1 type NA of an HPAIV were also not incorporated into particles of vectored vaccines based on the avian alphaherpesvirus, infectious laryngotracheitis virus (ILTV) (Pavlova et al., 2009).

Nevertheless, vaccination of chickens with HA-expressing ILTV conferred solid protection against HPAIV-infection (Pavlova et al., 2009). Concordantly, a single intranasal infection of swine with PrV-BaMI-synH1 induced HA-specific serum antibodies at levels similar to those observed after infection with a corresponding influenza virus. Thus, the HA expression in cells infected with the vectored live-virus vaccines was sufficient to induce a pronounced humoral immune response.

As expected, infection of pigs with the licensed live-virus vaccine PrV-Ba, or with the derived recombinant PrV-BaMI-synH1 did not cause any clinical signs of Aujeszky’s disease. Unlike PrV-Ba-vaccinated animals, the pigs immunized with PrV-BaMI-synH1 were protected against disease induced by challenge with H1N1 SoIV A/California/7/09. Moreover, vaccination with PrV-BaMI-synH1 significantly reduced the amount and duration of challenge virus replication and shedding. Due to the generally moderate courses of pandemic H1N1 SoIV infections in swine (Lange et al., 2009), precise comparison of the protective efficacies of different vaccine candidates is difficult unless they are tested in parallel experiments. However, in published studies, neither licensed inactivated swine influenza vaccines, nor homologous HA gene-based DNA- or vectored vaccines completely abolished clinical or pathological signs and productive replication of 2009 pandemic H1N1 SoIV isolates in challenged pigs (Gorres et al., 2011; Said et al., 2013; Vincent et al., 2010). Only an experimental, homologous, inactivated vaccine prevented nasal shedding of detectable amounts of infectious challenge virus (Vincent et al., 2010). Compared to an HA-expressing EHV-1 recombinant (Said et al., 2013), PrV-BaMI-synH1 reduced challenge virus RNA replication more efficiently as indicated by higher Ct values and lower proportions of positive swine at similar times after challenge. Presumably, the PrV-based vaccine replicated more efficiently in swine and thus produced more HA than the equine herpesvirus.

Although a single live-virus vaccination of pigs with H1 type HA-expressing PrV conferred protection against challenge with a heterologous but closely related H1N1 SoIV isolate, further improvement of efficacy is desirable, in particular with respect to challenge virus shedding. It has been reported that efficacy of inactivated swine influenza vaccines requires a high degree of identity to the HA of challenge virus (Ma & Richt, 2010; Vincent et al., 2010). In this study the incomplete inhibition of challenge virus replication was unlikely to have been due to antigenic differences between the HA proteins expressed by the vaccine and challenge virus, since they differ at only two amino acids. At position 100 SoIV A/California/7/09 contains a proline instead of serine, and at position 339 isoleucine substitutes for valine present in SoIV Regensburg/D6/09. However, if required, our BAC-based PrV vector permits an easy update of the transgene within a few weeks, and multiple insertions of several HA genes are also feasible.

Other prospects for improvement of the protective immune response are prime–boost vaccinations of the animals, optionally combined with intramuscular administration, which does not induce local mucosal responses in the respiratory tract but results in reliable delivery of the vaccine, and might help to reduce the variations in HA-specific antibody titres and challenge virus replication observed between individual pigs in the present study. Furthermore, co-expression of NA in the PrV-based vectored vaccine might enhance protection and suppress challenge virus replication more efficiently as shown previously with HA- and NA-expressing ILTV recombinants in chickens (Pavlova et al., 2009).

METHODS

Viruses and cells. Rabbit (RK13), porcine (PSEK) and canine (MDCK) kidney cell lines were cultivated at 37 °C in minimum essential medium (MEM) supplemented with 10% or 5% FBS and maintained in medium containing 5% FBS after transfection or infection. For plaque assays, the virus inoculum was removed 2 h after infection, and cells were overlaid with semisolid medium containing 6 g methylcellulose l−1. PrV-Ba and virus recombinants derived from it were grown in RK13 or PSEK cells. The pandemic H1N1 SoIV isolates A/Regensburg/D6/09 and A/California/7/09 were propagated in MDCK cells using serum-free medium containing 2 μg ml−1 trypsin.

Construction of expression plasmids. The HA ORF of H1N1 SoIV A/Regensburg/D6/09 was reverse-transcribed and amplified by PCR using primers SIRH1-R and SIRH1-F (Table 1). The primers contained engineered XhoI or EcoRI restriction sites for cloning into the correspondingly digested eukaryotic expression vector pcDNA3 (Invitrogen), which resulted in pcDNA3-sirH1 (Fig. 1b). To obtain vector pcDNA, the plasmid-cloned P-MCMV (Dorsch-Häsel et al., 1985) was amplified by PCR with primers PMCMV-F and PMCMV-R (Table 1) which contained artificial HindIII and MluI sites. Then the digested PCR product was used to replace a HindIII/MluI fragment of pcDNA3 which represented P-HCMV. To obtain

Influenza virus HA expression in a PrV vector
pcDNA3I or pcDNAMI, the synthetic IVS from pIRES1neo (Clontech) was inserted into the unique pC-III or pC-IM as described previously (Pavlova et al., 2009). The EcoRI/Xhol insert of pcDNA3-sirH1 was recloned into the EcoRI/Xhol digested novel vectors after Klenow treatment and religation (Fig. 1b). The resulting pcDNA3I or pcDNAMI-sirH1 (Fig. 1b). Furthermore, a synthetic HA ORF (synH1) was generated (Eurofins MWG), in which codon usage had been adapted to that of PrV-BaKJHXAE, and substituted by a BamHI fragment containing the gG gene of PrV-Ba, the BAC pPrV-Ba derived from pEGFP-N1 (Clontech). Using this transfer plasmid and an EGFP expression cassette (Fuchs et al., 2012) which contained the mini F-plasmid OMI fragment of pcDNA3 containing P-MCMV (PMCMV-F/R), and of the HA gene of SolV A/Regensburg/D6/09 (PSIRH1-F/R), contained additional nucleotides (lower case letters) and, like the codon-optimized HA gene (synH1), artificial restriction sites (underlined) for cloning. HA start and stop codons are printed in bold italics.

### Table 1. Synthetic oligonucleotide and DNA sequences

The oligonucleotide primers used for PCR amplification of P-MCMV (PMCMV-F/R), and of the HA gene of SolV A/Regensburg/D6/09 (PSIRH1-F/R), contained additional nucleotides (lower case letters) and, like the codon-optimized HA gene (synH1), artificial restriction sites (underlined) for cloning. HA start and stop codons are printed in bold italics.

<table>
<thead>
<tr>
<th>Name</th>
<th>Nucleotide sequence</th>
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<tbody>
<tr>
<td>PMCMV-F</td>
<td>5'-caagcGTAACTCCGCGCCCGCTTTATG-3'</td>
</tr>
<tr>
<td>PMCMV-R</td>
<td>5'-caggaattCTGACGCCGAGGCTG-3'</td>
</tr>
<tr>
<td>PSIRH1-F</td>
<td>5'-caggaattCTGACGCCGAGGCTG-3'</td>
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<tr>
<td>PSIRH1-R</td>
<td>5'-caagcGTAACTCCGCGCCCGCTTTATG-3'</td>
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<tr>
<td>synH1</td>
<td>5'-GAATTTCACCATGGAAGCCATCTCGTGTTGCTGCTGATACAGTTTGCACCCAGCCACACACGCTGAGCTGCTAGTGT</td>
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pcDNA3I or pcDNAMI, the synthetic IVS from pRES1neo (Clontech) was inserted into the unique HindIII sites of pcDNA3 or pcDNAMI as described previously (Pavlova et al., 2009). The EcoRI/Xhol insert of pcDNA3-sirH1 was recloned into the EcoRI/Xhol or EcoRI/Xbal digested novel vectors after Klenow treatment of non-compatible fragment ends, resulting in pcDNA3I-sirH1 and pcDNAMI-sirH1 (Fig. 1b).

Furthermore, a synthetic HA ORF (synH1) was generated (Eurofins MWG), in which codon usage had been adapted to that of PrV without altering the amino acid sequence (Table 1), and cloned into pcDNA3I and pcDNAMI via engineered EcoRI and Xhol restriction sites (Fig. 1b).

For prokaryotic expression, the EcoRI/Xhol insert of pcDNA3-sirH1 was recloned into the correspondingly digested vector pGEX-4T-3 (GE Healthcare). To obtain sufficient protein expression, the insert had to be 5'- and 3'-terminally substituted by subsequent digestions of the original plasmid with BamHI, and with Stul plus Xhol, each followed by Klenow treatment and religation (Fig. 1b). The resulting construct pGEX-sirH1BSX encoded HA amino acids 78–345 fused to glutathione S-transferase (GST).

**Generation of PrV recombinants.** A genomic KpnI fragment containing the gG gene of PrV-Ba was cloned into pUC19, and truncated by subsequent EcoRI/AatII and HindIII/XcmI double digestions, each followed by Klenow treatment and religation (Fig. 1a). This permitted deletion of a 196 bp BamHI fragment from pUC-BaKJHXAE, and substitution by a BamHI fragment of pBl-GFP/MBO/B (Fuchs et al., 2012) which contained the mini F-plasmid PMBO131 (O’Connor et al., 1989) and an EGFP expression cassette derived from pEGFP-N1 (Clontech). Using this transfer plasmid and genomic DNA of PrV-Ba, the BAC pPrV-BaAgGG (Fig. 1a) was generated and propagated in *Escherichia coli* as described previously (Fuchs et al., 2012; Kopp et al., 2003).

To obtain transfer vectors for HA insertion, pUC-BaKJHXAE was digested with BamHI and NotI, and the deleted fragments were substituted by a BglII/PspOMI fragment of pcDNA3 containing P-HCMV and the multiple cloning site. Subsequently, a *MluI/BamHI*...
fragment was replaced by corresponding Mlu/BamHI fragments of pcDNA3I or pcDNA-MI, providing P-HCMV or P-MCMV, each followed by an IVS (Fig. 1). The resulting vectors pUC-BaJPHI and pUC-BaMI1 were digested with EcoRI and XbaI, and used for recloning of the EcoRI/XbaI or EcoRI/Xbal fragments containing the sirH1 or synH1 genes, respectively (Fig. 1).

For generation of HA-expressing PrV recombinants, DNA of pPrV-BaAgGG was digested at unique EcoRI sites (Fig. 1b) and used for cotransfection (FuGene HD reagent; Promega) of RK13 cells together with the HA transfer plasmids. Homologous recombination between digested BAC DNA and plasmid DNA led to novel PrV recombinants in which the mini F-plasmid and the EGFP cassette were replaced by the HA expression cassettes. The recombinants PrV-BaH1-siRH1, PrV-BaMI-synH1, PrV-BaH1-synH1 and PrV-BaMI-synH1 could be identified and isolated from non-fluorescent plaques; presence of the desired mutations was verified by restriction and Southern blot analyses of genomic DNA, and also by amplification and sequencing of the mutated genome region.

One-step replication kinetics and plaque size determination.

To investigate virus replication, RK13 cells were infected with PrV-Ba or PrV-BaMI-synH1 at an m.o.i. of 5. After 1 h at 37°C, non-penetrated virus was inactivated by low pH treatment (Mettenleiter, 1989) and incubation was continued. After 0, 4, 8, 12, 24 and 48 h cells were scraped into the medium and lysed by freeze-thawing. Progeny virus titres were determined by plaque assays on RK13 cells. After 3 days cells were fixed with 2% formaldehyde and stained with 1% crystal violet in 50% ethanol. The mean titres of three experiments and the mean diameters of 50 plaques per virus, together with standard deviations, were calculated. The statistical significance of differences between the two viruses was evaluated by Student’s t-test.

Antiserum preparation, Western blot and IIF analyses.

The prokaryotic expression plasmid pGEX-sirH1BX was propagated in E. coli XL-1-Blue MRF’ (Agilent Technologies), and the GST-HA fusion protein was purified as described previously (Fuchs et al., 2002). A rabbit was immunized by subcutaneous application of 100 μg of the purified fusion proteins emulsified in mineral oil four times at 4 week intervals. Sera collected before and 4 weeks after the last immunization were analysed.

For Western blotting, RK13 cells were harvested 20 h after infection with PrV (m.o.i. of 2), or 48 h after transfection (X-tremeGENE HP; Roche) with eukaryotic HA expression plasmids. PrV particles were prepared from infected PSEK cells as described before (Klupp et al., 2000). Influenza virus particles were sedimented by ultracentrifugation of cleared supernatants of infected MDCK cells. Proteins of approximately 105 cells or 3 μg of virion proteins were separated by discontinuous SDS-PAGE, transferred to nitrocellulose membranes and incubated with antibodies as described previously (Pavlova et al., 2009). The HA-specific (z-GST-H1), and also PrV-gB- (Kopp et al., 2003), gG- (unpublished) and pUL34-specific (Klupp et al., 2000) rabbit sera were used at dilutions of 1:10000 (z-GST-H1) or 1:100000, respectively, and chemiluminescence reactions of peroxidase-conjugated secondary antibodies were recorded (VersaloDoc 4000MP; Bio-Rad).

For IIF tests, RK13 cells transfected with expression plasmid or infected with PrV under plaque assay conditions were fixed after 48 h with methanol and acetone (1:1) for 30 min at −20°C. The cells were blocked with 10% FBS in PBS, incubated with a 1:100 diluted antiserum from a pig which had been experimentally infected with H1N1 SoIV A/Regensburg/D6/09, and a fluorescein-conjugated secondary antibody (Dianova) for 1 h each. After each step the cells were repeatedly washed with PBS and analysed by fluorescence microscopy (Eclipse Ti; Nikon).

Evaluation of animal experiments.

Two groups of five 7-week-old pigs were vaccinated intranasally with 2 × 106 TCID50 of either PrV-Ba or PrV-BaMI-synH1. Three weeks after vaccination all animals were challenged intranasally with 2 × 106 TCID50 of pandemic H1N1 SoIV A/California/7/09. In another trial, three naïve pigs of the same age and from the same breeder were primarily infected with the same dose of the SoIV stock. Rectal temperatures were measured daily, and sera were collected before and at days 7 and 14 after infection. HI assays were performed according to standard procedures using pandemic H1N1 SoIV A/California/7/09 as antigen (Saïd et al., 2013; Vincent et al., 2010). HI titres were determined using log2 dilutions, and mean reciprocal values were reported. Nasal swabs were taken before and at days 1, 2, 3, 4, 6, 8 and 10 after challenge infection. Total RNA was prepared (NucleoSpin RNA kit; Macherey-Nagel) and analysed by real-time RT-PCR (AgPath-ID One-Step RT-PCR kit; Applied Biosystems) with primers targeting the influenza A virus M gene (Hoffmann et al., 2010). Virus titres were calculated (Reed & Muench, 1938) after infection of MDCK cells grown in 96-well plates with log10 dilutions of swab samples. Differences between the HI titres, G1 and TCID50 values of the two groups were evaluated using Student’s t-test.

ACKNOWLEDGEMENTS

This study was supported by the Seventh Framework Programme of the European Commission FLUPIG project. The authors thank G. M. Keil for providing the cloned MCMV promoter, O. and J. Stech for the cloned HA gene, and B. G. Klupp for PrV-specific antiserum. The technical assistance of C. Ehrlich, S. Sander and S. Schuparis is greatly appreciated.

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