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Short Communication

Full restoration of viral fitness by multiple compensatory co-mutations in the nucleoprotein of influenza A virus cytotoxic T-lymphocyte escape mutants

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Amino acid substitutions have been identified in the influenza A virus nucleoprotein that are associated with escape from recognition by virus-specific cytotoxic T lymphocytes (CTLs). One of these is the arginine-to-glycine substitution at position 384 (R384G). This substitution alone, however, is detrimental to viral fitness, which is overcome in part by the functionally compensating co-mutation E375G. Here, the effect on viral fitness of four other co-mutations associated with R384G was investigated by using plasmid-driven rescue of mutant viruses. Whilst none of these alternative co-mutations alone compensated functionally for the detrimental effect of the R384G substitution, the M239V substitution improved viral fitness of viruses containing 375G and 384R. The nucleoprotein displays unexpected flexibility to overcome functional constraints imposed by CTL epitope sequences, allowing influenza viruses to escape from specific CTLs.

Viruses can exploit a variety of different strategies to escape recognition by virus-specific cytotoxic T lymphocytes (CTLs), which play an important role in the control of viral infections (McMichael *et al.*, 1983; Yap *et al.*, 1978). The accumulation of amino acid substitutions in or adjacent to CTL epitopes is one such strategy (Koup, 1994; Oldstone, 1997) and has been described for a variety of viruses, including human immunodeficiency virus type 1 (HIV-1), simian immunodeficiency virus (SIV) (Goulder & Watkins, 2004), hepatitis C virus (Chang *et al.*, 1997; Erickson *et al.*, 2001; Weiner *et al.*, 1995) and influenza viruses (Berkhoff *et al.*, 2004; Boon *et al.*, 2002b; Gog *et al.*, 2003; Rimmelzwaan *et al.*, 2004a, b; Voeten *et al.*, 2000).

It is a general property of RNA virus quasispecies to adapt to changing environments and select for successful variants that have, for example, increased resistance to antiviral agents or altered interferon-inducing capacity, or have enabled escape from antibodies and also from virus-specific CTLs (Domingo, 2000; Domingo & Holland, 1997).

Amino acid substitutions at the anchor residue of a CTL epitope may prevent binding of the epitope to its corresponding major histocompatibility complex class I molecule, resulting in the loss of the epitope. Variation in T-cell receptor-contact residues may prevent recognition by specific T-cell receptors present on virus-specific T cells. Variation in CTL epitopes can thus result in effective immune evasion from CTLs and has been shown to play an important role in the pathogenesis of various virus infections. The selective pressure exerted by CTLs must be significant, as it has been demonstrated that escape can occur at the cost of viral fitness. This was shown recently for HIV escape mutants, which reverted upon transmission to new susceptible hosts in which HIV CTL immunity was not present (Friedrich *et al.*, 2004a; Leslie *et al.*, 2004). In addition, it has been demonstrated that, in some cases, viruses need to accumulate amino acid substitutions outside the epitope in order to restore viral fitness as a result of functional constraints imposed by the amino acid sequence in the epitope (Friedrich *et al.*, 2004a, b; Kelleher *et al.*, 2001; Leslie *et al.*, 2004; Peyerl *et al.*, 2003). Apparently, viruses can overcome these functional constraints, as there are examples of virus variants that have escaped from CTLs, but retained their viral fitness (Friedrich *et al.*, 2004b; Peyerl *et al.*, 2003). Amino acid substitutions in the influenza virus nucleoprotein (NP) have been described to be associated with escape from virus-specific CTLs (Berkhoff *et al.*, 2004; Boon *et al.*, 2002b; Gog *et al.*, 2003; Rimmelzwaan *et al.*, 2004a, b; Voeten *et al.*, 2000) and these mutations can become fixed rapidly, even when there are only small selective advantages – potentially due to population-dynamic effects (Gog *et al.*, 2003). The R384G mutation in the HLA-B*2705- and HLA-B*0801-restricted CTL epitopes $NP_{383–391}$ and $NP_{380–388}$, respectively, resulted in a marked reduction in the human influenza virus-specific CTL response *in vitro* (Berkhoff

et al., 2004; Gog *et al.*, 2003; Rimmelzwaan *et al.*, 2004a; Voeten *et al.*, 2000). Thus, the immunodominant nature of the epitope (Boon *et al.*, 2002a), prolonged viral shedding in the absence of these epitopes in HLA-B*2705- and HLA-B*0801-positive individuals and population dynamics could have contributed to the emergence of these CTL-escape variants. For these epitopes, it has also recently been demonstrated that an extra-epitopic co-mutation was required to compensate functionally for the detrimental effect of the R384G substitution. It was shown that the naturally occurring E-to-G substitution at position 375 partially restored viral fitness in viruses in which the detrimental R384G mutation was introduced by site-directed mutagenesis. Position 384 is the anchor residue of the epitopes and loss of the arginine resulted in loss of the anchor residue for binding to HLA-B*2705 and HLA-B*0801 (Rimmelzwaan *et al.*, 2004b; Voeten *et al.*, 2000). As the co-mutation at position 375 only partially compensated for the loss of viral fitness and multiple co-mutations can be identified associated with the mutation at position 384 (Macken *et al.*, 2001; Rimmelzwaan *et al.*, 2004a), we wished to study the contribution of these additional mutations (R65K, D127E, I197V and M239V) to viral fitness in viruses that had escaped from $NP_{383–391}$- and $NP_{380–388}$-specific CTLs.

To this end, recombinant influenza viruses were generated essentially as described previously (de Wit *et al.*, 2004; Pleschka *et al.*, 1996) by using reverse-genetics technology. For the generation of recombinant influenza viruses with amino acid substitutions at selected positions, the NP gene segment of influenza virus A/Hong Kong/2/68 (A/HK/2/68) was amplified by RT-PCR as described previously (Rimmelzwaan *et al.*, 2004a) and inserted between the human Pol I promoter and the hepatitis δ ribozyme sequence of plasmid pSP72-PhuThep (de Wit *et al.*, 2004). Site-directed mutagenesis of the NP gene was performed by PCR using a QuikChange Multi site-directed mutagenesis kit (Stratagene) according to the manufacturer's recommendations. Plasmids pHMG-NP, pHMG-PB1, pHMG-PB2 and pHMG-PA, encoding the NP and the polymerase proteins PB1, PB2 and PA of influenza virus A/PR/8/34, respectively, were kindly provided by Dr P. Palese (Pleschka *et al.*, 1996). The bi-directional reverse-genetics plasmids pHW181–pHW188, encoding the viral gene segments of influenza virus A/WSN/33, were kindly provided by Dr R. G. Webster (Hoffmann *et al.*, 2000). For the generation of recombinant viruses, the NP constructs of A/HK/2/68 were transfected into 293T cells together with pHMG-NP and the remaining genomic constructs of A/WSN/33 as described previously (de Wit *et al.*, 2004). Twenty-four hours after transfection, virus was passaged in Madin–Darby canine kidney (MDCK) cells and the infectious virus titre was determined as described previously (Rimmelzwaan *et al.*, 1998). The combinations of mutations that were tested are listed in Table 1. As shown in Fig. 1, of the single mutants, only the R384G substitution was detrimental to viral fitness. Each of the other amino acid substitutions that were associated with R384G mutation had very modest effects on virus titres compared with viruses containing the wild-type sequence. As we described previously (Rimmelzwaan *et al.*, 2004a), addition of the E375G substitution to the R384G substitution partially restored virus replication, which was confirmed in the present study. In contrast, none of the other co-mutations compensated functionally for the detrimental effect of the R384G substitution on viral fitness. This indicated that E375G is indeed of crucial importance for viruses with the R384G mutation. Next, we tested whether the other co-mutations played a role in the fitness of viruses with the double mutation E375G/R384G. As shown in Fig. 1(e–f), addition of the M239V mutation increased virus titres to the level of wild-type virus. Thus, viruses containing 384G in combination with 239V and 375G replicated to titres similar to those of wild-type virus. Addition of the co-mutation D127E had no effect, whereas R65K and I197V influenced the replication of viruses with 375G/384G negatively. These data suggested that positions 239, 375 and 384 are involved in the same function of NP. Surprisingly, however, 239V is not found in all viruses with the 375E and 384G mutations, for which sequences are available in the influenza sequence database at http://www.flu.lanl.gov/ (Macken *et al.*, 2001; Rimmelzwaan *et al.*, 2004a). We speculate that these viruses without 239V exist as intermediates. The effect of the respective mutations on viral fitness, exemplified in Fig. 1(g), was confirmed by determining multi-step growth kinetics of these viruses after infection of MDCK cells by using an equivalent m.o.i. of 0·001 $TCID_{50}$ per cell. Introduction of each of the single mutations (with the exception of R384G) did not alter the replication kinetics dramatically (Fig. 1h) compared with replication of the wild-type virus. In Fig. 1(i), the replication of viruses containing three amino acid substitutions

Table 1. Plasmid constructs and their amino acid substitutions compared with the wild-type A/HK/2/68 amino acid sequence

Plasmid construct	Amino acid position					
	384	375	239	197	127	65
WT	**R**	**E**	**M**	**I**	**D**	**R**
1	G					
2		G				
3			V			
4				V		
5					E	
6						K
7	G	G				
8	G		V			
9	G			V		
10	G				E	
11	G					K
12	G	G	V			
13	G	G		V		
14	G	G			E	
15	G	G				K

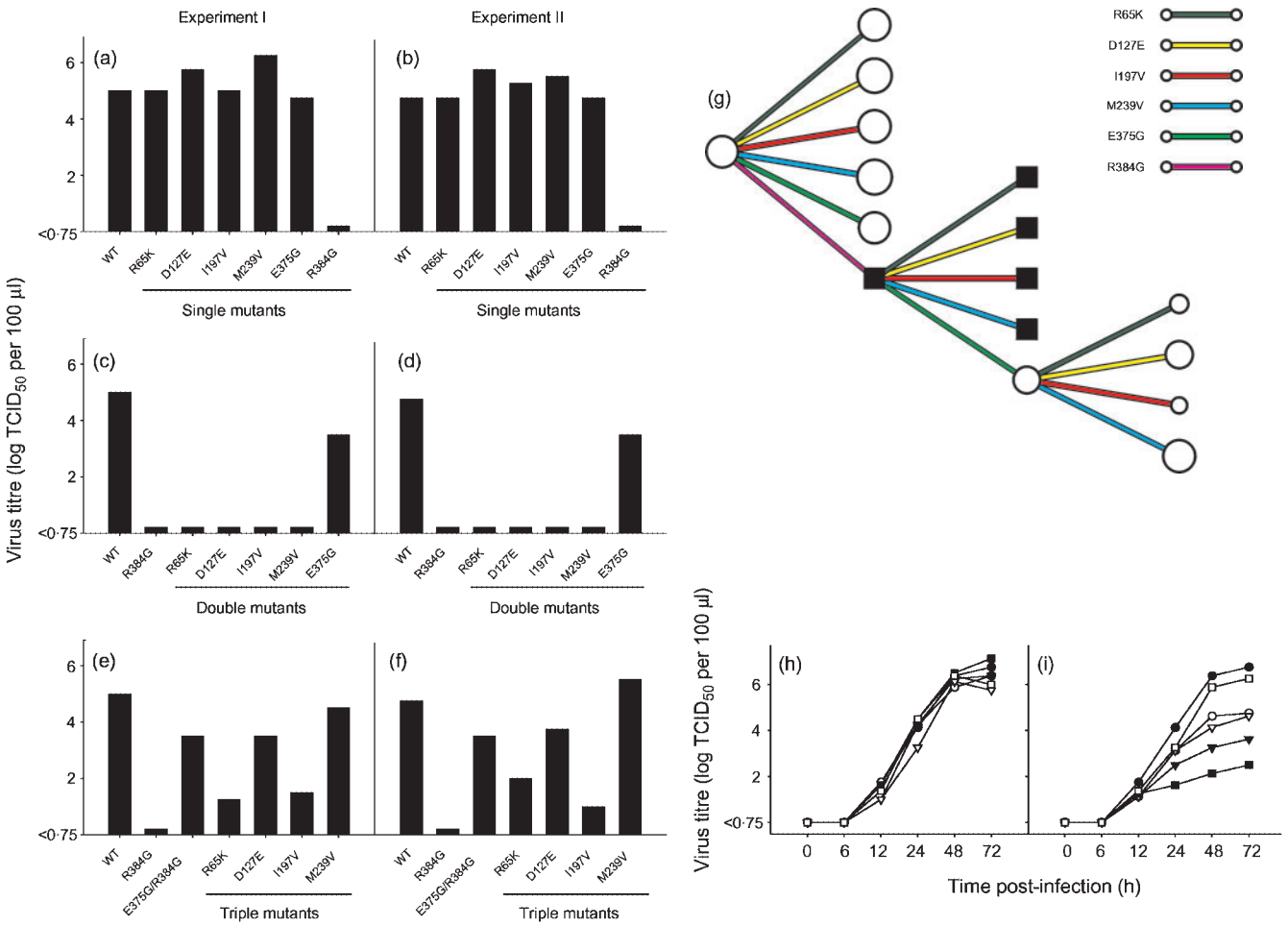

Fig. 1. Amino acid substitutions E375G and M239V restore the detrimental effect on viral fitness of the R384G substitution in virus containing the NP sequence of influenza virus A/HK/2/68. (a, b) Infectious virus titres are shown, obtained after infection of MDCK with viruses containing single amino acid substitutions (single mutants) or wild-type (WT) virus, as indicated. (c, d) Results obtained with viruses containing the R384G mutation in combination with one of the other amino acid substitutions as indicated (double mutants) or the R384G substitution only, and WT. (e, f) Results for viruses containing the E375G/R384G double mutation in combination with the other amino acid substitutions (triple mutants) or without, and WT. Data from two independent experiments are shown. (g) The tree displays all mutants that were generated and shows the effect of the respective mutations on viral fitness of the recombinant influenza viruses. The area of the circle is proportional to the mean viral titre obtained in the two experiments. Detrimental mutations preventing rescue of virus are indicated by solid squares. The mutations are indicated by line colour. (h, i) Replication kinetics of NP mutant influenza A viruses were determined by analysing multi-step growth curves of influenza viruses with single amino acid substitutions E375G (○), R65K (▼), D127E (▽), I197V (■) or M239V (□), or without an amino acid substitution (WT, ●) (h), or combinations of amino acid substitutions R384G/E375G without (○) or with R65K (▼), D127E (▽), I197V (■) or M239V (□) or without any substitution (WT, ●) (i), after infection of MDCK cells at an m.o.i. of 0·001 $TCID_{50}$ per cell. Data represent the means of two experiments.

was compared with wild-type viruses and the E375G/R384G double mutant. In agreement with the data presented in Fig. 1(e–f), introduction of 127E in the 375G/384G double mutant was neutral, whereas 65K and 197V had a negative effect on replication rate and 239V increased the replication rate to wild-type level.

The replication rates of the respective viruses correlated with the functionality of the NP variants as measured in an NP transcomplementation assay, performed as described previously (Voeten *et al.*, 2000). In brief, the NP-coding sequence of influenza virus A/HK/2/68, with or without selected mutations, was cloned into the eukaryotic expression plasmid pcDNA3. These plasmids were transfected into 293T cells along with pHMG-PB1, pHMG-PB1 and pHMG-PA, plus plasmid RF419, from which the green fluorescent protein (GFP) gene, flanked by the non-coding region of the NS gene segment, is transcribed in an antisense

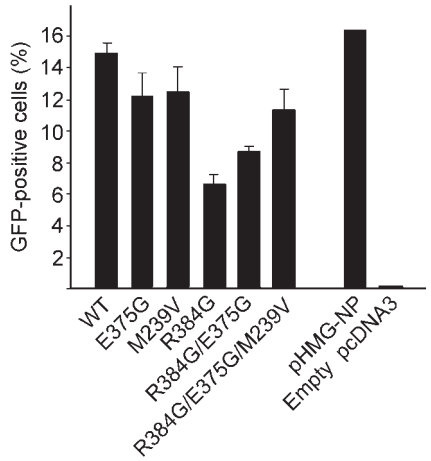

Fig. 2. Functional analysis of A/HK/2/68 NP with or without various amino acid substitutions or combinations thereof, as indicated. Functionality of the NP was tested in a minireplicon reporter assay. 293T cells were transfected with plasmids pHMG-PB1, pHMG-PB2, pHMG-PA and RF419 and 2 μg plasmid encoding the NP of A/HK/2/68 with or without amino acid substitutions as indicated. Cells transfected with empty pcDNA3 served as negative controls, whilst cells co-transfected with pHMG-NP were used as a positive control. The results are shown as the percentage of cells showing GFP expression, as measured by flow cytometry. The results represent the means of two independent experiments that were carried out in duplicate. Error bars indicate 1 SD. With the plasmid pEGFP-N1, transfection efficiencies were typically >90 %.

orientation. Transcription of the GFP minireplicon was assessed by GFP expression. As shown in Fig. 2, introduction of the E375G or M239V substitution alone did not have a significant effect on GFP expression in 293T cells. The R384G substitution reduced the functionality of the NP molecule markedly, as shown previously (Rimmelzwaan *et al.*, 2004a). The addition of E375G partially restored NP functionality, which was further improved by the introduction of the M239V substitution (Fig. 2). The number of GFP-positive cells obtained with this triple mutant was comparable to that obtained with the wild-type NP sequence, confirming that multiple co-mutations are required for full restoration of NP functionality. Although there was a correlation between GFP expression and replication rates, the NP with the R384G substitution showed residual transcriptional activity in the absence of demonstrable virus replication. It is unclear how much of this activity is required for virus replication. Apparently, there is no simple linear correlation between the two. Alternatively, other functional properties of the NP that are of importance at other stages of the virus-replication cycle may have been affected by the R384G substitution.

The NP has many different functions and can interact with a variety of viral and cellular proteins (Portela & Digard, 2002). Despite the many functional constraints on the protein, it shows a remarkable flexibility. Our data showed that the introduction of an otherwise detrimental amino acid substitution, which allows the virus to escape from virus-specific CTLs (Berkhoff *et al.*, 2004; Gog *et al.*, 2003; Rimmelzwaan *et al.*, 2004a; Voeten *et al.*, 2000), can be compensated for by the accumulation of a number of different co-mutations. Similar functionally compensating mutations have been observed in HIV and SIV CTL-escape mutants (Friedrich *et al.*, 2004a, b; Kelleher *et al.*, 2001; Leslie *et al.*, 2004; Peyerl *et al.*, 2003), indicating that this is a common strategy of RNA viruses. In contrast to the lentiviruses, which cause chronic infections in their hosts, the selective pressure against influenza virus CTL epitopes is probably exerted through herd immunity in the human population, induced by annual epidemics in a significant proportion of the population (Gog *et al.*, 2003).

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