Rubella virus-induced superinfection exclusion studied in cells with persisting replicons

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For the first time, homologous superinfection exclusion was documented for rubella virus (RUB) by using Vero cells harbouring persisting RUB replicons. Infection with wild-type RUB was reduced by tenfold, whereas Sindbis virus infection was unaffected. Replication following infection with packaged replicons and transfection with replicon transcripts was also restricted in these cells, indicating that restriction occurred after penetration and entry. Translation of such ‘supertransfecting’ replicon transcripts was not impaired, but no accumulation of supertransfecting replicon RNA could be detected. We tested the hypothesis favoured in the related alphaviruses that superinfection exclusion is mediated by cleavage of the incoming non-structural precursor by the pre-existing non-structural (NS) protease, resulting in an inhibition of minus-strand RNA synthesis. However, cleavage of a precursor translated from a supertransfecting replicon transcript with an NS protease catalytic-site mutation was not detected and the event in the replication cycle at which superinfection exclusion is executed remains to be elucidated.

Rubella virus (RUB) is a member of the genus Rubivirus within the family Togaviridae. RUB has a single-stranded, plus-sense RNA genome with two non-overlapping open reading frames (ORFs) (Frey, 1994). The 5′-terminal ORF (NS-ORF) encodes the non-structural proteins P150 and P90 and is translated from the genomic RNA as a polyprotein in the order NH2–P150–P90–COOH. Cleavage of P150 and P90 is mediated by a protease located at the C terminus of P150. These proteins undertake virus RNA synthesis in association with membranes of late endosomes and lysosomes (Magliano et al., 1998). The 3′-terminal ORF (SP-ORF) encodes the structural proteins and is translated from a subgenomic (SG) RNA as a polyprotein in the order NH2–capsid (C)–E2–E1–COOH. The RUB virion consists of the nucleocapsid and a lipid envelope in which the two envelope glycoproteins, E1 and E2, are embedded as a heterodimeric spike complex.

Superinfection exclusion or superinfection interference is a phenomenon common to most virus families by which an established virus infection blocks replication of a homologous superinfecting virus. Superinfection exclusion can be executed at different steps of the virus replication cycle, including attachment (Breiner et al., 2001a, b; Nethe et al., 2005; Schneider-Schaulies et al., 1995; Walters et al., 2004), penetration (Simon et al., 1990; Singh et al., 1997) and subsequent intracellular replication (Geib et al., 2003; Karpf et al., 1997; Lohmann et al., 2003; Schaller et al., 2007). Superinfection exclusion has also been studied with members of the alphaviruses, the other togavirus genus. Translation of the Sindbis virus non-structural proteins is sufficient to exclude superinfecting homologous virus (Adams & Brown, 1985) and the block occurs at an intracellular step, as replication of transfected Sindbis virus RNA is also restricted (Igarashi et al., 1977). During its maturation, nsP2 protease-mediated processing of the alphavirus non-structural precursor converts it first from an inactive enzyme to an active form that catalyses only minus-strand RNA synthesis, then to a form that catalyses both plus- and minus-strand synthesis and, finally, to the mature form that synthesizes only plus-strand RNA. It was thus suggested that the nsP2 protease of the resident virus is able to cleave the non-structural polyprotein precursor of the superinfecting virus prematurely, thereby terminating the replication of the superinfecting virus by inhibiting its ability to synthesize minus-strand RNA (Karpf et al., 1997).

RUB infection establishes resistance to superinfection with various heterologous viruses (Parkman et al., 1964), mediated through induction of interferon (Desmyter et al., 1968), which was the basis for the classical clinical assay for diagnosis of the virus (Carver et al., 1967). However, RUB-induced homologous superinfection exclusion has never been addressed. The asynchronous nature of RUB infection in cell culture (reviewed by Frey, 1994) complicates analysis of superinfection exclusion using RUB-infected cells. Cell cultures infected persistently by RUB are available (Abernathy et al., 1990), but it has been shown that the phenotype of superinfection exclusion can


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change in persistently infected cells (Lee et al., 2005). Therefore, we took advantage of the development of bicistronic RUB replicons expressing antibiotic-resistance genes (RUBrep/GFP/Neo; Fig. 1), through which cell cultures uniformly harbouring persistently replicating replicons can be selected and maintained (Fontana et al., 2007). Cells transfected with corresponding replicons of other viruses have recently been employed for analysis of superinfection exclusion (Lee et al., 2005; Sawicki et al., 2006; Tschirner et al., 2007). As shown in Fig. 2(a), plaque formation by RUB on cells infected persistently with this replicon (termed 'GFP-RUB-Vero' cells in this report) was reduced by tenfold and the plaques were smaller and opaque in comparison with plaques formed on Vero cells. Plaque formation by the heterologous Sindbis virus was similar on both Vero and GFP-RUB-Vero cells and, thus, homologous superinfection exclusion was at work.

Superinfection exclusion among alphaviruses effects a decrease in titre from approximately 100-fold in vertebrate cells to 105-fold in mosquito cells (Adams & Brown, 1985; Karpf et al., 1997), in comparison with the approximately 1 log restriction apparent in RUB-induced superinfection exclusion. A similar diversity in the extent of restriction is observed among the flaviviruses: superinfection exclusion by bovine viral diarrhea virus is associated with a 102-fold decrease in titres of the superinfecting virus (Lee et al., 2005), whilst with hepatitis C virus, the decrease is only approximately tenfold (Tschirner et al., 2007).

We continued our studies by using the replicon RUBrep/RFP (Fig. 1), which expresses red fluorescent protein (RFP) from the SG RNA and whose replication can thus be monitored by fluorescence microscopy of living cells. Packaged RUBrep/RFP particles were produced by transfecting a BHK cell line stably expressing the RUB SP-ORF (BHK-CE2E1) with RUBrep/RFP in vitro transcripts. When the same dilution of packaged RUBrep/RFP particles was used to infect Vero and GFP-RUB-Vero cells, the number of RFP-expressing cells in the GFP-RUB-Vero culture counted 2 days post-infection was only 20% of that in Vero cells (data not shown), a restriction similar in magnitude to that observed in the number of plaques produced when these cells were infected with RUB (Fig. 2a). As shown in Fig. 2(b), superinfection exclusion was exhibited regardless of whether RUBrep/RFP was introduced by superinfection with packaged particles or ‘supertransfection’ with in vitro transcripts. As the structural proteins are not expressed in GFP-RUB-Vero cells, superinfection exclusion is mediated by the non-structural proteins.

Superinfection exclusion of RUBrep/RFP in GFP-RUB-Vero cells was exhibited following both superinfection and supertransfection and thus occurs at a step in the replication cycle after adsorption and entry. As shown in Fig. 3(a), Northern blotting was used to assess RNA synthesis by supertransfecting RUBrep/RFP transcripts; however, no RNAs specific to this replicon were detectable, indicating that replication was blocked at an early step in the replication cycle prior to significant RNA accumulation. Predictably, P150 and P90 synthesis was detectable in Vero cells 3 days post-transfection, but not in GFP-RUB-Vero cells (Fig. 3b). It has been shown that, similar to the alphaviruses, minus-strand RNA synthesis in RUB-infected cells is catalysed by the uncleaved non-structural precursor and cleavage is necessary for plus-strand RNA synthesis to proceed (Liang & Gillam, 2000, 2001). Given the lack of detectable RNA accumulation by the supertransfecting replicon in GFP-RUB-Vero cells, it was therefore next of interest to determine whether superinfection exclusion was mediated by cleavage of the non-structural precursor translated from the supertransfecting replicon by the resident NS protease. To this end, GFP-RUB-Vero cells were supertransfected with RUBrep-HA/RFP, a replicon expressing a haemagglutinin (HA)-tagged P150, and RUBrep-HA/RFP-GDD*, a replicon with a substitution

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**Fig. 1.** Replicon constructs used in this study. The genomes of RUB and the replicons used in this study are shown. The first replicon, RUBrep/GFP/Neo, was used to establish a Vero cell line harbouring persisting replicons (GFP-RUB-Vero) by selection and maintenance in G418 after transfection (Fontana et al., 2007). The next four replicons, RUBrep/RFP and three variants, were used for superinfection and supertransfection. In all diagrams, ORFs are represented by open boxes and untranslated regions by lines. In the RUB genome, P150 and P90 are replicase proteins derived by translation of the non-structural ORF, whilst C, E2 and E1 are virion proteins derived from translation of the structural protein (SP) ORF from a subgenomic (SG) RNA. In replicons, the SP-ORF is replaced by a reporter gene such as green fluorescent protein (GFP) or red fluorescent protein (RFP). In RUBrep/GFP/Neo, expression of the neomycin-resistance factor, Neo*, is under control of an encephalomyocarditis virus internal ribosome entry site (IRES). Among the RUBrep/RFP variants, RUBrep-HA contains an HA epitope tag within the P150 gene that has no effect on replication (Tseng et al., 2006), C1152A contains a catalytic mutation in the non-structural protease that cleaves the non-structural precursor into P150 and P90 (Marr et al., 1994) and GDD* contains a GDD to AAA mutation in the RNA-dependent RNA polymerase that renders it non-functional (Wang & Gillam, 2001).
of GDD to AAA in the RNA-dependent RNA polymerase, rendering it unable to replicate (Fig. 1). As shown in Fig. 3(c), processed, HA-tagged P150 was detectable in both Vero and GFP-RUB-Vero cells 6 h post-transfection with both replicons. In itself, this result shows that superinfection exclusion was not mediated at the level of translation of the transfecting RNA. However, when \textit{in vitro} transcripts from RUBrep-HA/RFP C1152A, a mutant of one of the catalytic sites of the NS protease, were employed, cleavage was not observed in either Vero or GFP-RUB-Vero cells and, thus, \textit{trans}-cleavage of the non-structural precursor translated from the supertransfecting replicon by the resident NS protease could not be detected.
In summary, for the first time, we have documented the phenomenon of homologous superinfection exclusion with RUB. Similar to the related alphaviruses, superinfection exclusion occurred at a step in the virus replication cycle after adsorption, penetration and entry of the virion, but before accumulation of detectable RNA. It has been shown that expression of the RUB non-structural proteins does not interfere with replicon replication (Chen & Icenogle, 2004) and therefore active replication is required for superinfection exclusion to be elicited. We considered the alphavirus model in which superinfection exclusion is mediated by the resident nsP2 protease, which cleaves incoming non-structural precursors with an inactive NS protease. The results of a recent study using Semliki Forest virus and Sindbis virus suggested that inappropriate cleavage of non-structural precursors was not at work in modification of minus-strand RNA synthesis in cells infected with viruses and replications expressing different minus-strand synthetic phenotypes (Sawicki et al., 2006); however, this study did not rule out the possibility that alphavirus-mediated superinfection exclusion is due to a restriction of minus-strand synthesis by the superinfecting virus. Therefore, RUB-induced superinfection exclusion could also be mediated at the minus-strand RNA synthesis step. Other models for superinfection exclusion at intracellular steps in the replication cycle invoke competition for cell functions or replication sites between the resident and superinfecting viruses (summarized by Karpf et al., 1997) and these are also consistent with our observations thus far on the RUB-induced version of the event.

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References


Rubella virus superinfection exclusion


