

## Short Communication

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Received 1 March 2004  
Accepted 21 July 2004

# Effects of intrabodies specific for rotavirus NSP5 during the virus replicative cycle

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Intracellular antibodies or intrabodies (ICAbs) have great potential in protein knockout strategies for intracellular antigens. In this study, they have been used to investigate the role of the rotavirus non-structural protein NSP5 in the virus replication cycle. Intracellular antibody-capture technology was used to select single-chain Fv format (scFv) ICAbs against an NSP5 mutant. Five different specific ICAbs were selected and expressed in MA104 cells, in the scFv format, as cytoplasmic- and nuclear-tagged forms. By confocal microscopy, it was found that three of these ICAbs recognized the full-length wild-type NSP5 specifically, forming antigen-specific aggresomes in the cytoplasm of cotransfected cells. Expression of the ICAbs in rotavirus-infected cells largely reduced the assembly of viroplasms and cellular cytopathic effect. Replication of dsRNA was partially inhibited, despite there being no reduction in virus titre. These results demonstrate for the first time a key role for NSP5 during the virus replicative cycle.

Rotavirus is the most common diarrhoeal pathogen in children worldwide (Glass *et al.*, 1997) and in several other species. Replication occurs entirely in the cytoplasm of infected cells in characteristic, spherical structures called viroplasms, which appear soon after infection and in which virus replication and initiation of virus assembly occur. The non-structural rotavirus proteins NSP5 and NSP2 are two characteristic components of viroplasms (Afrikanova *et al.*, 1998; Berois *et al.*, 2003; Fabbretti *et al.*, 1999; Kattoura *et al.*, 1994; Petrie *et al.*, 1984). NSP5 is a highly conserved phosphoprotein. Its function has not yet been determined, although several properties have been elucidated; in particular, its hyperphosphorylation (Afrikanova *et al.*, 1996; Blackhall *et al.*, 1997; Eichwald *et al.*, 2002, 2004; Fabbretti *et al.*, 1999; Mohan *et al.*, 2003; Torres-Vega *et al.*, 2000), interaction with other viral proteins such as NSP2, VP1 and VP2 and its localization to viroplasms (Afrikanova *et al.*, 1998; Berois *et al.*, 2003; Eichwald *et al.*, 2002; Fabbretti *et al.*, 1999; Kattoura *et al.*, 1994).

As a reverse-genetic system is not yet available in rotavirus, we decided to use intracellular antibodies or intrabodies (ICAbs) as a protein knockout strategy to investigate the role of NSP5 during the virus replicative cycle. A novel and

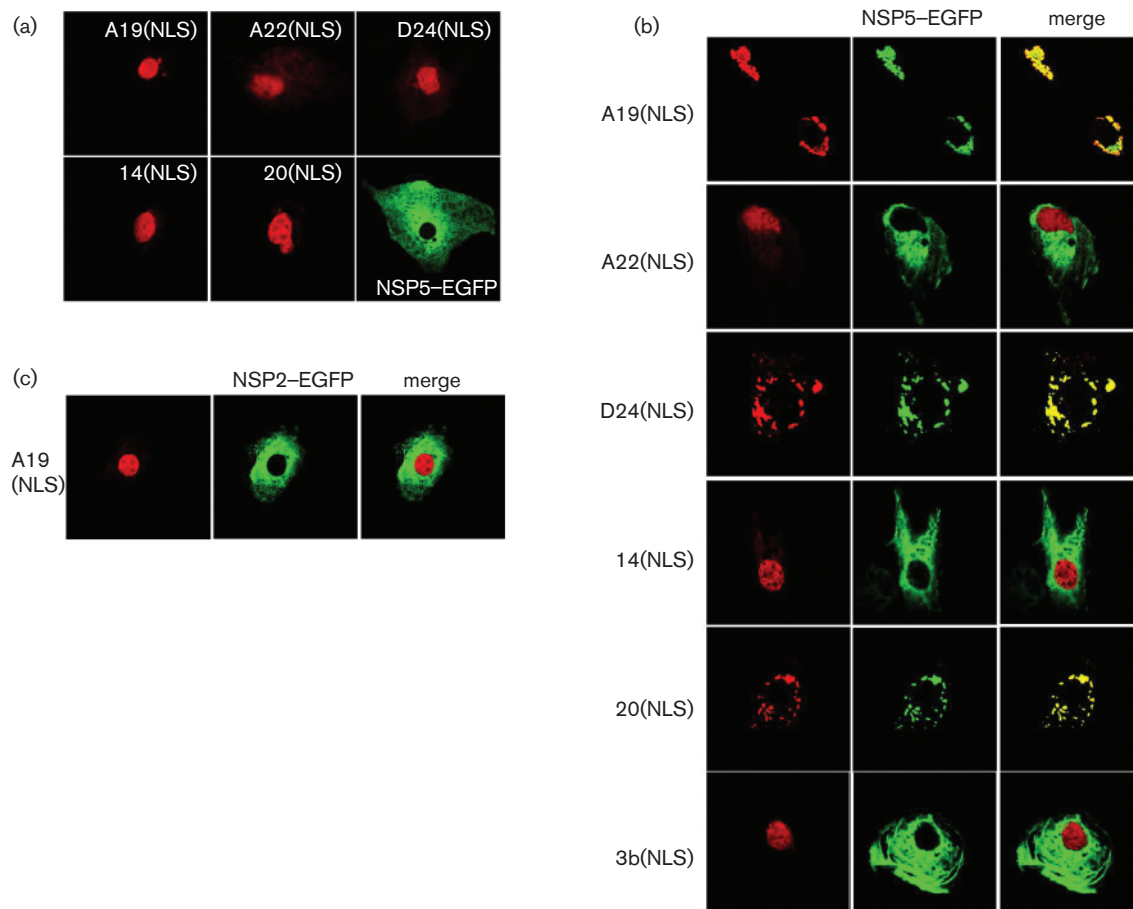
powerful technology to obtain intracellular-competent antibodies, termed intracellular antibody-capture technology, has recently been developed (Visintin *et al.*, 1999, 2002). It makes use of the two-hybrid system in yeast to select antibodies that are capable of binding antigens intracellularly. Although some recombinant antibodies expressed in the cell cytoplasm are able to maintain selective-binding properties against their antigens, a large number are unable to do so in the reducing cytoplasmic environment. Problems of folding and stability, mainly due to the lack of intrachain disulphide bonds of the light- and heavy-chain variable ( $V_L$  and  $V_H$ ) domains (Biocca *et al.*, 1995), occur very often, resulting in non-functional molecules with low expression levels and a short half-life (Cattaneo & Biocca, 1997, 1999). However, some  $V_L$  and  $V_H$  domains, despite a lack of disulphide bonds, retain their antigen-binding capacity in non-natural reducing environments, such as the cytoplasm and nucleus of the cell (Proba *et al.*, 1997, 1998). In particular, the single-chain Fv format (scFv) of antibodies (Bird *et al.*, 1988) has been shown to be suitable for intracellular expression (Marasco *et al.*, 1993; Tavladoraki *et al.*, 1993).

To select for anti-NSP5 ICAbs, we constructed an scFv library in the  $V_L$ -linker- $V_H$  orientation from mice that had been immunized with recombinant GST-NSP5. The immunization protocol is described previously (Eichwald *et al.*, 2002). The scFv library was fused to the transactivator

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protein VP16 and used in the yeast two-hybrid selection method as described by Hollenberg *et al.* (1995) and Visintin *et al.* (1999). The target antigen (fused to the DNA-binding domain of LexA) was  $\Delta 2$ , an NSP5 deletion mutant (lacking aa 34–80), because the wild-type NSP5 showed trans-activation of the two reporter genes histidine (HIS3) and  $\beta$ -galactosidase (data not shown) (F. Vascotto & O. R. Burrone, unpublished data). From two different selections, we obtained five clones that were specific for the target protein  $\Delta 2$ , named A19, A22, D24, 14 and 20, which were well-expressed in the cytoplasm of yeast cells. For expression in mammalian cells, the scFv cassettes were subcloned into pcDNA3 (Invitrogen). When cotransfected into MA104 cells as described previously (Eichwald *et al.*, 2002), all five selected ICABs retained specificity for the NSP5 mutant used for library selection (data not shown). However, to characterize their activity against the wild-type NSP5 protein, they were cotransfected with a construct encoding NSP5 fused to EGFP (NSP5–EGFP) (Eichwald *et al.*, 2004). To divert localization, ICABs were targeted to the nucleus by adding

a C-terminal nuclear localization signal (NLS) (Persic *et al.*, 1997). In addition, all scFvs contained the 12 aa SV5 tag (Hanke *et al.*, 1992) for detection by immunofluorescence, as described previously (Fabbretti *et al.*, 1999). As a negative control, we used an irrelevant ICAB [3b(NLS)], which was selected from the library and validated as an intracellularly competent scFv (M. Visintin & A. Cattaneo, unpublished data). As shown by confocal microscopy, on transfection in MA104 cells, all five ICABs showed nuclear localization, as expected, whereas NSP5–EGFP had a homogeneous cytoplasmic distribution (Fig. 1a). However, when ICABs and NSP5–EGFP were coexpressed in the same cell, three ICABs (A19, D24 and 20) showed a dramatic change in their original cellular distribution and complete colocalization: ICABs were retained in the cytoplasm and NSP5–EGFP was no longer distributed homogeneously (Fig. 1b). For these three selected ICABs, we clearly detected characteristic intracellular structures that were reminiscent of aggresomes (Kopito & Sitia, 2000), formed by their specific interaction with the antigen (NSP5–EGFP). Two other



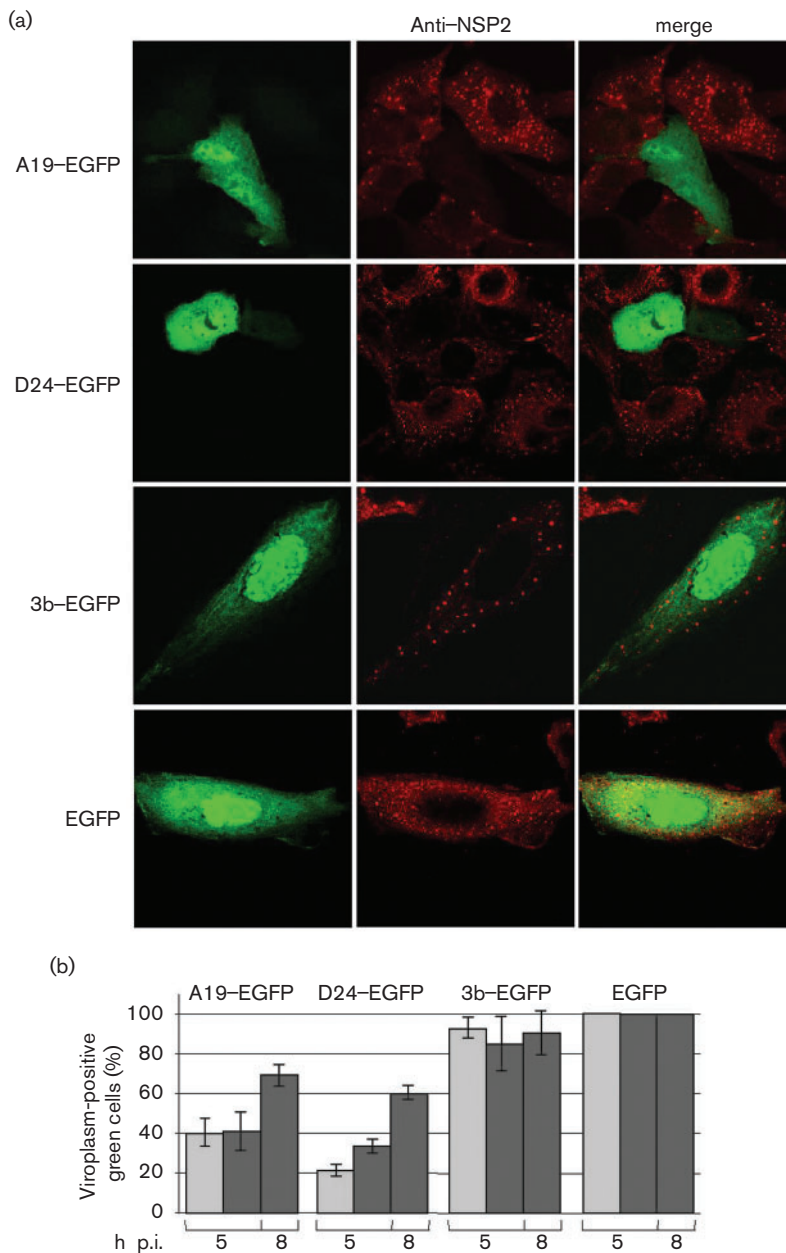
**Fig. 1.** Confocal immunofluorescence of transfected MA104 cells. (a) Cells transfected with scFv(NLS) (red) or NSP5–EGFP (green). (b) Cells cotransfected with scFv(NLS) (red) and NSP5–EGFP (green), showing colocalization and formation of aggresomes in three (A19, D24 and 20) of the five selected ICABs. ICAB 3b(NLS), negative control. (c) A19(NLS) coexpressed with the irrelevant antigen NSP2–EGFP.

ICABs, A22(NLS) and 14(NLS), as well as the negative control, 3b(NLS), did not show any interaction, with complete nuclear localization and NSP5-EGFP retaining its cytoplasmic distribution (Fig. 1b). Furthermore, as expected, all five ICABs selected did not recognize another non-structural rotavirus protein, NSP2 (NSP2-EGFP) (Eichwald *et al.*, 2004). Fig. 1(c) shows A19(NLS) co-expressed with NSP2-EGFP as an example.

We tested the ability of the three specific ICABs (A19, D24 and 20) to interact with NSP5 in the context of virus infection. The scFv cassettes were subcloned into pEGFP-N1 (Clontech) and transfected transiently into MA104 cells, in order to visualize their expression by the cells. Intrabody 3b-EGFP or EGFP alone were used as negative controls. At

30 h post-transfection of the ICABs-EGFP, cells were infected with rotavirus (strain OSU at an m.o.i. of approx. 10) for 5 h, as described previously (Afrikanova *et al.*, 1996), fixed and stained with anti-NSP2 (or anti-NSP5) to monitor formation of viroplasm (Fig. 2).

As not all cells were transfected, the state of rotavirus infectivity was analysed in green cells (i.e. those expressing ICAb-EGFP), which showed a similar distribution in mock-infected and virus-infected cells. We found a remarkable difference in the number of viroplasm-positive cells (detected with either anti-NSP2 or anti-NSP5) in cells expressing A1-EGFP or D24-EGFP, in relation to those expressing either EGFP or 3b-EGFP. Fig. 2(a) shows representative images of rotavirus-infected A19-EGFP and



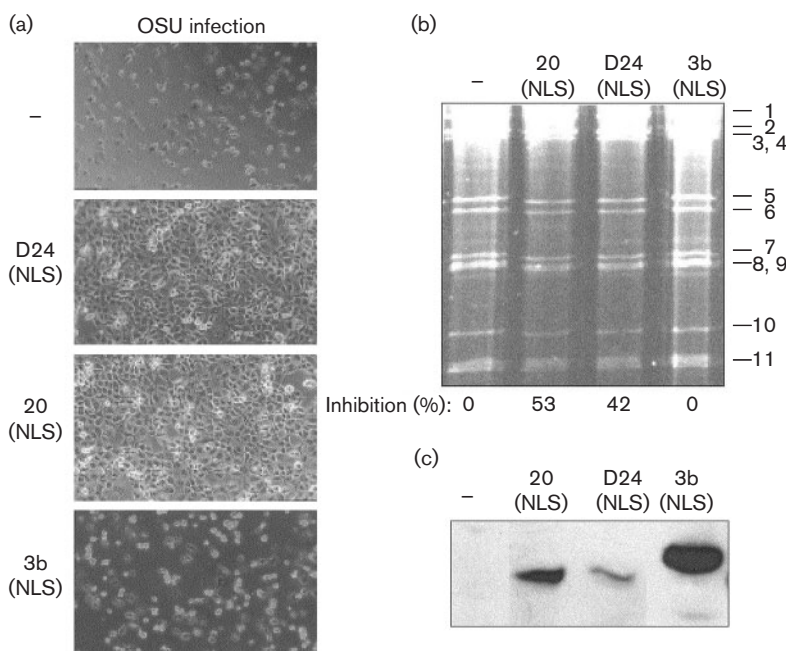
**Fig. 2.** Confocal immunofluorescence of ICAb-transfected and rotavirus-infected MA104 cells. (a) Cells were transfected with ICABs-EGFP (A19-EGFP, D24-EGFP, 3b-EGFP) or EGFP alone and subsequently infected with rotavirus (m.o.i. approx. 10). Viroplasms were detected with anti-NSP2 (red). (b) Percentage of viroplasm-positive green cells (corresponding only to transfected cells) for two anti-NSP5 ICABs, A19-EGFP and D24-EGFP, the irrelevant 3b-EGFP and the control EGFP. Viroplasms were detected with either anti-NSP2 (dark blue) or anti-NSP5 (light blue), at 5 or 8 h p.i. The number of viroplasm-positive green cells transfected with EGFP was considered to be 100% ( $n=3$ ).

D24-EGFP green positive cells, which did not have detectable viroplasm; in comparison, all neighbour cells that were not transfected showed a large number of viroplasms, indicating that normal infection had occurred. The inhibition of viroplasm formation was specific for the two anti-NSP5 ICABs (A19 and D24), as neither the negative control 3b-EGFP nor EGFP had any significant effect (Fig. 2a). Moreover, the strong inhibitory effect of both anti-NSP5 ICABs in about 70 % of the cells was obtained regardless of whether viroplasms were detected with anti-NSP2 or anti-NSP5. On the other hand, in nearly 30 % of green cells, the number of viroplasms per cell was close to normal, as in non-transfected cells or cells transfected with the negative controls EGFP or 3b-EGFP. The results plotted in Fig. 2(b) were obtained from three different experiments, counting 200 transfected green cells for each ICAB, taking as 100 % the number of viroplasm-positive green cells transfected with EGFP alone. When the experiments were performed over a longer period (8 h), there was still significant, but lower, inhibition of viroplasm formation (Fig. 2b). These results suggest that viroplasm formation cannot be inhibited when ICABs are unable to keep NSP5 below a certain threshold. In the immunofluorescence experiments, in those cells expressing the anti-NSP5 ICABs that showed inhibition of viroplasm formation, we always observed almost no background, not only for the target protein NSP5 but also for NSP2, suggesting that most of the viral proteins in infected cells are derived from mRNAs that were produced in viroplasms by newly formed particles. Thus, ICAB neutralization of NSP5 produced by the infecting particle(s) inhibits the formation of viroplasms which, in turn, could impair the general level of protein production.

To further investigate the effect of the selected ICABs in virus replication, we used the retrovirus expression system

(Zacchi *et al.*, 2002) to increase the percentage of cells expressing ICABs, as we were unable to select long-lived stable cell clones expressing ICABs, probably due to toxicity effects. Retroviruses encoding two anti-NSP5 ICABs, 20(NLS) and D24(NLS), and the negative control 3b(NLS), were used to infect the human lung carcinoma H1299 cell line. At 2 days post-infection, these cells were infected with rotavirus (m.o.i. approx. 1) and, 16 h later, cytopathic effect was analysed (Fig. 3a) and viral dsRNAs were collected from infected cells and detected as described by Chen *et al.* (1990). As shown in Fig. 3(a), the strong cytopathic effect observed in control cells and in cells expressing the irrelevant ICAB 3b was suppressed in the presence of the two anti-NSP5-specific ICABs. Moreover, they showed a partial, although significant, reduction (40–45 %) in production of dsRNA compared with control cells (considered as 100 %) (Fig. 3b). Despite this, we have not detected relevant differences in virus yields from cells expressing anti-NSP5 ICABs in comparison with the negative control (data not shown). The effects observed may depend on the expression level of the different ICABs, which was lower for the two anti-NSP5 ICABs (Fig. 3c).

NSP5 was previously shown to interact with the structural proteins VP1 and NSP2 in viroplasms (Afrikanova *et al.*, 1998; Fabbretti *et al.*, 1999). NSP5 and NSP2 were found to be components of replication intermediates that were purified from infected cells (Gallegos & Patton, 1989; Patton & Gallegos, 1988) and to interact, in the absence of other viral proteins, to form viroplasm-like structures (Eichwald *et al.*, 2004; Fabbretti *et al.*, 1999). NSP5 also associated with the major core protein VP2, an abundant protein of viroplasms, and was postulated to play a role in the switch from transcription to replication activity of newly formed viral particles (Berois *et al.*, 2003).



**Fig. 3.** Cytopathic effect and production of viral genomic dsRNA in H1299 cells infected with retrovirus-expressing ICABs (NLS). (a) Cytopathic effect on control cells and cells expressing the indicated ICABs at 16 h p.i. with rotavirus (OSU strain, m.o.i. approx. 1). (b) Viral dsRNA derived from rotavirus-infected cells at 16 h p.i., analysed by 10% SDS-PAGE. dsRNAs were quantified by using the Versadoc imaging system (Bio-Rad). The yield of dsRNA by H1299 cells obtained with the control empty retroviral vector (pLPC) or the irrelevant ICAB 3b(NLS) were considered to be 100%. (c) Western immunoblot (anti-SV5 tag mAb) of retrovirus-infected H1299 cellular extracts expressing the indicated ICABs(NLS).

Temperature-sensitive mutants, genome reassortment and *in vitro* reconstitution studies have been the main approaches used to understand the function of rotavirus proteins, because of the limitations imposed by the lack of reverse genetics. However, no conclusive data on the role of NSP5 protein have been obtained so far.

Here, we describe the application of intracellular antibody-capture technology to select active cytoplasmic ICABs as a protein knockout system, in order to investigate the function of rotavirus NSP5 (Visintin *et al.*, 1999, 2002). The phenotype observed upon NSP5 knockout by ICABs is similar to those reported for the temperature-sensitive (*ts*) mutants affecting the structural rotavirus proteins related to replication, VP1 (*tsC*) and VP3 (*tsB*) (Chen *et al.*, 1990), as well as the for the non-structural protein NSP2 (*tsE*), which showed a lack of viroplasm formation and impaired viral ssRNA and dsRNA production at the non-permissive temperature (Chen *et al.*, 1990; Ramig & Petrie, 1984; Taraporewala *et al.*, 2002). A similar conclusion was reported recently for NSP2 depletion by siRNA (Silvestri *et al.*, 2004). Our results indicate that NSP5 is also an essential element for the assembly of functional viroplasms and is therefore relevant for virus replication.

We have reached similar conclusions regarding the role of NSP5 in viroplasm formation and virus replication by means of an alternative knockout strategy using RNA interference specific for genomic segment 11 mRNA, which produced strong impairment of virus yield in MA104 cells (M. Campagna, C. Eichwald, F. Vascotto & O. R. Burrone, unpublished data). In contrast, ICABs were not as efficient in blocking infective virus release, probably due to low levels of expression. A recent study using ICABs specific for the NS1 protein of the bluetongue virus also demonstrated lack of virus inhibition, despite a clear reduction of the cytopathic effect (Owens *et al.*, 2004).

## Acknowledgements

We are thankful to Monica Gostissa for helpful advice on using the retroviral system. We are grateful to Roberta Contin for her technical help, to Catherine Eichwald and Elsa Fabbretti for their useful suggestions and to P. Zori for technical support. F.V. was partially supported by a SISSA predoctoral fellowship and by EU project QLK2-2000-00739 and M.C. was supported by a SISSA predoctoral fellowship.

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