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Prolonged Activity of the Pestiviral RNase E^{rns} as an Interferon Antagonist after Uptake by Clathrin-Mediated Endocytosis

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ABSTRACT

The RNase activity of the envelope glycoprotein E^{rns} of the pestivirus bovine viral diarrhea virus (BVDV) is required to block type I interferon (IFN) synthesis induced by single-stranded RNA (ssRNA) and double-stranded RNA (dsRNA) in bovine cells. Due to the presence of an unusual membrane anchor at its C terminus, a significant portion of E^{rns} is also secreted. In addition, a binding site for cell surface glycosaminoglycans is located within the C-terminal region of E^{rns}. Here, we show that the activity of soluble E^{rns} as an IFN antagonist is not restricted to bovine cells. Extracellularly applied E^{rns} protein bound to cell surface glycosaminoglycans and was internalized into the cells within 1 h of incubation by an energy-dependent mechanism that could be blocked by inhibitors of clathrin-dependent endocytosis. E^{rns} mutants that lacked the C-terminal membrane anchor retained RNase activity but lost most of their intracellular activity as an IFN antagonist. Surprisingly, once taken up into the cells, E^{rns} remained active and blocked dsRNA-induced IFN synthesis for several days. Thus, we propose that E^{rns} acts as an enzymatically active decoy receptor that degrades extracellularly added viral RNA mainly in endolysosomal compartments that might otherwise activate intracellular pattern recognition receptors (PRRs) in order to maintain a state of innate immunotolerance.

IMPORTANCE

The pestiviral RNase E^{rns} was previously shown to inhibit viral ssRNA- and dsRNA-induced interferon (IFN) synthesis. However, the localization of E^{rns} at or inside the cells, its species specificity, and its mechanism of interaction with cell membranes in order to block the host's innate immune response are still largely unknown. Here, we provide strong evidence that the pestiviral RNase E^{rns} is taken up within minutes by clathrin-mediated endocytosis and that this uptake is mostly dependent on the glycosaminoglycan binding site located within the C-terminal end of the protein. Remarkably, the inhibitory activity of E^{rns} remains for several days, indicating the very potent and prolonged effect of a viral IFN antagonist. This novel mechanism of an enzymatically active decoy receptor that degrades a major viral pathogen-associated molecular pattern (PAMP) might be required to efficiently maintain innate and, thus, also adaptive immunotolerance, and it might well be relevant beyond the bovine species.

Bovine viral diarrhea virus (BVDV) is a pathogen of cattle that is spread worldwide. Together with the classical swine fever virus (CSFV) and border disease virus (BDV), this positive-sense, single-stranded RNA (ssRNA) virus belongs to the genus *Pestivirus* of the family *Flaviviridae* (1). BVDV infections are either transient or persistent. Persistent infections may occur when the fetus is infected by a noncytopathogenic (ncp) biotype of virus early in its development (2, 3). The persistent virus elicits immunotolerance that is specific to the infecting strain. In contrast to other genera of the family *Flaviviridae* family, like the hepaciviruses, pestiviruses express two unique proteins to block type I interferon (alpha/beta interferon [IFN-α/β]) induction, i.e., the N-terminal protease N^{pro} and the structural glycoprotein E^{rns}. Both proteins are required to establish persistent infections (4). The nonstructural protein N^{pro} targets the transcription factor IRF3 for proteasomal degradation (5), thus antagonizing interferon induction, e.g., by double-stranded RNA (dsRNA), in virus-infected cells (6, 7). E^{rns} harbors an RNase active domain belonging to the T2 RNase superfamily (8), and this enzymatic activity is essential for its ability to block the induction of IFN-α/β (9–11). Together with viral glycoproteins E1 and E2, E^{rns} forms the envelope of the virus, but a significant portion of the E^{rns} protein is also secreted into the extracellular space (8). Attachment of E^{rns} to cell membranes is mediated by an amphipathic helix that acts as an unusual membrane anchor at the C terminus that embeds the protein in plane into cell membranes (12, 13), which might explain its dual function as an envelope glycoprotein and a secreted RNase. The cell tropism of pestiviruses has been attributed to E2, which binds to its receptor, CD46 (14–16), followed by cellular uptake by clathrin-mediated endocytosis (17–19). In contrast, E^{rns} may bind to a different receptor (20), but this might not be required for virus particles to infect their host cells, as E1- and E2-pseudotyped viruses are sufficient to mediate cell entry (21). Although binding of E^{rns} to glycosaminoglycans and immobilized heparin has been shown, the possibility that a cell- or species-specific receptor existed could not be excluded. Thus, binding of E^{rns} was saturable to fetal bovine epithelial or porcine PK15 cells, indicative of receptor-mediated attachment, but not to porcine SK6, hamster BHK-21, or insect Sf21 cells (20). On the basis of the broad pH optimum of its RNase activity (22) and the ability to cleave dsRNA only at

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low pH values, it was proposed that E^{rns} might be active mainly in endolysosomal compartments (23). However, the latter restriction was recently extended, as dsRNA is also cleaved at neutral pH (11). Previous experiments showed that extracellularly added E^{rns} blocks IFN induction by ss- and dsRNA in bovine cells and that E^{rns} could be removed just prior to the addition of dsRNA, which suggested the possibility of an intracellular activity of this viral RNase (10, 11). Nevertheless, the location of E^{rns} at or inside a cell is still unknown, and its exact role in the evasion of the innate immune system remains elusive so far.

Here we provide evidence that soluble E^{rns} protein enters cells within minutes in an energy-dependent fashion via clathrin-dependent endocytosis and then remains active for several days. The activity of the protein was observed not only in bovine cells but also in caprine, ovine, canine, and human cells. Heparin effectively competes with the binding of E^{rns} to extracellular glycosaminoglycans, and thus, cell-bound E^{rns} could be removed by washing with heparin, which impeded its ability to block the interferon expression induced by dsRNA. E^{rns} protein lacking the C-terminal membrane anchor retained RNase activity but had a strongly reduced activity in blocking dsRNA-induced IFN synthesis.

MATERIALS AND METHODS

Reagents. Cell culture media were purchased from Seromed (Biochrom, Munich, Germany). Fetal calf serum (FCS) confirmed to be free from BVDV and from antibodies to BVDV was from Sigma (Buchs, Switzerland) or Oxoid GmbH (Wesel, Germany).

Chlorpromazine (CPZ), monodansylcadaverine (MDC), methyl-β-cyclodextrin (MβCD), heparin sodium salt from porcine intestinal mucosa, chondroitin sulfate A sodium salt from bovine trachea, and the synthetic dsRNA poly(I·C) were purchased from Sigma, whereas 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA) was from Enzo Life Sciences (Lausen, Switzerland).

Cells. Bovine turbinate (BT) cells, goat synovial membrane (GSM) cells, and lamb synovial membrane (LSM) cells were prepared at the Institute of Veterinary Virology from bovine fetuses or animals obtained from a local abattoir and were maintained in Earle's minimal essential medium (MEM) supplemented with 15% FCS (2% during experiments), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified 5% CO_2 atmosphere. Human umbilical vein endothelial cells (HUVECs) were kindly provided by Stefano Di Santo (University Hospital Inselspital, Bern, Switzerland) and cultured using a standard protocol (24). Canine keratinocytes, provided by Philippe Plattet (Division of Experimental Clinical Research, University of Bern, Bern, Switzerland), were maintained as described in reference 25.

Western blotting. Cells, cultured in 24-well plates, were washed with phosphate-buffered saline (PBS) and directly lysed in the wells with 30 μl M-PER mammalian protein extraction reagent (Pierce, Socochim SA, Lausanne, Switzerland) containing complete protease inhibitor cocktail (Roche Diagnostics, Rotkreuz, Switzerland). Protein separation was performed on 10% SDS-polyacrylamide gels (Bio-Rad, Reinach, Switzerland), and proteins were electroblotted on nitrocellulose membranes (Amersham Biosciences, GE Healthcare, Glattbrugg, Switzerland) in a Mini Trans-Blot cell (Bio-Rad). Membranes were blocked in PBS containing 5% low-fat dry milk and 0.1% Tween 20 before probing for the expression of the Mx protein using a mouse monoclonal antibody against human MxA (kindly provided by Jovan Pavlovic, Institute of Medical Virology, University of Zurich, Zurich, Switzerland) collected from hybridoma supernatants (diluted 1:10). β-Actin, detected with a mouse anti-β-actin antibody (diluted 1:50,000; Sigma), served as loading control for the individual lanes. Peroxidase-labeled donkey anti-mouse antibody (Jackson ImmunoResearch Laboratories, Milan Analytica AG, Rheinfelden, Switzerland) was used as a secondary antibody (diluted 1:5,000), and proteins were visualized using WesternBright enhanced chemiluminescence horseradish peroxidase substrate (Witec AG, Lucerne, Switzerland) according to the manufacturer's protocol. Signal intensities were quantified using the advanced image data analyzer software AIDA (Raytest, Wetzikon, Switzerland).

Assays for E^{rns} import. BT cells were seeded in 24-well plates at a density of 7.5×10^4 cells/well. The confluent cell monolayer was preincubated with the various inhibitors, as indicated in the appropriate figures, for 30 min prior to the addition of 2.5 μg/ml E^{rns}, still in the presence of the inhibitors, for 60 min in 250 μl of cell culture medium. Cells were then washed twice with MEM containing 0.1 mg/ml heparin and once with MEM only. Control samples were washed three times with MEM. Thereafter, 2 μg/ml poly(I·C) was incubated with the cells for 18 to 20 h before collecting them for Western blot analysis.

Expression of E^{rns} variants. Wild-type (wt) E^{rns} and the RNase-inactive mutant H30F were expressed in MDBK Tet-On cells using a tetracycline-inducible expression plasmid as described previously (10). Supernatants were collected and concentrated using Vivaspin 20 10,000-molecular weight cutoff (MWCO) filter tubes (Milian AG, Wohlen, Switzerland) before use.

The E^{rns} mutant lacking the C terminus was expressed in 293T/17 cells (kindly provided by Philippe Plattet) that had been transfected with pCI-E^{rns} or empty vectors (pCI mammalian expression vector; Promega, Dübendorf, Switzerland) by use of the Fugene HD transfection reagent (Roche Diagnostics) and a 3:1 ratio of transfection reagent to cDNA. The supernatant was harvested after 3 days, followed by concentration with Vivaspin 6 30,000-MWCO filter tubes. The concentration of E^{rns} in the preparations was quantified by a commercially available enzyme-linked immunosorbent assay (ELISA; IDEXX BVDV Ag/Serum Plus; IDEXX Switzerland AG, Bern-Liebefeld, Switzerland) as described previously (10).

RNase activity assay. A total of 250 ng of *in vitro*-transcribed 300-bp dsRNA fragments from the 5′ untranslated region (UTR) of BVDV strain Ncp7 (prepared as described in reference 11) was incubated for 1 h at 37°C with the supernatant of E^{rns}-expressing cells in the presence of 40 U RNasin (Promega), to block unspecific RNase activity (11, 23), in 100 mM Tris-acetate buffer at pH 6.5. Digested RNA was diluted in 2× RNA loading dye solution (Fermentas, Fisher Scientific, Wohlen, Switzerland) and separated on a 1% agarose gel for 25 min at 100 V. Gels were stained with ethidium bromide, and visualization and image capture were done with a U:Genius gel imaging system (Syngene, Biolabo Scientific Instruments SA, Châtel-St-Denis, Switzerland).

RESULTS

E^{rns} is active in nonbovine cells. The activity of extracellularly supplied BVDV E^{rns} protein against the synthetic dsRNA poly (I·C) in bovine cells has been established previously (10). To investigate whether this activity is restricted to bovine cells and to provide evidence that E^{rns} does not require a species-specific receptor for its activity, we used cells from a variety of host species. Only cells that were able to express Mx protein, a widely used, sensitive, and reliable marker for the presence of IFN-α/β (26), upon stimulation by extracellularly added poly(I·C) were used to test the effect of E^{rns}. Caprine GSM and ovine LSM cells, canine keratinocytes, and HUVECs of human origin all showed robust Mx protein expression in response to poly(I·C). In these cell types, E^{rns} dose-dependently inhibited dsRNA-induced Mx synthesis (Fig. 1). Whereas GSM cells (Fig. 1A), LSM cells (Fig. 1B), and canine keratinocytes (Fig. 1C) showed full inhibition of Mx expression at a similar ratio of E^{rns} to poly(I·C), HUVECs were less responsive to the inhibitory effect of E^{rns} but still maintained a dose-dependent inhibition of the Mx signal (Fig. 1D). Mutant E^{rns} (E^{rns} H30F), which represents the most appropriate negative control exclusively lacking the RNase activity of the wt protein (10),

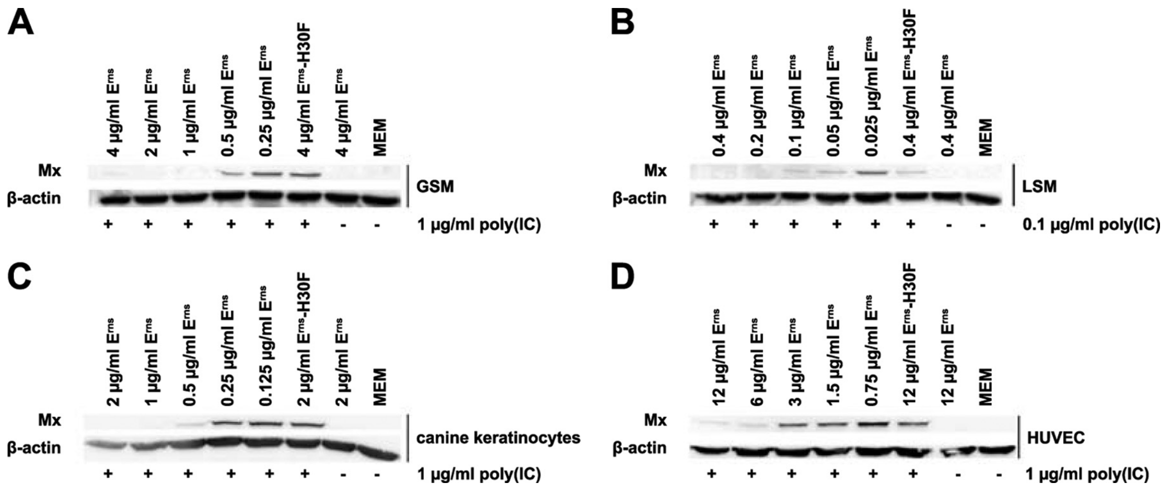

FIG 1 Extracellularly added E^{rns} inhibits Mx induction by poly(I·C) in nonbovine cells. Confluent GSM cells (A), LSM cells (B), canine keratinocytes (C), and HUVECs (D) were stimulated with poly(I·C) for 20 h in the presence or absence of E^{rns} or the RNase-inactive mutant E^{rns} H30F at various concentrations, as indicated. Cell culture medium (MEM) and E^{rns} applied at the highest concentrations used, but in the absence of poly(I·C), served as negative controls. Cytosolic extracts were assayed for Mx and β-actin expression by Western blotting. Typical results out of three independent experiments are shown.

was tested at the highest concentration applied with wt E^{rns} and did not inhibit dsRNA-induced Mx synthesis in any of the cell types tested.

E^{rns} enters the cells within minutes and remains active for several days. As the E^{rns}-containing supernatant could be removed just prior to the addition of dsRNA (10; unpublished observation), we investigated whether E^{rns} was actively taken up by the cells and how long its inhibition of IFN synthesis induced by dsRNA continued.

Notably, the ability of E^{rns} to block Mx induction after poly (I·C) stimulation remained intact for up to 3 days in BT cells after the cells were incubated for 1 h in the presence of wt E^{rns} but not mutant H30F with inactive RNase (Fig. 2). The E^{rns} protein was therefore firmly associated with the cells, which allowed us to temporally separate the incubation of the cells with E^{rns} from the one with poly(I·C) for further investigation. To assess whether E^{rns} remains on the cell surface or is taken up, we washed the cells with soluble heparin, which can compete for E^{rns} binding to the cell surface glycosaminoglycans (27). The activity of E^{rns} that might remain cell associated after the wash procedure was assayed by determination of its ability to block Mx induction upon subsequent addition of extracellular dsRNA (Fig. 3). E^{rns} lost most of its activity when the viral RNase was preincubated with soluble heparin, which indicates that heparin effectively prevents E^{rns} from binding to the cells (Fig. 3A). Similarly, washing the cells with heparin after they were preincubated with E^{rns} reduced the ability of E^{rns} to block the Mx synthesis induced by poly(I·C) in a time-dependent manner. Thus, washing the cells with heparin after 15 min of incubation with E^{rns} was able to reduce its activity to only about half of its maximal activity, and after 1 h, heparin was only marginally able to prevent the IFN-antagonistic properties of E^{rns} (Fig. 3B). Further reduction of the time interval between the addition of E^{rns} and washing with heparin showed that already at 1 min after E^{rns} addition, heparin was not able to completely remove E^{rns} from the cell surface (Fig. 3C). As the variability of the effect within the first 10 min of incubation was rather high (not shown), a quantitative assessment of the Western blot was not feasible. In contrast, heparin was still able to remove cell-bound E^{rns} when the latter was incubated for up to 6 h with the cells at 4°C instead of 37°C (Fig. 3D), revealing the involvement of an energy-dependent mechanism of uptake of extracellular E^{rns} protein into the cells. The notion of a washing effect by heparin was challenged by the fact that the E^{rns} RNase activity was decreased in the presence of high concentrations of heparin in *in vitro* experiments (data not shown). The reduction of the RNase activity was about 100 times lower in the presence of chondroitin sulfate, another glycosaminoglycan that, however, does not bind to E^{rns} (27). To verify that the inhibitory effect observed with heparin is based on its ability to wash away cell surface-bound E^{rns} and not inhibition of E^{rns} RNase activity, we either preincubated E^{rns} or washed E^{rns}-treated cells with heparin or chondroitin sulfate at concentrations that revealed the same inhibition of E^{rns} in the *in vitro* RNase assays. No reduction of the E^{rns} activity was observed when the protein was preincubated or washed with 100 μg/ml chondroitin sulfate, while even at 1 μg/ml, heparin retained the full activity required to interfere with the action of E^{rns} (Fig. 3E). Accordingly, at this low concentration, which is 100-fold lower than that used in the standard assays, heparin did not inhibit or only marginally reduced the RNase activity of E^{rns} in the *in vitro* RNase assays (not shown). These results clearly indicate that the effect of heparin on E^{rns}-

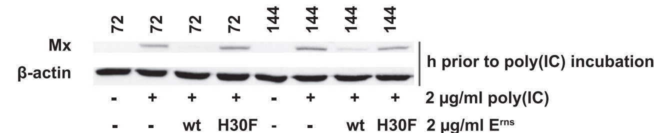

FIG 2 E^{rns} remains active for at least 72 h. BT cells were incubated in the presence of wt E^{rns} protein or the RNase-inactive mutant H30F for 1 h prior to washing the cells with MEM. Thereafter, cells were incubated for 3 days (72 h, as indicated above the lanes) or 6 days (144 h) in fresh medium. Samples incubated for 6 days were detached with trypsin-EDTA and passaged at a ratio of 1 to 2 in new flasks after 3 days. Subsequently, poly(I·C) stimulation was performed for 20 h. The samples were probed for Mx and β-actin protein expression by Western blotting as described in the legend to Fig. 1. A typical result out of three independent experiments is shown.

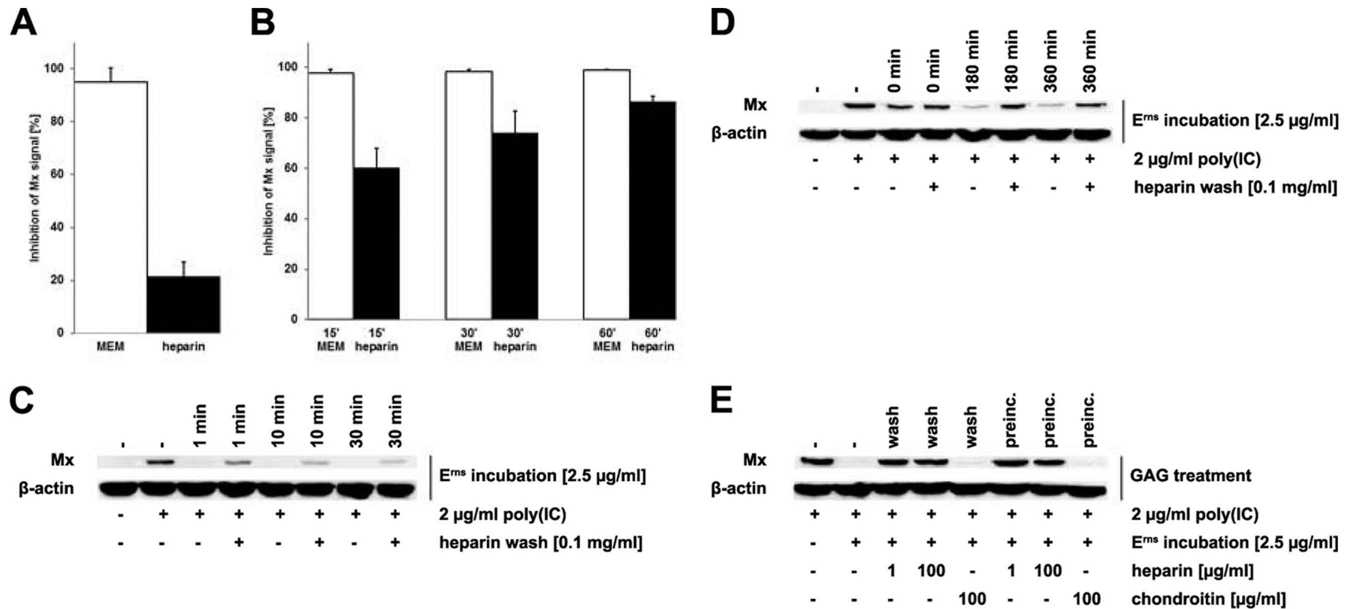

FIG 3 E^{rns} bound to the cell membrane can be washed off with soluble heparin. E^{rns} was preincubated in wash medium (0.1 mg/ml heparin in MEM) for 30 min before incubation on BT cells for 30 min at 37°C (A). Alternatively, E^{rns} was incubated with BT cells for 15 to 60 min (B) or 1 to 30 min (C), as indicated in the figure, in the absence of heparin prior to treatment with wash medium. Thereafter, BT cells were stimulated with 2 μg/ml poly(I·C) for 18 h at 37°C and cytosolic extracts were assayed as described in the legend to Fig. 1. The signal intensities of Mx synthesis were quantified relative to the levels of β-actin expression (A, B), with complete inhibition of Mx expression being set equal to 100% (mean ± SD, $n = 3$). Similarly, E^{rns} was incubated for up to 6 h prior to washing, as described above for panels B and C, but at 4°C instead of 37°C (D). To compare the inhibitory activity of heparin and chondroitin, E^{rns} was either preincubated at 4°C in the presence or absence of glycosaminoglycans (preinc.) or incubated on BT cells for 15 min at 4°C prior to washing with either heparin or chondroitin sulfate, followed by Western blotting as described in the legend to Fig. 1 (E). Typical results out of two (C) or three (D, E) independent experiments are shown.

treated cells is indeed related to the removal of the E^{rns} protein bound to the cell surface instead of the inhibition of its RNase activity.

Extracellularly added E^{rns} enters the cells via clathrin-dependent endocytosis. With the energy-dependent uptake of E^{rns} into the cells having been established, we further investigated the specific mechanism of this uptake process. Chlorpromazine (CPZ), an inhibitor of the assembly of clathrin-coated pits (28), and monodansylcadaverine (MDC), an agent blocking the transglutaminase (29), were used to block clathrin-dependent endocytosis. Caveolin-dependent endocytosis was inhibited with methyl-β-cyclodextrin (MβCD), an agent that extracts cholesterol from membranes (30, 31). 5-(*N*-Ethyl-*N*-isopropyl)amiloride (EIPA), a specific inhibitor of the Na^+/H^+ antiporter, was used to block macropinocytosis (32).The inhibitory activities of the agents in BT cells were confirmed by fluorescence-activated cell sorter analysis (not shown) of Alexa 488-labeled transferrin, cholera toxin subunit B, and dextran, markers for clathrin- and caveolin-dependent endocytosis (33, 34) and for macropinocytosis (35), respectively. In our bovine cells, MβCD indeed inhibited caveolin-dependent endocytosis but, to a lesser degree, also inhibited the clathrin-dependent pathway, which is in accordance with reports that showed that it also perturbs clathrin-coated vesicles (36). All these endocytosis inhibitors showed only minimal cytotoxicity in BT cells at the concentrations used in the assays.

To test the effects of the inhibitors on the uptake of the viral RNase, the efficacy of inhibition of dsRNA-induced Mx synthesis by cell surface-bound E^{rns} was assessed by determination of the ability of heparin to remove any residual E^{rns} proteins prior to the addition of dsRNA. Thus, E^{rns} preincubated on BT cells in the presence of CPZ and MDC for 1 h was partially washed away by heparin-containing medium, as verified by the loss of activity against Mx induction by poly(I·C). In the absence of any drugs, there was no or very little interference with E^{rns} activity by heparin washes (Fig. 4A). No decrease in the activity of E^{rns} was observed in the presence of MβCD and EIPA

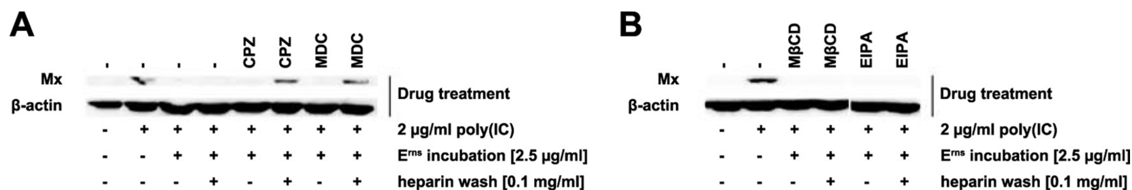

FIG 4 Inhibitors of clathrin-dependent endocytosis interfere with E^{rns} uptake. BT cells were preincubated in the presence of 25 μM CPZ or 0.2 mM MDC (A) and 10 mM MβCD or 20 μM EIPA (B) for 30 min. Thereafter, cells were supplemented with E^{rns} and incubated in the presence of the inhibitors for another hour. Washing of the cells with soluble heparin or medium was performed prior to stimulation of the cells with poly(I·C) for 18 h, followed by analysis for Mx and β-actin expression by Western blotting. Typical results out of three independent experiments are shown.

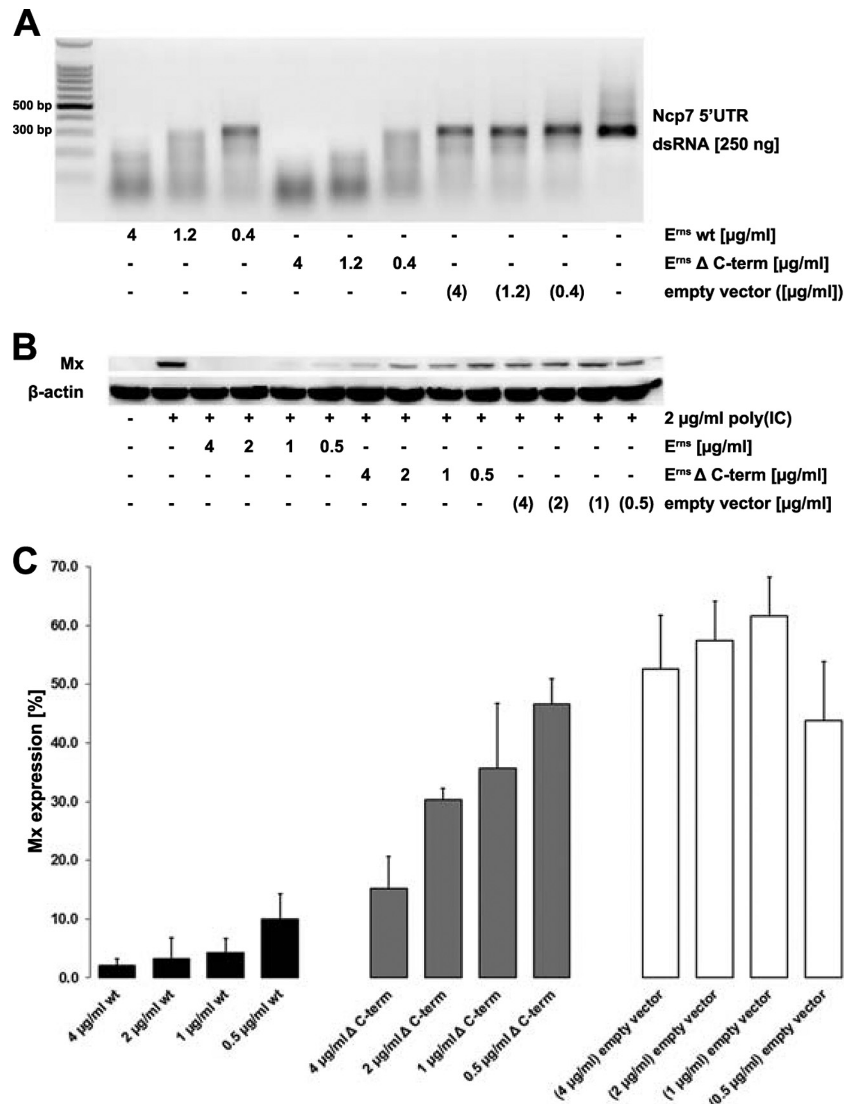

FIG 5 The activity for inhibition of IFN synthesis, but not the RNase activity, of E^{rns} is reduced in the absence of its C terminus. (A) A 300-bp dsRNA fragment from the 5′ UTR of BVDV strain Ncp7 was incubated in 100 mM Tris-acetate buffer in the presence of wt E^{rns}, E^{rns} lacking the C terminus (E^{rns} ΔC-term), or concentrated supernatant of cells transfected with the empty vector for 1 h at 37°C. RNA was separated on 1% agarose gels and visualized by UV light after staining with ethidium bromide. (B) E^{rns} was incubated on BT cells for 30 min before the cells were washed and incubated with 2 μg/ml poly(I·C) for 18 h at 37°C. Cytosolic extracts were assayed for Mx as described in the legend to Fig. 1. Typical results out of three independent experiments are shown. (C) Three independent replicates performed as described for panel B were quantified for the efficiency of E^{rns} inhibition of dsRNA-induced Mx expression by AIDA software, as described in the legend to Fig. 3, with the level of Mx expression induced by poly(I·C) alone being set equal to 100% (mean ± SD, $n = 3$).

(Fig. 4B), indicating that E^{rns} is basically taken up via clathrin-dependent endocytosis.

The IFN-antagonistic activity but not the RNase activity of E^{rns} is strongly reduced in the absence of its C terminus. The membrane association of E^{rns} has been attributed to an unusual membrane anchor at the C terminus of the protein (12). An E^{rns} mutant lacking the 37 C-terminal residues (E^{rns} ΔC-term) was constructed and tested for its ability to degrade dsRNA and to block IFN induction in BT cells stimulated by poly(I·C). Dose-dependent degradation of 300-bp dsRNA fragments of BVDV was observed to occur at an even slightly better efficiency with E^{rns} ΔC-term than with the wt enzyme (Fig. 5A), but the small difference might originate in the variability of the quantification of the protein concentration by ELISA rather than in a true difference in enzyme activity. The possibility of unspecific RNase activity in the supernatant of the cells expressing the E^{rns} protein was excluded, as equal amounts of supernatant concentrated from cells expressing the empty vector showed no activity against the dsRNA (Fig. 5A). In contrast, 1 μg/ml wt E^{rns} was sufficient to completely block Mx induction by poly(I·C), whereas 4 μg/ml of E^{rns} ΔC-term was only partially able to do so (Fig. 5B). Quantitative analysis of these Western blots revealed that wt E^{rns} is approximately 1 order of magnitude more efficient than E^{rns} ΔC-term in inhibiting the IFN synthesis induced by extracellularly added dsRNA (Fig. 5C). Again, cells incubated with supernatant concentrated from cells expressing the empty vector only slightly reduced the expression of the Mx protein in response to poly(I·C), and this was possibly caused by the large

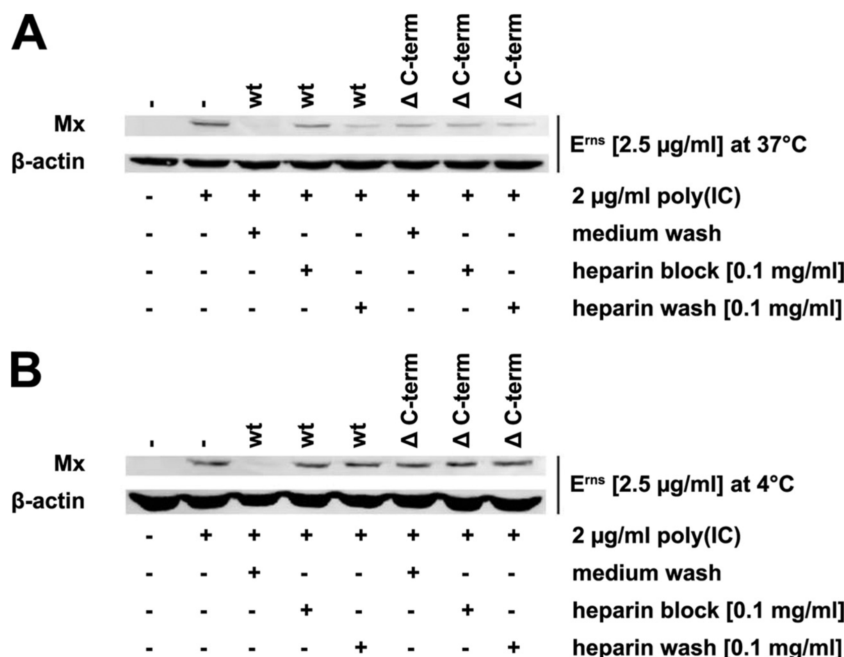

FIG 6 E^{rns} ΔC-term is not affected by heparin treatment at 37°C and is unable to bind to the cell surface at 4°C. E^{rns} was either preincubated in the presence of 0.1 mg/ml heparin for 15 min (heparin block) or left untreated before incubation on BT cells at 37°C (A) or 4°C (B) for 30 min. Unattached protein was removed by washes with medium (medium wash) or with medium containing 0.1 mg/ml heparin (heparin wash). Incubation of the cells in the presence of poly(I·C) at 37°C for 20 h was followed by extraction of the cytosol to evaluate Mx and β-actin expression by Western blotting. Typical results out of three independent experiments are shown.

amount of various serum components present in the concentrated cell culture supernatants (Fig. 5B and C).

Finally, as it was reported that a strong heparin-binding site is located within the C-terminal domain (9), we monitored the effect of the heparin washes on the inhibition of Mx synthesis by the E^{rns} ΔC-term mutant protein. In accordance with the findings shown in Fig. 5, the E^{rns} ΔC-term mutant did not completely inhibit the Mx synthesis induced by poly(I·C) (Fig. 6A). However, incubation of E^{rns} ΔC-term, but not wt E^{rns}, in the presence or absence of heparin for 15 min or washing with heparin-containing medium thereafter at 37°C had no discernible effect on the partial inhibition of dsRNA-induced IFN synthesis (Fig. 6A). Moreover, binding of E^{rns} ΔC-term to the cell surface was very weak and, thus, could be washed away at 4°C with cell culture medium even in the absence of heparin (Fig. 6B).

DISCUSSION

Pestiviruses express the three envelope glycoproteins E^{rns}, E1, and E2, with the first one being the most unusual, as it is the only viral structural protein to possess RNase activity (37). In addition, E^{rns} is also secreted from infected cells and was shown to cleave ss- and dsRNA and, thus, to antagonize the IFN synthesis induced by extracellularly added viral RNA (10, 11, 23). However, details of the mechanism of this innate immune evasion are not yet known. Here, we show that soluble E^{rns} is quickly taken up by cells (within approximately 1 h after addition) and remains active to inhibit dsRNA-induced IFN synthesis for several days. Uptake appears to be energy dependent, involves elements of clathrin-mediated endocytosis, and is much less efficient when E^{rns} lacks its C-terminal membrane anchor. These data provide strong evidence that E^{rns} is mainly active intracellularly, e.g., in endolysosomal compartments, to inhibit the effect of extracellularly added viral RNA that might trigger the host's pattern recognition receptors (PRRs) and activate the innate immune response. However, the precise intracellular location where E^{rns} resides and where it is active at degrading ss- and dsRNA remain to be determined.

The E^{rns} expressed in bovine cells was also able to efficiently block IFN induction by poly(I·C) in nonbovine cells, i.e., in cells of caprine, ovine, canine, and human origin (Fig. 1). This is in accordance with reports demonstrating that E^{rns}, mostly expressed in insect cells, is able to bind to the surface of a large variety of cell types from different species (20, 38). This also confirms that E^{rns}, in contrast to the envelope glycoprotein E2 (14), does not attach to the cells via a specific receptor, which would be expected to be more species specific. Thus, the activity of E^{rns} was similar in GSM and LSM cells and canine keratinocytes, all of which were from species that were shown to be at least partially susceptible to BVDV infection (39–42). In contrast, human cells are reported to be resistant to pestivirus infections, but E^{rns} was nevertheless able to inhibit dsRNA-induced Mx expression in HUVECs, though at concentrations somewhat higher than those used for the other cell types. The reason for this difference is not known but might be related to different numbers of E^{rns} binding sites (whether saturable or not) on the various cell types (20). In addition, further investigations are required to establish a possible link of cell permissibility for BVDV infection and the activity of E^{rns}.

The activity of the E^{rns} protein was surprisingly robust over time, maintaining its full activity even after 72 h (Fig. 2). Notably, even passaging of BT cells that were treated with E^{rns} for 1 h, but not the inactive mutant H30F, at 3 days posttreatment and further incubation in fresh medium for another 3 days only slightly reduced the inhibition of poly(I·C)-induced Mx synthesis. In con-

trast, E^{rns}-treated MDBK cells lost the capacity to inhibit dsRNA-stimulated IFN expression within 5 days even without passaging (not shown). This strongly indicates that a certain amount of E^{rns} must reside inside a cell at a given time point in order to be able to act as IFN antagonist, as MDBK cells proliferate much faster than BT cells, and over time, the viral RNase becomes thereby much more diluted in MDBK cells than in BT cells. This also suggests that the viral RNase must be present over the whole time period in order to block dsRNA-induced IFN synthesis rather than induce a state of unresponsiveness in the cell by an unknown mechanism. The former is not an implausible possibility, as E^{rns} is a very stable protein that displays RNase activity even at high salt concentrations or in 7 M urea (22, 43; unpublished observation). Accordingly, spiking of E^{rns}-treated BT cells with naive cells dose-dependently reduced the block in Mx synthesis, and this blocking activity could not be transferred via the supernatant (not shown), further indicating that E^{rns} mainly resides inside the cells. Notwithstanding its long-lasting effect, the uptake of E^{rns} was rather rapid, with maximal activity being reached after 30 to 60 min of incubation (Fig. 3). The protein was not internalized at 4°C, even when the incubation time was extended to 6 h, as shown by the strong washing effect of the heparin solution. For that reason, E^{rns} is clearly taken up by an energy-dependent pathway.

The uptake of E^{rns} was efficiently inhibited by inhibitors of clathrin-mediated endocytosis, CPZ and MDC, but not by inhibitors of caveolin-dependent endocytosis and macropinocytosis, MβCD and EIPA, respectively (Fig. 4), further confirming the energy dependence of the uptake mechanism. Interestingly, CPZ and MβCD were shown to completely inhibit BVDV infections (18, 44), but only CPZ was able to inhibit E^{rns} uptake in our experiments. However, unlike CPZ, MβCD was also able to partially inhibit infection with bovine herpesvirus 1 (BHV-1 [18]), and even though MβCD is widely used to block caveolin-dependent endocytosis (31, 45, 46), it might also perturb the formation of clathrin-coated endocytic vesicles (36). CPZ inhibited E^{rns} activity more efficiently than MDC, which might be based on its ability to bind to glycosaminoglycans, thereby competing for binding sites on the cell membrane (47), in addition to its activity as an inhibitor of clathrin-mediated endocytosis. Despite the limitations on the specificity of endocytosis inhibitors (48, 49), clathrin-dependent endocytosis is the most likely pathway for E^{rns} cell entry. The insensitivity to MβCD might indicate that the uptake of the E^{rns} protein differs from the uptake of the complete virion after binding of E2 to its receptor, CD46 (15). The latter is in accordance with the report that E2, but not E^{rns}, is able to prevent the cell-to-cell spread of CSFV (20). Nevertheless, the use of a similar uptake mechanism for E^{rns} and virus particles might well be beneficial for the virus, as any genomic viral RNA inadvertently released from virions or defective particles (50) into endolysosomal compartments might be degraded by free E^{rns} present at the same location or even by viral RNase originating from the virion prior to the activation of intracellular PRRs.

Removal of the C-terminal membrane anchor of E^{rns} strongly reduced its IFN-inhibitory activity without inhibiting its RNase activity (Fig. 5). Thus, the intracellular activity of E^{rns} ΔC-term was reduced by about 1 order of magnitude compared to that of the wt enzyme. Notably, the remaining activity as an IFN antagonist in cell culture could not be blocked by heparin treatment at 4°C and at 37°C (Fig. 6), indicating that the heparin-binding site that was reported to be present within the C-terminal membrane anchor (9) is important for cell attachment prior to internalization. It remains to be established whether other positively charged regions on the surface of the E^{rns} protein (9, 51) account for residual binding to and uptake into the cells. Remarkably, C-terminal peptides of E^{rns} were also reported to translocate across the cell membrane within minutes and to be targeted to, among other locations, the nucleoli, with peptides encompassing the heparin-binding site being the most efficient ones (38). The peptides were able to carry active enzymes, including full-length E^{rns}, into the cells, but this could not be reproduced even at high concentrations of E^{rns} (20; unpublished observation). In addition, penetration of the C-terminal peptides was reported to occur not only at 37°C but also at 4°C, indicating an energy-independent translocation, which is in contrast to what we observed in this study using full-length and C-terminally truncated E^{rns} (Fig. 3D and 6, respectively). The reasons for these discrepancies are not known, but an independent confirmation of the cell-penetrating properties of the C-terminal peptide fragments of E^{rns} is currently lacking.

The PRRs that are activated by extracellularly added dsRNA, such as poly(I·C), in various cell types are not known in every case. Knockdown of scavenger receptors that act as extracellular receptors for dsRNA affected activation of Toll-like receptor 3 (TLR-3) in endolysosomes and the cytosolic RIG-I-like receptors (RLRs) RIG-I and Mda-5 in a dsRNA length-dependent manner (52). However, the mechanism of how poly(I·C) escapes the endosomal compartment after clathrin-mediated endocytosis to activate the cytosolic RLRs is not yet known (52). As the pestiviral E^{rns} is remarkably stable, i.e., the RNase activity is not affected by temperatures of up to 60°C, pH values from 7 to as low as 4.5, or the presence of dithiothreitol (22, 37, 53), it can be envisioned that E^{rns} is able to degrade the RNA substrates in the extracellular space as much as in endolysosomal compartments prior to their possible escape to the cytosol, thus effectively preventing the activation of cytosolic and endolysosomal PRRs. Accordingly, intracellular E^{rns} of CSFV was shown to potently inhibit TLR-7-dependent IFN-α expression in plasmacytoid dendritic cells induced by cell-cell interaction with cells infected with RNA viruses, such as CSFV, foot-and-mouth disease virus (FMDV), or transmissible gastroenteritis virus (TGEV) (54). As the cell types employed in our study probably express TLR-3 rather than TLR-7 (55–57) and as E^{rns} is an effective ss- and dsRNase (11), degradation of viral ss- and dsRNA by E^{rns} might be able to effectively prevent the activation of TLR-3 and TLR-7/8, depending on the cell type analyzed.

Taken together, we show that extracellularly added E^{rns} has to enter the target cells in order to efficiently block the IFN synthesis induced by ss- and dsRNA. Quantification by ELISA of E^{rns} added to the cells and removed after 1 h of incubation indicated that, within the limits of quantification by this method, all of the E^{rns} was removed again, and thus, only a few molecules entered the cells. This further suggests that the enzymatic function of this protein, rather than a stoichiometric interaction with the viral RNA, may be responsible. The fate of the internalized E^{rns} protein is as yet unknown, but localization in an endolysosomal compartment appears to be likely. Immunofluorescent-antibody localization of E^{rns} after extracellular addition was not successful, possibly because the method is not sensitive enough to detect the few molecules that entered the cells, whereas the same antibody brightly stained the viral RNase in BVDV-infected cells.

As a nonstructural protein, BVDV N^{pro} efficiently blocks IFN synthesis, e.g., that induced by replication intermediates, in in-

fected cells. However, it is well established that not all host cells are infected with BVDV even in persistently infected animals. Genomic viral RNA replicating in the cytosol or being protected by the viral envelope is usually not accessible to the E^{rns} that is located extracellularly or intracellularly in endolysosomal compartments. However, any viral RNA that reaches these functionally extracellular compartments, either in a free form that is resistant to digestion by serum RNases (11) or in infected cells or cell fragments, might be a potential danger signal for uninfected cells that have an intact IFN signaling pathway. Therefore, we propose that the main function of E^{rns} as a viral RNase is to act as an enzymatically active decoy receptor that avoids the recognition of viral ss- and dsRNA by intracellular pattern recognition receptors and their subsequent activation of the innate immune response in uninfected cells. As a result, BVDV is able to establish persistent infection and to maintain the strain-specific B- and T-cell tolerance by perpetuating an innate immunotolerance while concurrently avoiding the detrimental effects of the systemic expression of type I IFN (for reviews, see references 26 and 58).

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