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Synthetic RNA derived from the foot-and-mouth disease virus genome elicits antiviral responses in bovine and porcine cells through IRF3 activation

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ABSTRACT

Foot-and-mouth disease virus (FMDV) is the causative agent of a highly transmissible disease affecting wild and domestic animals including pigs, cattle and sheep. The ability of synthetic RNA transcripts mimicking distinct domains in the non-coding regions of the FMDV genome (ncRNAs) to induce a potent innate immune response in swine cultured cells and mice has been previously described, as well as their enhancing effect on conventional inactivated FMD vaccines. Here, we provide evidence of the activation of interferon regulatory factor 3 (IRF3), a key transcriptional regulator of type I interferon (IFN)-dependent immune responses after transfection of swine and bovine cells with transcripts corresponding to the FMDV 3´ non-coding region (3´NCR). Induction of IFN-β and Mx1expression, concomitantly with antiviral activity and IRF3 activation was observed in bovine MDBK cells transfected with the 3´NCR. Our results link the stimulation of the innate immune response observed in 3´NCR-transfected cells to the intracellular type I IFN signaling pathway and suggest the potential use of these molecules for antiviral strategies in cattle.

1. Introduction

Foot-and-mouth disease virus (FMDV) is the etiologic agent of a disease affecting cloven-hooved wild and domestic animals worldwide. FMD is a major concern in animal health due to the rapid spread of the virus and the devastating economic consequences of the outbreaks involving restrictions in international trade of animals and animal products (Knight-Jones et al., 2016). Current FMD vaccines are based on chemically inactivated virus and many efforts are being invested on vaccine improvement aimed to achieve earlier, more robust and long-lasting immune responses (Robinson et al., 2016). The innate immune system stands as the fist-line defense recognizing viral infection through different pattern-recognition receptors (PRRs) able to detect the pathogen-associated molecular patterns (PAMPs), and then activate type I interferon (IFN) and proinflammatory cytokines responses leading to the antiviral state (Gebhardt et al., 2017). Endogenous or therapeutically induced early type I IFN responses triggered by immunopotentiators may confer protection until adaptive immunity is activated to an extent that the pathogen can be eliminated.

We have previously reported the immunomodulatory properties of small RNA transcripts corresponding to different structural and functional domains in the 5´and 3´-non-coding regions flanking the FMDV genome (ncRNAs). These synthetic non-infectious molecules exerted a broad antiviral effect against different viruses *in vivo* and their sensing as PAMPs by PRRs involved in antiviral immunity has been described (Borrego et al., 2015; Kloc et al., 2017; Lorenzo et al., 2014; Rodriguez-Pulido et al., 2012; Rodriguez-Pulido et al., 2011b). The enhancing effect of the ncRNAs on a conventional FMD vaccine in pigs has also been described (Borrego et al., 2017).

With the aim of extending the encouraging results obtained with the ncRNAs in swine to other farm species, we have explored the ability to elicit innate immune responses in bovine MDBK cells of the 3´non-coding region (3´NCR) transcripts, previously found to exhibit the highest IFN induction profile in porcine cultured cells among all the different ncRNAs assayed. To gain insight on the molecular mechanisms involved in the immunostimulatory effect of the ncRNAs, we analyzed the activation of the IFN regulatory factor 3 (IRF3), the crucial transcription regulator of type I IFN genes and IFN-stimulated genes (ISGs) in swine and bovine cells transfected with the 3´NCR transcripts.

2. Materials and methods

2.1. Cells and viruses

Swine kidney epithelial cells (SK6 and IBRS-2 lines) and Madin-Darby bovine kidney epithelial cells (MDBK line) (obtained from Centro

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de Investigación en Sanidad Animal, CISA-INIA, Valdeolmos, Spain) were grown in Dulbecco´s modified Eagle´s medium supplemented with 10% fetal calf serum, penicillin-streptomycin and L-glutamine. FMDV O1K isolate was used for infection experiments in SK6 and IBRS-2 cells; vesicular stomatitis virus (VSV) Indiana was used to infect MDBK cells.

2.2. RNA synthesis, transfection and RT-PCR

RNA transcripts corresponding to the 3´NCR of the FMDV O1K genome (186 nt including a 58-nt polyA tail) were synthetized by *in vitro* transcription with T3 RNA polymerase (NEB) from previously described plasmid linearized with *Not*I (Rodriguez-Pulido et al., 2011a). Then, RNAs were treated with RQ1 DNase (1 U/ μg; Promega), extracted with phenol-chloroform, and precipitated with ethanol, resuspended in water and quantified by spectrometry. The RNA integrity and size were analyzed by electrophoresis. Prior to transfection, RNAs were heated at 92 °C for 5 min, cooled down to room temperature for 10 min, and then chilled on ice. Poly(I:C) (Sigma-Aldrich) and *E. coli* MRE600 tRNA (Roche) were used as controls in some experiments. Approximately 1×10^6 SK6 or MDBK cells were transfected with 20 μg/ml 3´NCR transcripts, poly(I:C) or tRNA using Lipofectamine 2000 (Invitrogen). Cells were lysed at different times after transfection and total RNA was extracted, quantified by spectrometry and DNase-treated with Turbo DNA-free kit (Ambion). Then, aliquots of 500 ng RNA were used for RT-PCR analysis. Previously reported primers were used for amplification of GAPDH and bovine Mx1 (Borrego et al., 2015). Primers for amplification of a 252-bp fragment of bovine IFN-β were: 5´-TTAT CCACGAGATGCTCCAGCAG-3´ and 5´-AGGCACAGCTTCTGTACTCCT TGG-3´; the amplification products were analyzed on 2–2.5% agarose gels.

2.3. Immunoblot analysis

Total IRF3, phospho-IRF3 and Mx1 were detected by SDS-PAGE. For that, SK6 or MDBK cells transfected with 3´NCR transcripts or FMDV-infected were washed twice in ice-cold PBS and lysed at different times after transfection/infection in PBS containing 1% NP-40, 1 mM DTT and 1X Complete protease inhibitor cocktail (Roche). 20 μg of cell extracts was run on 10% SDS-PAGE gels, transferred onto nitrocellulose membrane and probed with the corresponding primary antibody. Blots were then incubated with the corresponding secondary antibody HRP conjugate (Thermo Scientific Pierce). Protein bands were visualized using Western Lightning Plus-ECL detection reagents (Perkin Elmer Inc.) followed by exposure to X-ray film.

IRF3 dimers were analyzed by native PAGE. Briefly, transfected or infected SK6 and MDBK cells were harvested in TEN buffer (40 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA), lysed in buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% NP40 and 1X Complete protease inhibitor cocktail (Roche), incubated for 5 min at RT and clarified by centrifugation. Supernatants were loaded onto 7.5% native gels. PAGE was performed as described (Iwamura et al., 2001). Gels were transferred onto nitrocellulose membrane and probed as above.

Primary antibodies used were: rabbit polyclonal anti-IRF3 (FL-425, Santa Cruz Biotech), rabbit monoclonal anti-Phospho-IRF3 (Ser 396) (4D4G, Cell Signaling), mouse monoclonal anti-Mx1 (AM39, Acris Antibodies) and rabbit polyclonal anti-βII tubulin (Armas-Portela et al., 1999).

2.4. Antiviral activity assays

The autocrine antiviral activity induced in MDBK cells by transfection with the FMDV 3´NCR was assessed by a VSV infection inhibition assay. 24 h after transfection with 20 μg/ml of RNA transcripts MDBK cells were infected with 50–100 PFU of VSV. Cytophatic effect (CPE) 24 h later was monitored by plaque assay on semi-solid medium, and compared with that in mock-transfected cells.

Paracrine antiviral activity of the supernatants from transfected MDBK cells was determined as described (Rodriguez-Pulido et al., 2011a). Antiviral activity against VSV or FMDV was assayed on MDBK or IBRS-2 cells, respectively. Briefly, MDBK cells were transfected for 24 h with 20 μg/ml of the 3´NCR transcripts, poly(I:C), tRNA or mock-transfected with PBS. Fresh monolayers of MDBK or IBRS-2 cells were incubated with different dilutions of transfection supernatants for 24 h, washed, and infected with 50–100 PFU of VSV or FMDV, respectively for plaque assay. Plaques were counted 24 h post-infection. In some cases, supernatants from MDBK transfected cells were incubated previously with 5–10 μg of polyclonal antibodies against bovine IFN-α (AHP2370, Bio-Rad). Antiviral activity was expressed as the reciprocal of the highest supernatant dilution needed to reduce the number of plaques by 50%.

2.5. Indirect immunofluorescence microscopy

SK-6 and MDBK cells were seeded on sterile glass coverslips and incubated at 37 °C for 24 h in 6-well cell culture plates (Nunc) and transfected with the 3´NCR RNA transcripts (as described in 2.2.). Cells were then washed with PBS, fixed with 4% paraformaldehyde for 20 min, blocked and permeabilized with PBTG buffer (0.1% Triton X-100, 1% BSA, 1 M glycine in PBS) for 15 min. Samples were incubated with a rabbit anti-IRF3 polyclonal antibody (FL-425 from Santa Cruz Biotech) for 1 h, washed with PBS, and incubated for 30 min with a donkey anti-rabbit IgG (H + L) Highly Cross-Adsorbed Alexa Fluor 488 conjugate. Both antibodies were diluted in 1% BSA PBS and nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) at 1 μg/ml (Invitrogen) for 5 min. All the steps were performed at RT. Coverslips were mounted on glass slides using Fluoromount-G (Southern Biotech). Fluorescence microscopy was carried out using an Axioskop (Zeiss) fluorescence microscope coupled to a Coolsnap FX monocrome camera (Roper Scientific). Vertical images were acquired at 63X magnification using a 1.4 Plan-Apochromat DICIII oil objective (Zeiss) using RS Image v.1.9.2 Software (Roper Scientific, Inc.) and processed with Adobe Photoshop 7 software (Adobe Systems, Inc.).

3. Results and discussion

3.1. Activation of IRF3 in porcine cells transfected with the 3´NCR transcripts

We have previously shown the induction of antiviral activity associated with IFN-β and Myxovirus resistance gene 1 (Mx1) mRNAs up-regulation, as well as activation of PRR signaling pathways, in 3´NCR-transfected swine cells (Borrego et al., 2015; Rodriguez-Pulido et al., 2011a). We sought to analyze the role of IRF3 in the potent antiviral response triggered in swine SK6 cells by the 3´NCR, and compare it with that elicited during FMDV infection.

Following viral infection, as a result of PRR signaling, the inactive form of IRF3 found in the cytoplasm undergoes phosphorylation of a series of serine residues (including Ser396) by TBK1 and IKKε kinases, inducing a conformational change leading to its dimerization and nuclear localization to form a complex which activates the transcription of the type I IFN and ISG genes (Ikushima et al., 2013). When lysates from SK6 cells transfected with the 3´NCR were analyzed by native PAGE, IRF3 dimers were observed from 2 to 6 h after transfection (Fig. 1A), being undetectable at 24 h post-transfection. In native PAGE monomers and dimers of IRF3 exhibit clearly distinct mobilities, being this method useful to monitor the early stage of IRF3 activation and to discriminate active and abortive phosphorylation of IRF3. The levels of total and phosphorylated IRF3 were also analyzed. While the levels of total IRF3 remained stable over time, the phospho-IRF3 fraction could only be detected from 2 to 6 h following transfection, fully overlapping with the time window of detection of dimeric IRF3 forms (Fig. 1A). Induction of