Short Communication

The p59 oligoadenylate synthetase-like protein possesses antiviral activity that requires the C-terminal ubiquitin-like domain

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Viral infection of mammalian cells prompts the innate immune system to initiate an antiviral response. The recognition of the virus triggers several antiviral signalling pathways, which among others include the family of 2'-5' oligoadenylate synthetase (OAS) proteins. The p59 protein encoded by the OAS-like (OASL) gene is an atypical member of the OAS family in the sense that it lacks the characteristic 2'-5' oligoadenylate synthetase activity. We decided to investigate the putative antiviral activity of p59 by ectopically expressing this protein in Vero cells and then infecting these cells with virus. We demonstrate that OASL has an antiviral effect against the single-stranded RNA virus picornavirus, encephalomyocarditis virus, but not against a large DNA virus, herpes simplex virus 1. Importantly, this antiviral activity was lost in a truncated version of p59 lacking the ubiquitin-like C-terminal domain of p59. Taken together our results indicate that p59 is indeed an antiviral protein that works through a novel mechanism distinct from other OAS proteins.

Interferons (IFN) are signalling molecules that constitute an important part of the vertebrate innate immune system (Stark et al., 1998). IFN induce an antiviral state via binding to their cognate receptor present on the cell surface. Receptor engagement triggers a signalling cascade mediated by Janus kinases (Jak) and signal transducers and activators of transcription (STAT) that leads to the transcription of hundreds of genes (Der et al., 1998). For the majority of IFN-induced proteins (ISGs), the downstream mechanism by which they inhibit viral replication is poorly understood. Nevertheless, for a few selected ISGs there is some understanding of the mechanism by which they block viral replication. For example, the double-stranded (ds) RNA-activated protein kinase (PKR) (Williams, 1999) and the p56 protein (Guo et al., 2000) inhibit translation in virus-infected cells by preventing initiation of protein synthesis. The family of 2'-5' oligoadenylate synthetase proteins (OAS) target translation indirectly via activation of a latent RNase (RNase L) that degrades both mRNA and rRNA in infected cells (Justesen et al., 2000).

The OAS proteins were originally discovered as dsRNA-induced inhibitors of protein synthesis (Hovanessian et al., 1977; Roberts et al., 1976). The activity of OAS proteins is under strict control. While the transcription of the OAS gene family is induced by IFN, the translated proteins are latent enzymes that require dsRNA for activation. The activation of the OAS enzymes results in the synthesis of 2'-5' linked oligoadenylates (2-5A) from ATP (Justesen et al., 2000). In turn, these 2-5A can bind to RNase L, which subsequently dimerizes into its active form. Activated RNase L then degrades viral and cellular RNAs suppressing protein synthesis and viral growth (Dong & Silverman, 1995).
The fourth member of the OAS family is the OAS-like gene product (p42, p44, p46 and p48). In this study, we used the p42 isoform of the OAS1 gene, referred to as p42. The fourth member of the OAS family is the OAS-like (OASL) gene located at locus 12q24.2 (Hartmann et al., 1998). The OASL gene encodes a two-domain protein of 59 kDa (hence referred to as p59). The p59 protein is composed of an N-terminal OAS domain fused to a 164 aa C-terminal domain, which has a weak sequence similarity (approx. 30% sequence identity) to a tandem repeat of ubiquitin. Notably, p59 lacks 2-5A synthetase activity (Hartmann et al., 1998; Reboyllatt et al., 1998), due to a number of mutations in the active site. Using the crystal structure of porcine OAS1 protein as a template for site-directed mutagenesis, it was possible to show that the active site of OAS is located in a cleft situated between the N- and C-terminal domains of OAS (Hartmann et al., 2003). The N-terminal domain is a five-stranded anti-parallel β-sheet domain, encoded by exons A and B. The majority of active site residues are contributed by the N-terminal domain of OAS. Most notably three aspartic acid residues coordinating the active site magnesium ion (Sarkar et al., 1999) and a short helical turn motif, known as the P-loop, which can wrap around the phosphates of the triphosphate of the incoming nucleotide. The lack of 2-5A synthetase activity in the p59 protein can be attributed to changes in the amino acid sequence within the active site. Mutations are found in both the P-loop sequence and the active site aspartic acids.

A thorough investigation of the OASL loci in mice revealed two genes, mOasl1 and mOasl2. The mOasl1 gene is the orthologue of the human OAS1 gene and like the human orthologue the gene product of the mOasl1 is devoid of any 2-5A synthetase activity (Eskildsen et al., 2003). The paralogous gene mOasl2 has retained its 2-5A synthetase activity and appears to be an evolutionary intermediate between the original OAS1 and the OAS1L gene family (Eskildsen et al., 2003). Throughout this paper OASL is referring to the human OAS1L gene unless otherwise stated. Full-length sequences encoding protein orthologues to the human OAS1L can be found in the database for a number of mammalian species (Perelygin et al., 2006). We aligned OAS1L from human, horse, dog, rat, mouse and cattle with the mOasl2 and OASL (isoform A) chicken. Both the mOasl2 and the chicken OASL genes encode active 2'-5' OAS enzymes (Tatsumi et al., 2000), whereas all the OASL1 proteins are presumed inactive. Fig. 1 shows the alignment where changes in the active site are highlighted, blue indicates residues found in active enzymes and yellow indicates changes incompatible with activity (as described below). Particularly, four conserved changes in the putative active site of OASL1 distinguish it from its active counterparts. Lysine 65 (K65) in the active mOasl2 is replaced by asparagine in all of the OASL1 proteins. K65 is a crucial part of the P-loop that is involved in binding of the donor ATP (Hartmann et al., 1998, 2003). Furthermore, the binding site for the catalytic metal ion is destroyed by two substitutions, since aspartate 83 (D83) in mOasl2 is replaced by a glutamate and aspartate 154 (D154) is replaced by a threonine. It is unlikely that these are merely random mutations due to the fact that these changes are well conserved within different OASL1 proteins in mammals. Rather these changes suggest that the OASL1 protein has acquired a novel function. Furthermore, the ubiquitin-like motif is strongly conserved among the mammalian OASL1 genes and divergent from the mOasl2 and chicken OASL. In this study, we demonstrate that human OASL1 protein has antiviral properties and that its ubiquitin-like motif is required for this antiviral function. Indeed the high degree of conservation of this domain among the OAS1L family suggests it plays an important role in the function of OAS1L.

Since the OASL gene is strongly induced by IFN (Hartmann et al., 1998; Reboyllatt et al., 1998), we speculated that this gene could be involved in antiviral defence. The expression of the human OAS1 gene (p42 isoform) in CHO cells resulted in an increased resistance towards picornaviruses (Chebath et al., 1987) and a similar antiviral effect of OAS2 was also observed (Ghosh et al., 2000; Marie et al., 1999). Therefore, to analyse the effect of p59 on viral replication we measured encephalomyocarditis virus (EMCV) yields from HEK293T transiently transfected cells with vectors encoding either p59, p59ΔUbi (a deletion mutant of the OAS1L protein without the ubiquitin-like domain) or the p42 encoded by the OAS1 gene. Cells were seeded in six-well plates and transfected with 2 μg DNA per well using the calcium phosphate method. Twenty-four hours post-transfection, cells were infected with EMCV at an m.o.i. of 0.01 TCID50.
per cell. Twenty-four hours post-infection with EMCV, cells were subjected to freeze–thaw and the virus containing-supernatant was harvested. The virus yield was determined by the TCID method using serial dilutions. Cells transfected with the vector encoding p59 or p42 gave a reduced viral yield compared with cells transfected with the empty vector (33 and 47 %, respectively) (Fig. 2a). Interestingly, cells transfected with the vector encoding p59ΔUbi showed little difference in virus yields compared to control cells. The expression of the different proteins was verified by Western blotting (data not shown).

Assaying viral replication in cells that are transiently transfected can cause several problems both due to variation in the transfection efficiency and due to normal viral replication in non-transfected cells. To avoid this we established stable pools of cells expressing the same construct described above. To bypass possible interference with the antiviral assays due to endogenous IFN production by infected cells, we utilized Vero cells that lack the entire type I IFN chromosomal region (Emeny & Morgan, 1979). After G418 selection, pools of resistant cells were propagated. The expression of either p42, p59 or p59ΔUbi in Vero cells resistant pool was verified by Western blotting (Fig. 2d). The different pools were infected with EMCV at an m.o.i. of 0.01 and virus yields were determined. Mean and standard deviation of four independent experiments are shown. Statistical analysis of these results is discussed in the text. (c) Using the same stable transfected pools of Vero cells as in (b), we performed direct titration of our standard EMCV stock. Titres obtained from cells expressing the indicated construct are shown. Data from two independent experiments are shown. (d) Western blot on whole-cell extracts from the stable pools of Vero cells using V5 antibody showing the expression of p42, p59 and p59ΔUbi.

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**Fig. 2.** EMCV is inhibited by p59 and p42 but not by p59ΔUbi. HEK293T cells were transfected with a vector encoding the different OAS proteins p42, p59 or a truncated version lacking the ubiquitin-like domain (p59ΔUbi) and a vector control plasmid (pcDNA3.1, Neo). All constructs were tagged with a V5 epitope. (a) Cells were infected with EMCV, 24 h post-transfection the virus yields were measured as described in the text. Mean and standard deviation of three independent experiments are shown. (b) Vero cells were transfected with the same constructs described above and selected for stable integration of the constructs. These cells were infected with EMCV at an m.o.i. of 0.01 and virus yields were determined. Mean and standard deviation of four independent experiments are shown. Statistical analysis of these results is discussed in the text. (c) Using the same stable transfected pools of Vero cells as in (b), we performed direct titration of our standard EMCV stock. Titres obtained from cells expressing the indicated construct are shown. Data from two independent experiments are shown. (d) Western blot on whole-cell extracts from the stable pools of Vero cells using V5 antibody showing the expression of p42, p59 and p59ΔUbi.
We also measured the antiviral effect using a direct approach by analysing the sensitivity of our different pools of Vero cells to our standard stock of EMCV (Fig. 2c). In this experiment, p59-expressing cells returned a virus titre that was 13-fold lower than those transfected with the empty pcDNA vector. The titre is measured as the minimal dose of virus needed to kill the cells, also known as TCID50. Thus, as expression of p59 decreases the apparent titre of the virus stock (Fig. 2c), we assume that the expression of p59 increases the cellular resistance towards the cytopathic effect of EMCV. Again, we observed no significant antiviral activity by expressing p42 in Vero cells. This might be a specific effect observed in Vero cells since we saw comparable antiviral activity of p42 and p59 by transient transfection in HEK293T cells (Fig. 2a).

Both OAS1 and OAS2 show restricted antiviral activity, inhibiting the replication of EMCV, but not vesicular stomatitis virus (VSV) (Chebath et al., 1987; Ghosh et al., 2000). To test if the antiviral activity of p59 was restricted to some viruses such as the picornavirus, we performed similar experiments as described above using a dsDNA virus from the family Herpesviridae, herpes simplex virus (HSV)-1. The same stable pools of Vero cells expressing the different OAS constructs were infected with HSV-1, at an m.o.i. of 0.01 and the virus yield was measured after 24 h by plaque assay (Fig. 3). In contrast to the results observed with EMCV, no significant differences in HSV-1 yields were observed with pools expressing any of the constructs compared to controls. This experiment was conducted at a range of different m.o.i. values from 0.001 to 1 with all showing no effect upon HSV-1 replication (data not shown). Therefore, p59 does not seem to have any antiviral effect on HSV-1 replication. This might be explained by the fact that large DNA viruses encode several proteins that inhibit antiviral pathways. Specifically, the HSV-1 Us11 protein is capable of inhibiting the induced 2-5A synthetase activity in infected cells (Sanchez & Mohr, 2007). Whether the Us11 protein is active against the OASL protein remains to be tested.

The discovery of p59 as an antiviral protein naturally leads to the consideration of the mechanism behind this activity. It has been shown that p59 expressed in mammalian cells is not capable of synthesizing 2-5A (Rebouillat et al., 1998). However, as a control we performed immunoprecipitation with an antibody directed against the V5 epitope found in the pcDNA vector, we found 2-5A synthetase activity associated with the p42 isofrom of OAS1 but not with the p59 protein (data not shown). Therefore, the mechanism behind the antiviral effect displayed by p59 must be independent of the synthesis of 2-5A and the subsequent activation of RNase L by 2-5A. In summary, the p59 protein exhibits its antiviral activity by a novel and yet undisclosed mechanism requiring its unique ubiquitin-like domain.

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References


