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A potential robust antiviral defense state in the common vampire bat: expression, induction and molecular characterization of the three interferon-stimulated genes - OAS1, ADAR1 and PKR

Sarkis Sarkis^{a,*}, Stéphanie Dabo^b, Marie-Claude Lise^a, Christine Neuveut^b, Eliane F. Meurs^b, Vincent Lacoste^a and Anne Lavergne^{a,*}

^a Laboratoire des Interactions Virus-Hôtes, Institut Pasteur de la Guyane, Cayenne, French Guiana

^b Hepacivirus and Innate Immunity, Institut Pasteur, 75015 Paris, France

* Corresponding authors.

Laboratoire des Interactions Virus-Hôtes, Institut Pasteur de la Guyane, 23 avenue Pasteur, 97300 Cayenne, French Guiana.

E-mail addresses : sarkis.sarkis@hotmail.com (S. Sarkis); alavergne@pasteur-cayenne.fr (A. Lavergne)

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Abstract

Bats are known to harbor many zoonotic viruses, some of which are pathogenic to other mammals while they seem to be harmless in bats. As the interferon (IFN) response represents the first line of defense against viral infections in mammals, it is hypothesized that activation of the IFN system is one of the mechanisms enabling bats to co-exist with viruses. We have previously reported induction of type I IFN in a cell line from the common vampire bat, *Desmodus rotundus*, upon polyinosinic:polycytidylic acid (poly(I:C)) stimulation. To deepen our knowledge on *D. rotundus*' IFN-I antiviral response, we molecularly characterized three interferon-stimulated genes (ISGs), *OAS1*, *PKR* and *ADAR1*, closely implicated in the IFN-I antiviral response, and tested their functionality in our cellular model. We first found that *D. rotundus* encoded two *OAS1* paralogs, *OAS1a* and *OAS1b*, and that the functional domains of the four ISGs characterized were highly conserved with those of other mammals. Despite their significant transcription level in the absence of stimulation, the transcription of the four ISGs characterized was enhanced by poly(I:C). In addition, the transcription of *OAS1a* and *OAS1b* appears to be differentially regulated. These findings demonstrate an active ISG antiviral response in *D. rotundus* in which *OAS1b* may play an important role.

Highlights

- Four ISGs implicated in the antiviral response were molecularly characterized.
- Two *OAS1* paralogs were characterized in *D. rotundus*.
- The functional domains of the characterized ISGs are highly conserved relative to their mammalian homologs.
- Significant induction of *Oas1b* was observed after stimulation with poly(I:C).

1. Introduction

The interferon (IFN)-mediated innate immune response provides a robust first line of defense against invading pathogens. Pathogen-associated molecular patterns' (PAMP) recognition by pattern recognition receptors (PRRs) leads to a subsequent production of IFN, especially of types I (IFN α/β) and III (IL28A, IL28B, IL29), considered as the main antiviral IFNs (Borden et al., 2007). Newly synthesized IFN molecules up-regulate the transcription of hundreds of interferon-stimulated genes (ISGs) resulting in the production of various antiviral effectors (Der et al., 1998). This cascade leads to a remarkable antiviral state of infected and neighboring cells and limits the spread of infectious agents. Considering the large number of ISGs activated upon IFN signaling, considerable redundancy in the system is likely. Among the molecules with important biological functions induced by IFN are the 2'5' oligoadenylate synthetase/ribonuclease L (OAS/RNase L) system, the adenosine deaminase RNA-specific (ADAR) as well as the dsRNA-activated protein kinase (PKR), which play a critical role in the host's antiviral defense mechanism and are among the most extensively studied ISGs (Der et al., 1998; George and Samuel, 1999; Levy et al., 2002; Sen, 2001). Even though the specific functions of mammalian OAS1, ADAR and PKR have yet to be clarified, their structural (architectural) domain organizations are well characterized and show a high degree of similarity among species.

The OAS gene family comprises three classes of genes in human, *OAS1*, *OAS2* and *OAS3*, in addition to an "OAS-like" (*OASL*), which is catalytically inactive. The mouse OAS cluster, the second most well known after the human OAS cluster, contains one *OAS2* and one *OAS3* gene; two *OASLs*, of which one is a pseudogene; and eight paralogs of *OAS1*, *Oas1a*–*Oas1h* (Kristiansen et al., 2011). Activation of the OAS proteins in presence of single-stranded (ss) or double-stranded (ds) RNA triggers the synthesis of 2'5' oligoadenylates. These in turn bind to and activate the latent RNaseL, which restricts viral propagation through direct and indirect

70 mechanisms, including i) viral genome degradation (RNA and DNA), ii) cellular mRNA
71 degradation, including mitochondrial RNA followed by apoptosis, as well as iii) amplification
72 of IFN signaling through the release of additional PAMPs activating cytoplasmic helicases,
73 which in turn activate type I IFN synthesis and create a positive feedback loop (Justesen et al.,
74 2000; Malathi et al., 2007, 2005; Silverman, 2003; Xiang et al., 2003). Regarding its domain
75 organization, on its N-terminus OAS1 comprises a nucleotidyl transferase domain (NTP-
76 transf_2) constituted of a dsRNA-binding domain flanked by two ATP-binding domains,
77 which plays a critical role in OAS1 activation. On its C-terminus, OAS1 presents the
78 OAS1_C domain including the region of enzymatic activity thought to activate latent
79 RNaseL (Sarkar et al., 2002).

80 ADAR belongs to a family of proteins that modulate nucleic acid integrity and play an
81 important role in the defense against viral RNAs. All ADAR family members present double-
82 stranded RNA-binding domains (dsRBDs) at their N-termini and a catalytic deaminase
83 domain located at their C-termini (Mannion et al., 2015). A unique feature of ADAR1, when
84 compared to other members of the ADAR family, is the presence of Z-DNA-binding domains
85 (ZBDs) at its N-terminus with a $Z\alpha$ and a $Z\beta$ domain (Kim et al., 1999). In addition to the
86 capacity of the $Z\alpha$ domain to bind to Z-DNA/RNA (Herbert et al., 1997), it has been reported
87 that all proteins containing this domain are implicated in the type I IFN response pathway
88 (Athanasiadis et al., 2005). Furthermore, the $Z\alpha$ domain is essential for the location of
89 ADAR1 to cytoplasmic stress granules following activation. Upon binding to dsRNA,
90 ADAR1 induces editing of adenosine to inosine, one of the most abundant modifications of
91 mammalian RNA (Bazak et al., 2014; Goodman et al., 2012). However, depending on the
92 viral species, the editing event can make ADAR1 act as either a proviral or an antiviral factor.
93 Indeed, whereas editing by ADAR1 of lymphocytic choriomeningitis virus introduces
94 mutations in viral proteins and reduces infectivity, editing of HIV-1 rev and tat proteins leads

to increased infectivity (Doria et al., 2009). In addition, ADAR1 can also exert a proviral function through its Z-DNA and dsRBD domains by interacting with PKR, another dsRBD-containing protein. Such interaction negatively regulates the antiviral function of PKR (Clerzius et al., 2009).

PKR (protein kinase dsRNA-dependent) is one of the four eIF-2 α serine-threonine kinases that controls protein synthesis through the phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF-2 α). PKR is ubiquitously expressed and can be induced upon IFN treatment (Meurs et al., 1990, 1992; Williams, 2001). PKR becomes activated upon binding to dsRNA, as during viral infection, or to cellular activators in response to stress (Dabo and Meurs, 2012). Because of PKR's role in controlling protein synthesis, it can inhibit infection by a number of viruses. Nevertheless, other viruses, such as the hepatitis C virus, benefit from the general shut-down of protein synthesis, which promotes their own eIF-2 α -independent translation (Arnaud et al., 2010; Garaigorta and Chisari, 2009). In addition, PKR modulates signaling pathways involved in inflammation independently of its enzymatic activity (Bonnet et al., 2006; García et al., 2007). PKR consists of two functionally distinct domains: a N-terminal dsRNA binding regulatory domain (dsRBD) and a C-terminal kinase catalytic domain. The dsRNA-binding domain contains two dsRNA-binding motifs (dsRBMs). These motifs are rich in basic residues and confer to PKR the capability of binding to dsRNA independently of the sequence. As a member of the kinase proteins, PKR presents all the conserved catalytic kinase subdomains. In addition, the homology within subdomain VI identifies PKR as a serine/threonine kinase (Meurs et al., 1990). PKR also possesses an insert region C-terminal of Domain IV. This insert region and the dsRBD are key determinants of the PKR function and regulation.

Bats are known to harbor many zoonotic viruses, some of which are pathogenic to other mammals while they seem to be harmless or only rarely associated with clinical signs in bats

(Moratelli and Calisher, 2015). Strikingly, mechanisms that allow bats to coexist with these viruses have not yet been fully researched. It is currently acknowledged that early control of viral replication by their innate immune system might be one of the mechanisms implicated, enabling bats to coexist with viruses (Baker et al., 2013). Following the establishment of a *Desmodus rotundus* cell line, FluDero, and the molecular characterization of the RIG-I-like receptor members and of type I interferons (Sarkis et al., 2018), we studied several ISGs from this bat species given that they are responsible for the antiviral activity of IFNs in mammals (Der et al., 1998; He et al., 2014; Levy et al., 2002; Sen, 2001; Zhou et al., 2013). Here, we describe the molecular characterization of three ISGs, *OAS1*, *ADAR1* and *PKR*, from *Desmodus rotundus* and report differential expression profiles for these genes upon stimulation of the FLuDero fetal lung cell line with the synthetic analog of dsRNA, the polyinosinic:polycytidylic acid, poly(I:C).

2. Materials and Methods

2.1. Cell line, cell culture and poly(I:C) stimulation

Establishment of the FluDero cell line has been described previously (Sarkis et al., 2018). The cell line was cultured in prewarmed DMEM/Ham's Nutrient Mixture F12 (Sigma) supplemented with 10% fetal bovine serum (FBS) (Sigma), 100 units/mL penicillin and 100 µg/mL streptomycin and was maintained in a humidified incubator with 5% CO₂ at 37°C. For poly(I:C) stimulation, FLuDero cells were seeded at a density of 2.5×10^5 cells/well in 12-well plates. Cells were stimulated by transfection with 20 µg/mL of poly(I:C) (Sigma, cat #P9582) by calcium phosphate transfection (Invitrogen), 20 h after seeding. Cells were harvested in TRIzol Reagent at 0, 3, 6, 9, 12, 24, 48 and 72 h post-transfection and stored at -80°C until RNA extraction. The experiment was performed three times.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from FLuDero cells transfected with poly(I:C) using TRIzol Reagent (Invitrogen) as recommended by the manufacturer. cDNAs were synthesized using Superscript[®] III reverse transcriptase (Invitrogen) and random hexamers following the manufacturer's instructions.

2.3. Sequence identification and domain characterization of *OAS1*, *ADAR1* and *PKR*

To generate the full-length coding sequences of *OAS1*, *ADAR1* and *PKR*, all amplifications were performed on cDNA obtained from poly(I:C)-transfected FLuDero cells. Internal coding regions were amplified by PCR using the AmpliTaq Gold DNA polymerase kit (Thermo Fischer Scientific). Different combinations of consensus and specific degenerate primers were designed using Primer3 (v.0.4.0) based on alignments of orthologous mammal sequences, which were available in the GenBank database (**Supplementary Table 1**). The 5'- and 3'-terminal sequences were obtained by rapid amplification of cDNA ends (RACE) using the FirstChoice[®] RLM-RACE Kit (Ambion, Inc.). PCR and RACE products were cloned using the pCR[™]4-TOPO[®] TA CLONING[®] KIT (Invitrogen). The sequences obtained were confirmed by similarity analysis using the NCBI BLAST search tool (<http://www.ncbi.nlm.nih.gov/BLAST>). The structural domains of each protein were identified using SMART software (<http://smart.embl-heidelberg.de/>) (Letunic et al., 2015). Multi-alignment was performed with the ClustalW program (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalw.html) using default parameters.

2.4. Phylogenetic analysis

The complete coding sequences for *ADAR1* and *PKR* of 18 and 17 representatives Laurasiatherian and Euarchontoglires, respectively, were downloaded from the NCBI database. Available sequences included those from the bat species *Eptesicus fuscus*, *Miniopterus natalensis*, *Myotis brandtii*, *Myotis davidii*, *Myotis lucifugus*, *Pteropus alecto*,

Pteropus vampyrus, *Rhinolophus sinicus* and *Rousettus aegyptiacus* (**Supplementary Table 2**). The ISG sequences of *D. rotundus* obtained in this study were further added to each data set. After alignment and editing, the MEGA version 6.0 software (Tamura et al., 2013) was used to compute pairwise comparisons in the nucleotide and amino acid using uncorrected p-distances. The best-fit amino acid evolutionary substitution model (Jones-Taylor-Thornton + G + F) was determined using MEGA version 6.0. Bayesian phylogenetic analyses were performed using MrBAYES 3. Markov Chain Monte Carlo (MCMC) simulations were run for 10,000,000 generations, with four simultaneous chains, using a sample frequency of 500 and a burn-in of 25,000. Majority rule consensus trees were obtained from the output. Validation of the inference was assessed based on the standard deviation of split frequencies, which was less than the expected threshold value of 0.01 (calculated value of 0.002). ADAR1 and PKR trees were rooted with the rodent *Mus musculus* sequences. Coding sequences from 33 representative mammalian species retrieved in NCBI were used for OAS1 pairwise comparisons and phylogenetic analysis (**Supplementary Table 2**). Eight corresponded to the bat species *E. fuscus* (two paralogs), *M. natalensis* (two paralogs), *M. brandtii* (two paralogs), *M. davidii*, *M. lucifugus* (two paralogs), *P. alecto*, *P. vampyrus* and *R. aegyptiacus*. An OAS1 tree was constructed based on the alignment of amino acid residues and was rooted with OASL sequences from *M. natalensis*, *P. alecto*, *P. vampyrus* and *R. aegyptiacus*.

2.5. Quantitative PCR

Quantitative PCR (qPCR) was performed on a StepOne™ Real-Time PCR System using the TaqMan® Universal PCR Master Mix (Applied Biosystems). Primers and TaqMan® probes were designed using Primer Express 3.0 (Applied Biosystems) (**Supplementary Table 1**). Each reaction was performed in duplicate. The cycling conditions were those recommended by the manufacturer: 55°C for 2 min, 95°C for 2 min followed by 40 cycles at 95°C for 15 s, 60°C for 1 min. For absolute quantification, the exact number of copies of the

gene of interest was calculated using a plasmid DNA standard curve. Individual expression values were normalized and compared to mRNA encoding β -actin. Values are shown graphically as fold induction compared to the mock sample (0 h transfection) for each experiment.

3. Results

3.1. Sequence characterization of *D. rotundus* *OAS1a*, *OAS1b*, *ADAR1* and *PKR* genes

Primers derived from alignments of the orthologous mammalian *OAS1*, *ADAR1* and *PKR* sequences were used to characterize *OAS1*, *ADAR1* and *PKR* genes from mRNA extracted from the FluDero cell line. The full-length coding sequences for *ADAR1* and *PKR* were identified. Sequence analysis revealed that their coding regions were 3444 and 1611 base pairs (bp) long, respectively, encoding proteins of 1149 and 537 amino acids (aa), respectively. In addition, we characterized two *OAS1* paralog genes in *D. rotundus*, which we named *OAS1a* and *OAS1b*. The partial coding sequence of *OAS1a* was 1056 bp in length (352 aa). On the other hand, the complete coding region of *OAS1b* was 1101 bp long encoding a 367-aa protein. When comparing the *OAS1a* and *OAS1b* with their mammalian counterparts, *OAS1a* seems to lack 17aa at its C-terminal.

Pairwise comparison done on 897 nucleotides corresponding to 299 amino acid residues showed that the two *OAS1* paralogs shared 81.6 and 77.3% of sequence identity at the nucleotide and amino acid levels, respectively. However, when compared to their mammalian homologs, *OAS1a* showed a higher level of sequence identity at the nucleotide and amino acid levels than *OAS1b*. For instance, *OAS1a* exhibited 84% and 80% identity at the nucleotide and amino acid levels, respectively, and *OAS1b* shared 78 and 73% identity with the *OAS1* sequence of the *Pteropus* species. In the same way, *OAS1a* shared 73–80% amino acid identity with its equine, canine, bovine and human homologs, while *OAS1b* presented 69–

75% sequence identity (**Supplementary Table 3**). *D. rotundus*' *ADAR1* and *PKR* were also subjected to pairwise comparison with their mammalian homologs. Among the four ISGs characterized, the complete amino acid sequence of *ADAR1* displayed the highest level of sequence identity with its mammalian counterparts, while *PKR* shared the lowest. For instance, *ADAR1* and *PKR* displayed 85.4–89.6% and 64.7–71% sequence identity at the protein level with their bat homologs, respectively (**Supplementary Table 3**). In addition, when comparing *D. rotundus* *PKR* with other mammalian homologs the identity at the protein level was slightly lower.

3.2. Conserved domain organization in *D. rotundus* ISGs

One of the hallmarks of the ISGs is their domain architecture responsible for their function. Sequence analysis of the two OAS1 paralogs identified in *D. rotundus* using the SMART software showed that these two proteins presented a similar structural domain organization to that described in their mammalian homologs. They possess the two characteristic functional domains of OAS1, *i.e.*, the NTP-transf_2 domain on their N-termini and C-terminal OAS1_C domain. These domains are located between aa 36–121 and 162–345, respectively (all values are given relative to *H. sapiens* sequences) (**Figure 1**). The *D. rotundus* OAS1a and OAS1b putative proteins showed conservation of the hallmark motifs crucial for OAS1 activity, *i.e.*, the LxxxP sequence required for enzymatic activity, a P-Loop motif with a (G[G/S]xx) sequence, which precedes the first triad residue corresponding to the second signature of the nucleotidyl transferase superfamily formed by Asparagine residues (represented with an inverted triangle), a K-R rich region and a CFK motif located in the C-terminal domain (**Figure 1**). Similarly, the *D. rotundus* *ADAR1* protein contained all domains necessary for its function, including at its N-terminus the two $Z\alpha$ domains, a central dsRBD with three dsRBM, and a catalytic deaminase domain at its C-terminus. These domains and motifs are located between aa 132–202, 291–360, 504–569, 615–680, 727–793 and 840–

1222, respectively (**Figure 2a**). Finally, the deduced protein sequence of *D. rotundus* PKR showed the conservation of all domains relative to its function including the N-terminus dsRBD with the two dsRBM (dsRBM1 and dsRBM2) between aa 9–81 and 102–169 (yellow and light gray boxes, respectively) and all subdomains (open boxes) in the catalytic region. A variable region was also found in *D. rotundus* PKR between the dsRBM2 and the basic region (in dark gray) between aa 229 and 251. In addition, *D. rotundus* PKR presented a conserved insert region at the C-terminal of subdomain IV (blue) between aa 362 and 370. However, *D. rotundus* PKR did not possess the repeat sequence (green) found in the catalytic region of the human PKR, a common feature observed in other bat species and mice (**Figure 2b**).

3.3. The *D. rotundus*-characterized ISGs are closely related to their mammalian counterparts

To elucidate the evolutionary relationships of OAS1a and OAS1b, ADAR1 and PKR, phylogenetic trees were constructed based on multiple amino acid alignments constructed with other published mammal sequences (**Figure 3**). The OAS1 phylogenetic topology obtained was in agreement with the different mammalian orders, showing that OAS1a and OAS1b from *D. rotundus* belonged to a single clade of chiropters. Within this clade, OAS1a and OAS1b clustered with OAS1-like and OAS1 of microbats, respectively, and were supported with high posterior probability values (**Figure 3a**). On the other hand, the phylogenetic analyses of ADAR1 (**Figure 3b**) and PKR (**Figure 3c**) showed that all bat sequences belonged to a clade distinct from other mammal species, with the microbats (*D. rotundus*, *M. natalensis*, *E. fuscus* and *Myotis* species) diverging from the megabats (*Pteropus* species, *R. aegyptiacus*, and *R. sinicus*).

3.4. *D. rotundus* OAS1, ADAR1 and PKR can be induced in response to poly(I:C) stimulation

To investigate the functionality of these ISGs in FLDero, we studied their expression profile after poly(I:C) transfection (**Figure 4**). The kinetics of the expression of OAS1a,

266 OAS1b, ADAR1 and PKR mRNA revealed a similar induction profile. The induction was
267 observed 6 h post-transfection, reaching a peak at 24 h followed by a decline at 48 h. Among
268 the ISGs studied, *OAS1b* was the most inducible gene (400-fold up-regulation at 24 h) at all
269 time points of the kinetics. In contrast, *ADAR1* and *PKR* were the least induced genes,
270 showing a peak of induction at 24 h with a 15- and nine-fold induction, respectively.
271 Strikingly, in the absence of stimulation, transcription of the four ISGs characterized was
272 already significant, with a strong expression of PKR mRNA (**Figure 5**, 0 hrs).

273

4. Discussion

Viral infection in mammals often triggers the IFN-I-mediated frontline host defense mechanism, leading to the transcription of a wide range of ISGs (Randall and Goodbourn 2008). These ISGs confer an antiviral state to the host and neighboring cells by several mechanisms, including inhibition of viral transcription, translation and replication, as well as degradation of viral nucleic acids. We previously demonstrated that FLDero cells were competent to mount high levels of IFN-I after transfection with poly(I:C) (Sarkis et al., 2018). To deepen our understanding of the *D. rotundus*' IFN-I antiviral response, we molecularly characterized three ISGs, *OAS1*, *PKR* and *ADAR1*, closely implicated in the IFN-I antiviral response and looked at their expression upon poly(I:C) stimulation of FLDero cells.

Contrary to other mammals such as in human, which only possesses one *OAS1* gene, we characterized two *OAS1* paralogs (*OAS1a* and *OAS1b*) in *D. rotundus*, a feature shared with some bat species such as *Myotis lucifigus*, *M. natalensis* and *E. fuscus*, and other mammals such as the mouse, which has several *OAS1* paralogs. Nevertheless, even if the mouse expresses eight *OAS1* genes, only two (*OAS1a* and *OAS1g*) conserve their activities (Elkhateeb et al., 2016). The other paralogs are non-functional due to mutations or deletions of at least one of their functional domains. Multiple sequence alignment of *D. rotundus* *OAS1a* and *OAS1b* with their mammalian ortholog sequences demonstrated that they have the same structural organization and are thus likely to possess similar enzymatic and antiviral activities. For instance, the P-Loop motif, which is an ATP-binding site (Saraste et al., 1990; Tag-El-Din-Hassan et al., 2012), the DAD motif in D box, which is a Mg^{2+} -binding site (Sarkar et al., 2002; Yamamoto et al., 2000), the KR-rich region, which is an oligoadenylate and ATP-binding site (Marié et al., 1997), and the CFK motif located in the C-terminal domain, required for the tetramerization of the proteins, are all conserved (Ghosh et al., 1997). In a previous study on *P. alecto*, Zhou and colleagues characterized a single *OAS1* gene.

However, they did not exclude the possible existence of additional *OAS1* paralogs given that their analysis was based on a *P. alecto* genome assembly not fully achieved at the time of the study (Zhou et al., 2013). In the database, many bat *OAS1* genes have been annotated as “*OAS1-like*” such as in *M. lucifigus*, *P. vampyrus*, *M. natalensis* and *E. fuscus*. However, these genes do not show any mutation on their functional sites, which could result in a loss of function (Guénet et al. 2015). Moreover, the *OAS1* phylogenetic tree reveals that some bat *OAS1* and *OAS1-like* sequences are intermixed within different clades (Fig. 3a). A consensus on “*OAS1*” naming is therefore needed. In this regard, the generation of more *OAS1* sequences from other bat species should extend our understanding of their evolutionary history.

The molecular characterization and domain prediction of PKR show that the regulatory and kinase subdomains of the *D. rotundus*’ PKR protein are remarkably conserved with respect to those of other mammals. For instance, the N-terminal domain of *D. rotundus*’ PKR includes a repeated motif (dsRBD) that possesses dsRNA-binding activity and the C-terminal half of PKR includes the 11 conserved catalytic subdomains characteristic of kinases. Our multiple sequence alignment of mammals’ PKR shows the conservation of the major residues crucial to the function of the protein such as the highly conserved lysine K⁶⁴ of the RNA-binding domain required for its RNA-binding activity, the K²⁹⁶ in the catalytic subdomain II required for its kinase activity and the T⁴⁴⁶ within the activation loop that confers to PKR its specificity as a serine/threonine kinase (Dabo and Meurs, 2012). In addition, the salt bridge interaction of R²⁶² and D²⁶⁶ at the dimer interface of PKR, which is critical to eIF2 α phosphorylation and PKR activation, is also conserved (Dey et al., 2007).

Similarly, depending on the sequence alignment, ADAR1 is also highly conserved with those of other mammals. For instance, we found that residues H⁹¹⁰ and E⁹¹² of the CHAE motif, considered as a key element of the catalytic core characteristic of deaminases, are

conserved. It has been shown that their mutations completely abolish A-to-I editing activity (Lai et al., 1995; Liu and Samuel, 1996). Further, C⁹⁶⁶ and C¹⁰³⁶, which are believed to play a role in zinc coordination along with H⁹¹⁰, are necessary for ADAR1 deaminase activity and are also conserved in *D. rotundus* (Lai et al., 1995). Moreover, as in other mammals, the lysine residues crucial for both RNA binding and enzymatic activity, present in the core sequence of each of the dsRBD domains (K⁵⁵⁴, K⁶⁶⁵, and K⁷⁷⁷) are conserved (Fierro-Monti and Mathews, 2000; Liu and Samuel, 1996; McCormack et al., 1994).

To test the functionality of the ISG system upon viral infection in our bat model, expression of the characterized ISGs was examined following transfection of FLuDero cells with poly(I:C). The induction profile of the ISGs characterized in our bat model does not match that obtained by Zhou and colleagues (Zhou et al., 2013) on lung cells from *P. alecto* after poly(I:C) transfection. Although we also showed an induction of OAS1 and PKR following poly(I:C) transfection, they found higher and earlier induction of PKR as compared to OAS1. The discrepancy between this previous study and our results is probably due to intrinsic factors specific to the bat species leading to distinct expression profiles. Indeed, it has recently been reported that the up-regulation of ISGs was not only lineage- but also species-specific when comparing the megabats and the microbats (Shaw et al., 2017). In addition, the higher induction of OAS1a and OAS1b, as compared to ADAR1 and PKR, may be due to the presence of multiple IFN-stimulated response elements (ISREs) in the OAS1 promoter, as described in *P. alecto* (Zhou et al., 2013). This can conveniently allow bats to mount a more robust expression of OAS1 rather than of ADAR1 or PKR. In fact, we previously showed an induction of expression of IFN β in poly(I:C)-transfected FLuDero, reaching a peak at 24 h (Sarkis et al., 2018). Interestingly, the expression kinetics of the three ISGs characterized was similar to that of IFN β , suggesting that these proteins are induced in an IFN β -dependent manner, acting on the ISREs present in their promoters. It is conceivable that the similar

expression levels of ADAR and PKR observed at the RNA level are also observed at the protein level. Accordingly, given the negative regulation of PKR by ADAR, as indicated above, an active PKR might not be in sufficient quantity to exert a proper antiviral action. Therefore, the higher induction of OAS observed, as compared to PKR and ADAR, tends to show the particular importance of the OAS proteins in the antiviral action of IFN in bats. Another interesting observation was the differential expression of OAS1a and OAS1b in our cellular model after poly(I:C) transfection. Interestingly, this feature has already been described in mice (Pulit-Penaloza et al., 2012). It has been shown that the ISRE as well as the overlapping STAT sites are required for Oas1a promoter induction by IFN- β while Oas1b expression requires only the ISRE. Furthermore, while OAS1a requires both STAT1 and STAT2 for its upregulation by IFN- β , OAS1b only requires STAT2. Therefore, analyzing the promoter region of these two paralogs should clarify their differential expression in our bat species.

We found that the basal transcription level of the ISGs characterized was elevated in the FLuDero cells. This observation is consistent with previous results showing that ISGs are constitutively expressed in bats and suggests that bat cells are equipped to mount a rapid defense against viral infections (Cruz-Rivera et al., 2018; Shaw et al., 2017; Zhou et al., 2016). In addition to their already significant levels of expression in the absence of treatment, these ISGs can be further enhanced in response to poly(I:C). Together these results tend to prove that these ISGs are functional and must play an important antiviral role in *D. rotundus*. Further work is therefore necessary to elucidate their role during viral infection, explore the mechanisms underlying their unique expression profiles and determine whether or not they contribute to the unique ability of bats to manage viral infection.

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Competing Interests

The authors declare that they have no competing interests.

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Figure 1. Amino acid alignment of *D. rotundus* OAS1 paralogs and other mammalian species. The two important signature domains, NTP-transf_2 and OAS1_C in *D. rotundus* OAS1a and OAS1b are highlighted with solid arrows. Amino acid sequence motifs important for the enzyme activity are shown in boxes. The orange rectangle represents the Serine residue in the putative helical turn, the adjacent Serine, which is either Serine or Threonine (Rune Hartmann et al., 2003 Molecular cell). Inverted triangle: one of the signature features of the nucleotidyl transferase superfamily is a conserved triad of aspartic acids predicted to coordinate the catalytically active magnesium ions. The multi-alignment was performed with the ClustalW program using default parameters. Dashes indicate gaps in the amino acid alignment. Abbreviations: MIOAS1: *Myotis lucifigus* OAS1 (XM_014464612), MIOAS1l: *Myotis lucifigus* OAS1-like (XM_014462497), HsOAS1: *Homo sapiens* OAS1 (BC000562), SsOAS1: *Sus scrofa* (NM_214303), MmOAS1a-h: *Mus musculus* OAS1a-h (XM_006530330, BC012877, NM_033541, BC052835, BC053721, BC116433, BC018470, NM_145228).

Figure 2. Amino acid alignments of *D. rotundus* ADAR1 and PKR with their homologs from other mammalian species. a) All motifs required for ADAR1 activity are highlighted with solid arrows, including two Z α domains at the N-terminus, followed by three double-stranded RNA-binding motifs (dsRBM), dsRBM1, 2 and 3, and a catalytic deaminase domain at its C-terminus. **b)** PKR contains two dsRBM highlighted in yellow (dsRBM1) and light gray (dsRBM2) followed by a basic region (dark gray) and subdomain sequences in boxes found in the catalytic region highlighted with solid arrows, green represents the repeat sequence in human PKR not present in *D. rotundus*, other bat species and mice PKR. An insert region at the C-terminal of the subdomain IV is highlighted in blue. The multi-alignments were performed with the ClustalW program using default parameters. Dashes indicate gaps in the amino acid alignments. Abbreviations: PaADAR1/PKR: *Pteropus alecto*

ADAR1/PKR (XM_015598363/NM_0012910168), HsADAR1/PKR: *Homo sapiens* ADAR1/PKR (NM_001111/XM_011532987), MmADAR1/PKR: *Mus musculus* ADAR1/PKR (NM_019655/NM_011163).

Figure 3. Phylogenetic trees generated from amino acid sequence alignments of OAS1, ADAR1 and PKR from *D. rotundus* and other mammals. Phylogenetic trees were generated based on (a) the alignment of partial amino acid sequence of the mammalian OAS1, and the full-length protein sequence of (b) ADAR1 and (c) PKR. The trees were inferred using the Bayesian method with the JTT + G model. Sequence identifiers include the NCBI accession number. Posterior probabilities of the Bayesian analysis (>50%) are shown next to each node. The scale bar indicates amino acid substitutions per site.

Figure 4. Poly(I:C) transfection induces expression of ISGs. Time-course fold induction of (a) OAS1a, (b) OAS1b, (c) ADAR1 and (d) PKR in FLuDero cells upon poly(I:C) transfection. ISGs mRNA were assessed by RTqPCR. Data were normalized against β -actin. Cells were stimulated with 20 μ g/mL of poly(I:C) and harvested at the indicated time points. Data represent the mean values of three separate experiments, and the error bars represent standard deviations (SD).

Figure 5. Quantitative mRNA expression of OAS1a, OAS1b, ADAR1 and PKR. Time course mRNA expression levels of (red) OAS1a, (green) OAS1b, (blue) ADAR1 and (purple) PKR were assessed by RTqPCR in FLuDero cells upon poly(I:C) transfection. Cells were stimulated with 20 μ g/mL of poly(I:C) and harvested at the indicated time points. The expression was normalized to the housekeeping gene β -actin. Data represent the mean values of three separate experiments.

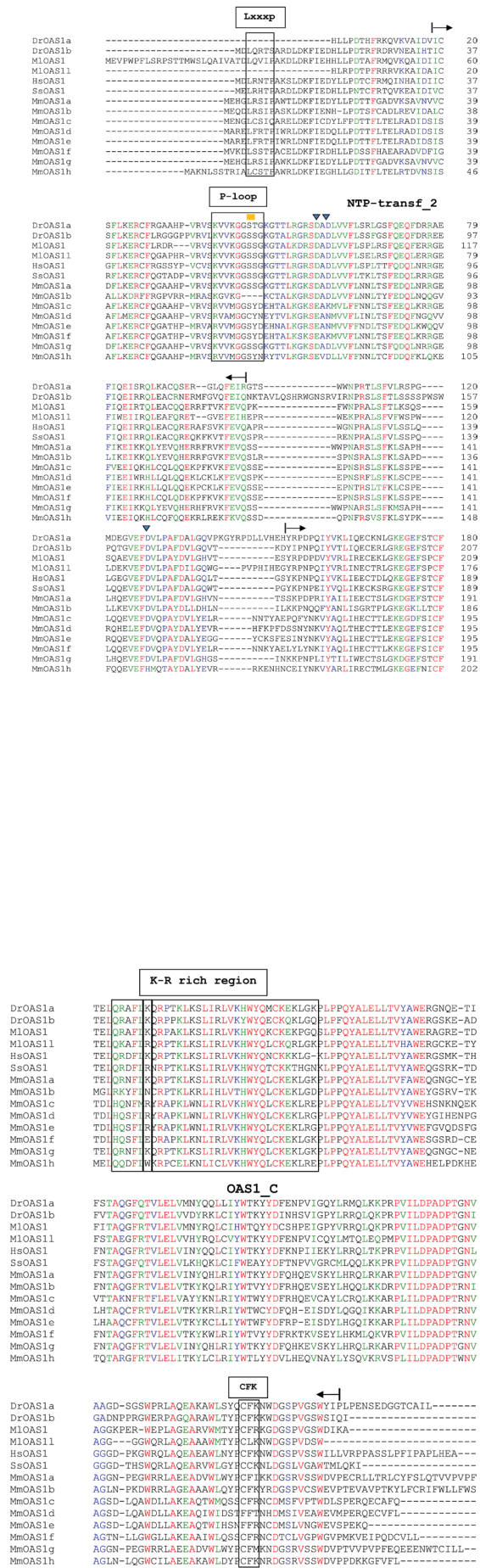


Figure 1.

A

DrADAR1	----NSRQCITLKNYHNFLQRYEHSRLRQGL--GRSYVNSFQLOQIEFLKQGLFVP	55
PaADAR1	----MEFRQCFLRYPS----FFPGYELGHQK--GLGAYPNRQLOQIEFLKQGLFVP	51
HaADAR1	----MNFRCQYSLGYTHFPQGYEHRQYQPP--GGSSFSFSLKQIEFLKQGLFVP	55
MmADAR1	MSQGFPGTCTVPHQTQSYLDPSSHEHSKRWYPPQPGQPSYFSFQLOQIEFLKRLFLFVP	60
DrADAR1	LIRKQAPVFLPLPEFWRFP---GPPARGRQPEINGIPRGVPLRSQALQSSTQPPFPQG	112
PaADAR1	LIGQTSLSLFFPYGLMWRFP---GPPARGRQSENGVPRPVLRSQAQGRGFLPLCRG	108
HaADAR1	VIGQTSLSLFLGLRNFVLLASSTRGRVDYGVNPGVGLGHQGLQNGQGFPSFG	115
MmADAR1	LIGIQTSLSLFFPLFGHWRFP---GPPAQDRGLEIMWFPSVTLRNQGFHIGPLPFHS	117
DrADAR1	RIPFWRGVRLSSHFQRLSDQQRVRLLELQELGAGQVTTAQDLAKLQAPKEINRI	172
PaADAR1	KTRPWRGVDTLSARLQELYISQEQORVRLLELQELGAGKAITAHLARRLQASKEVNSV	168
HaADAR1	RSLPQRGVCLSSHFQELSYDQQRILKFLLELGEKATTADHLGKGLGTPKKEINRV	175
MmADAR1	ROTFRWAGDGLSHFRELISISQGVQVNLNLEELGEKATTAVHLARELIPFRDINRI	177
DrADAR1	LYSLAKKGLHQAACKPFLNRLAASVPRWNQSQASRSDSCSQAPHSDBLETEDRD--	230
PaADAR1	LYSLAKKGLLREGVTFPLRRAVSAQANDRQQAADQWSRPAVSDGLETEDRN--	226
HaADAR1	LYSLAKKGLQEAQCTPFLRLAVSTQAWNHSQGVPRDSQGAQNSGLETEDRNST	235
MmADAR1	LYSLKKGKLIHGRGKPFLLSLVPLSQAWTOPGVVNPDSCTQEPFRGEGLDSEDDG--	235
DrADAR1	-----PPGLEDPTEAD	243
PaADAR1	-----PTSGVEGPEPLD	239
HaADAR1	SVSEDLLEFFIAVSAQAMNHSQGVPRPDSHSQSPNSDPGLEPEDSNSTSALEDPLEFLD	295
MmADAR1	-----PASLEGPSPGLD	248
DrADAR1	MAETKEICHYLNVFKSSALNKNIGLTKARDVNAVILDLERQGVYRGQTPTTNYTL	303
PaADAR1	MAETKEICELYHAFSSSALNKNIGLTKARDVNAVILDLERQGVYRGQTPTTNYTL	299
HaADAR1	MAETKEICDYLNVDSGSALNKNIGLTKARDNAVILDMERQGVYRGQTPTTNYTL	355
MmADAR1	MAETKEICDYLNVNSSSALNKNIGLTKARDVTVLILDLERQGVYRGATPTTNYTL	308
DrADAR1	TDKKRERIQLRNNTSTLETTAAIPETQRNVELPCNLPSASDAS--HNVTGNLENGQEP	362
PaADAR1	TDKKRERIQIKNNVNSVETTTQAAVPETKRSVEPPHNSAVPRAS--DETAAPKANGQGL	358
HaADAR1	TDKKRERIQIKNNVNSVETTPAALPETKNNSELTCTIPFMSASNNVTVTEKVENQEP	415
MmADAR1	TDKKRERLQKKRSTHSAFAPPTTAVPEATRSFPPFACHPFAGASSVAASKRVENQEP	368
DrADAR1	VIKLES--QGVTFEIKFPVRDNGSPKTVYDFNGQWATDDIPDLNLSIAAPGEFR	421
PaADAR1	VILKLESPEVVTTFEYTPKFPVRDNGSPRAGYVDFNGQWATDDIPDLNLSIAAPGEFR	418
HaADAR1	VIKLENR--QEARFELARKFPVHYNGSPKAGYVDFNGQWATDDIPDLNLSIAAPGEFR	474
MmADAR1	ATIKLES--HEARFPGRLRPHAYHNGSPKAGYVASENGQWATDDIPDLNLSIHTAPGEFR	427
DrADAR1	AIMEMSPFYSHGLFRCSFYKILTECOLKNPISGLLEYAQFASQTCFNLIQSGPPHPR	481
PaADAR1	AIMEMSPFYSHWRKCSFYKILTECOLKNPVSGLLEYAQFASQTCFNLIQSGPPHPR	478
HaADAR1	AIMEMSPFYSHGLFRCSFYKILTECOLKNPISGLLEYAQFASQTCFNLIQSGPPHPR	534
MmADAR1	AIMEMSPFYSFTLFRCSFYKILTECOLKNPVSGLLEYAQFASQTCDFNLIQSGSPHPR	487
DrADAR1	FKFQVVMINGREFFPFAEAGSKKVAQDAATKNAILLERAKARDGSPESHSVCSEKAG	541
PaADAR1	FKFQVVISGREFFPFAEAGSKKVAQDAATKANTILLEAKARDGSRPESHYCTERESE	538
HaADAR1	FKFQVVMINGREFFPFAEAGSKKVAQDAAMKANTILLEAKARDGSKSESSEHYCTERESE	594
MmADAR1	FKFQVVMINGREFFPFAEAGSKKVAQDAVNAAILLEAKARDGSPQDLSHCMEDESE	547
DrADAR1	ETAESQPTTFSATSFSGKNPVTTILECVIKHIGSSCEFRLLSREGPAHDPFQVCVAMGN	601
PaADAR1	KTAESQPTTFSATSFSGKNPVTTILECVIKHIGSSCEFRLLSREGPAHDPFQVCVAMGT	597
HaADAR1	KTAESQPTTFSATSFSGKSPVTTILECMHKHIGNSCEFRLLSREGPAHEPFQVCVAVGA	654
MmADAR1	KPAEQAQSSSATLSFGKSPVTTILECMHKHIGNSCEFRLLSREGPAHDPFQVCVAVGA	607
DrADAR1	HTFPTASAPSKKVAQMAEAMKALHGEATSTSPEDQPGSTNTIESFONLESAMNKRVR	659
PaADAR1	HTFPTASAPSKKAAQMAEAMKALHGEATDLSPENQGSASSEPFUSLESAPSKVR	657
HaADAR1	OTTFVSAPSKKVAQMAEAMKALHGEATNSMADQPEGMISSELNLESMMNKRVR	714
MmADAR1	OTTFVSAPSKKVAQMAEAMKALGEAASE--DDQSGSANTDGL--ESMAFLR	663
DrADAR1	RIGELVRYLNTNPGVGLLEYARSHGFAAFKFLVDQSGPHEPFYVQAKVGRWFFAVCA	721
PaADAR1	RIGELVRYLNTNPGVGLLEYARSHGFAAFKFLVDQSGPHEPFYVQAKVGRWFFAVCA	717
HaADAR1	KIGELVRYLNTNPGVGLLEYARSHGFAAFKFLVDQSGPHEPFYVQAKVGRWFFAVCA	774
MmADAR1	RIGELVRYLNTNPGVGLLEYARSHGFAAFKFLVDQSGPHEPFYVQAKVGRWFFAVCA	723
DrADAR1	HSKKQKGQEAADALRVLIGENEKAERMGTTEL-----P	755
PaADAR1	HSKKQKGQEAADALRVLIGESEKAERMGTTEL-----P	751
HaADAR1	HSKKQKGQEAADALRVLIGENEKAERMGTTEVTPVTGASLRRTMLLSRSPEAQPKTLP	834
MmADAR1	HSKKQKGQDAADALRVLIGESEKAELGLFAEL-----P	757
DrADAR1	LTGSTFHDQIAMLSHRCFNALTNSFQPSLLGRKILAAIIMKKDSEDLGVVVS LGTGNRVC	815
PaADAR1	LTGSTFHDQIAMLSHRCFNALTNSFQPSLLGRKILAAIIMKKDSEDLGVVVS LGTGNRVC	811
HaADAR1	LTGSTFHDQIAMLSHRCFNLTNSFQPSLLGRKILAAIIMKKDSEDMGVVVS LGTGNRVC	894
MmADAR1	LSGSTFHDQIAMLSHRCFNALTNSFQPSLLGRKILAAIIMKRDPEDMGVVVS LGTGNRVC	817
DrADAR1	KGDSLSLKGETVNDCHAEIISRGGFIRFLYNELMKYSPQTAKDSIFEARGGEKLQIKKT	875
PaADAR1	KGDSLSLKGETVNDCHAEIISRGGFIRFLYSELMKYNFQTAKDSIFEARGGEKLQIKKT	871
HaADAR1	KGDSLSLKGETVNDCHAEIISRGGFIRFLYSELMKYNQTAKDSIFEARGGEKLQIKKT	954
MmADAR1	KGDSLSLKGETVNDCHAEIISRGGFIRFLYSELMKYNHTAKNSIFEARGGEKLQIKKT	877
DrADAR1	VSFHLIYSTAPCGDGLFDKSCSDRAVESTDSRHPVPFENPKQGLRRTKVENGEGTIPVE	935
PaADAR1	VSFHLIYSTAPCGDGLFDKSCSDRAVESTDRHPVPFENPKQGLRRTKVENGEGTIPVE	931
HaADAR1	VSFHLIYSTAPCGDGLFDKSCSDRAVESTSRHPVPFENPKQGLRRTKVENGEGTIPVE	1014
MmADAR1	VSFHLIYSTAPCGDGLFDKSCSDRAVESTSRHPVPFENPKQGLRRTKVENGEGTIPVE	937
catalytic deaminase domain		
DrADAR1	SSDIVPTWDGIRLGERLRTMSCSDKILRWNVLGLQALLTHFLQPVYLKSVTLGYLFSQG	995
PaADAR1	SSDIVPTWDGIRLGERLRTMSCSDKILCWNVLGLQALLTHFLQPVYLKSVTLGYLFSQG	991
HaADAR1	SSDIVPTWDGIRLGERLRTMSCSDKILRWNVLGLQALLTHFLQPIYLKSVTLGYLFSQG	1074
MmADAR1	SSDIVPTWDGIRLGERLRTMSCDKILRWNVLGLQALLTHFLQPVYLKSVTLGYLFSQG	997
DrADAR1	HLTRAICCRVTRDGSAFEDGLRHPPFIVNHPKVGRVSYDSKRQSGKTKETSVNWC LADGY	1055
PaADAR1	HLTRAICCRVTRDGSAFEDGLQPPFTVNHPKVGRVSYDSKRQSGKTKETSVNWC LADGC	1051
HaADAR1	HLTRAICCRVTRDGSAFEDGLRHPPFIVNHPKVGRVSYDSKRQSGKTKETSVNWC LADGY	1134
MmADAR1	HLTRAICCRVTRDGFADFDGLRYPPFIVNHPKVGRVSYDSKRQSGKTKETSVNWC LADGY	1057
DrADAR1	DLEILDGTRGTVDGPRNELSRVSKKNIFLLFKRLCSFRARRDLLKLSYGEAKKAARDYET	1115
PaADAR1	DLEILDGTRGTVDGPRNELSRVSKKNIFLLFKRLCSFRARRDLLKLSYGEAKKAARDYEA	1111
HaADAR1	DLEILDGTRGTVDGPRNELSRVSKKNIFLLFKRLCSFRARRDLLKLSYGEAKKAARDYET	1194
MmADAR1	DLEILDGTRGTVDGPKELSRVSKKNIFLQFKRLCSFRARRDLLQLSYGEAKKAARDYDL	1117
DrADAR1	AKNYFKKSLKDMGYGNWISKPOEEKNFYLCVP	1147
PaADAR1	AKNYFKKGLKDMGYGNWISKPOEEKNFYLCVP	1143
HaADAR1	AKNYFKKGLKDMGYGNWISKPOEEKNFYLCVP	1226
MmADAR1	AKNYFKKSLRDMGYGNWISKPOEEKNFYLCVP	1149

B		dsRBM1		
DrPKR	MADNHIRSFICAIWKYSCKNNVNVYRELERTGPPHDSIKKFOVILDEREYPAEGKSK	60		
PaPKR	MADDLSGFFLEELNKYRKNNVEIEYRELKRGPPHLSRTTFQVVI GEREPFAEGKSK	60		
HePKR	MAGDLSAGFPMRELNTYRKQGVVLKYQELFNSGPPHDSRTTFQVVI DGEREPFEGEGRSK	60		
MmPKR	MASDTP-GTYMOKLNKYRMHGVAITYKELSTSGPPHDSRTTFQVLI DEKEFPFAEGRSK	59		
DrPKR	REAKDAAKLAFAELKE-KEEVSASVLLTDTSDDLMSGNYVGQINRLTQKANLSVNYQQ	119		
PaPKR	RGAKNAAKLAVEIINEEKEEVSSLSTTDTSDLSIGNYIGLVNRTAQKALPVNYQL	119		
HePKR	REAKNAAKLAVEIINKEKAVSPLLLTTNSSEGLMSGNYIGLVNRTAQKRLTVNYEQ	120		
MmPKR	QEARNAAKLAVDILDN---ENKVDCHTSASEQGLFVGNVIGLVNFAQKKLSVNYEQ	115		
		dsRBM2		
DrPKR	HESYTNQPERFSCCKIIGQKEYSSAVGSTQKQAKQLAAKLAYEKILAEISSSTKDDSVLLG	179		
PaPKR	GLG-AGEPGRFYTCIIGQKYNVAVGSTQKQAKQLAAKLAYEQILSAKTS-VDDLASLG	177		
HePKR	CASGVHGFEGFHYCKRMGQKEYSIGTGSTQKQAKQLAAKLAYLQILSEETSVKSDYLSGG	180		
MmPKR	CEPNSLEFQRICKCKIGQTMGTGSGVTKQKQAKQLAAKLAYQELKSEPK----TAGTS	171		
		basic		
DrPKR	SSPAGPGDYKGSFRT--GGSESPSENGFLENGESNDNSGSFINTPSSSPDLRFSSGR	237		
PaPKR	SPSAASSDYGSNSMTNMCDSESPCENGFLANGSERNDNSDFSNLSSPSVSVRRSLGR	237		
HePKR	S-FATTICESQSNLVTSTLASESSSEDDYSAUTSEINNSDS-LNYSLLANGLRNQRM	238		
MmPKR	S-----SVVT-----ST--FSGFSSSSMTSNGVQSAPGSPSSENVVTNGLGE	213		
		region		
DrPKR	GR--RSIAAKFDSPLIGRNKYTMNERFIKDFSEITQIGGGYGRVFKAKHRIDGKTVIK	295		
PaPKR	TRIFNLAPHLNVLBAARNEYMDHRFLENLEITSLSGGTGVFKAKHRIDGKTVIK	297		
HePKR	AK--RSLAFPTDPPMKETKYTVDKRYGMDFKIELSSGGFGVFKAKHRIDGKTVIK	296		
MmPKR	NK--RKSGVKVSPDQVQRNKYTLDAFNSDFEDIEELGGEGGVFKAKHRIDGKRAIK	271		
		III		
DrPKR	RKYNTKNKEKVERETIVLSALDHRNIVRYNNDGIDDAVESPRD-----	341		
PaPKR	RKYDN--EKVVETKALAAISHNIVRYNNDGIDYASEDSNGR-----	342		
HePKR	RKYNN--EKAERETKALAKLDHNVIVHYNCWDGFTDPEISDSLESDYDEPNKSK	353		
MmPKR	RKYNT--EKAERETQALAEI LNHNIVQYHSCWEGVDYDPEHMSMDT-----	316		
		catalytic kinase domain		
DrPKR	----SPECLEFQMECEGTLEQWINKRRDQTKDKHLSLELFEQIAHGVNYIHSKALIHR	397		
PaPKR	-FESCTKCLFIQMECEGTLEQWINKRRDQTKDKHLSLELFEQIAHGVNYIHSKALIHR	401		
HePKR	SSRKTKCLFIQMECEGTLEQWINKRRGEKLDKVLALSLFEQITKGVDIYHSKALIHR	413		
MmPKR	-SRVTKCLFIQMECEGTLEQWNRNRNQSKVDKALILDLYEQIVGVYVYHSKALIHR	375		
		VII		
DrPKR	DLKPSNIFLVDTKQIKIGDFGIVTFOEYDEK-RTDRDQFLYMSPEQISPEYGVNVDIF	456		
PaPKR	DLKPSNIFLVDTKKIKIGDFGIVTFLKNDK-RTNNRGTFRYMSPEQLSSTEYGVNVDIY	460		
HePKR	DLKPSNIFLVDTKQVKIGDFGIVTSLKNDGK-RTRSKGTFRYMSPEQISPDYGVKEVDLY	472		
MmPKR	DLKPGNIFLVDERHIKIGDFGIVTALENDGKSRTRTGTQYMSPEQLFKHYGVKEVDIF	435		
		IX		
DrPKR	ALGLIIAELLYISPTHLEKSKIFQDLRSCVVP-DTYDDEKSLLEKLSAEPKKRINTSE	515		
PaPKR	PLGLIIAELLYISRTLETCKIFEDLKKGVL-DVFDDEKILLQKLSKDPKKRINTCE	519		
HePKR	ALGLIIAELLHVCDTAFETSKFFDLDGDIIS-DIFDKKETLLQKLSKKPEDRINTSE	531		
MmPKR	ALGLIIAELLHTCTESEKIKFFESLRKGFSDNDFDKKESLLEKLSKKPKDRINTSE	495		
		XI		
DrPKR	ILKTLQEWKTVVREKMRHTY 536			
PaPKR	ILTTLEWKNVV-EKKKRNTC 539			
HePKR	ILRTLIVWKKSP-EKKRHTC 551			
MmPKR	ILKTLAEWRNIS-EKKKRNTC 516			

Figure 2.

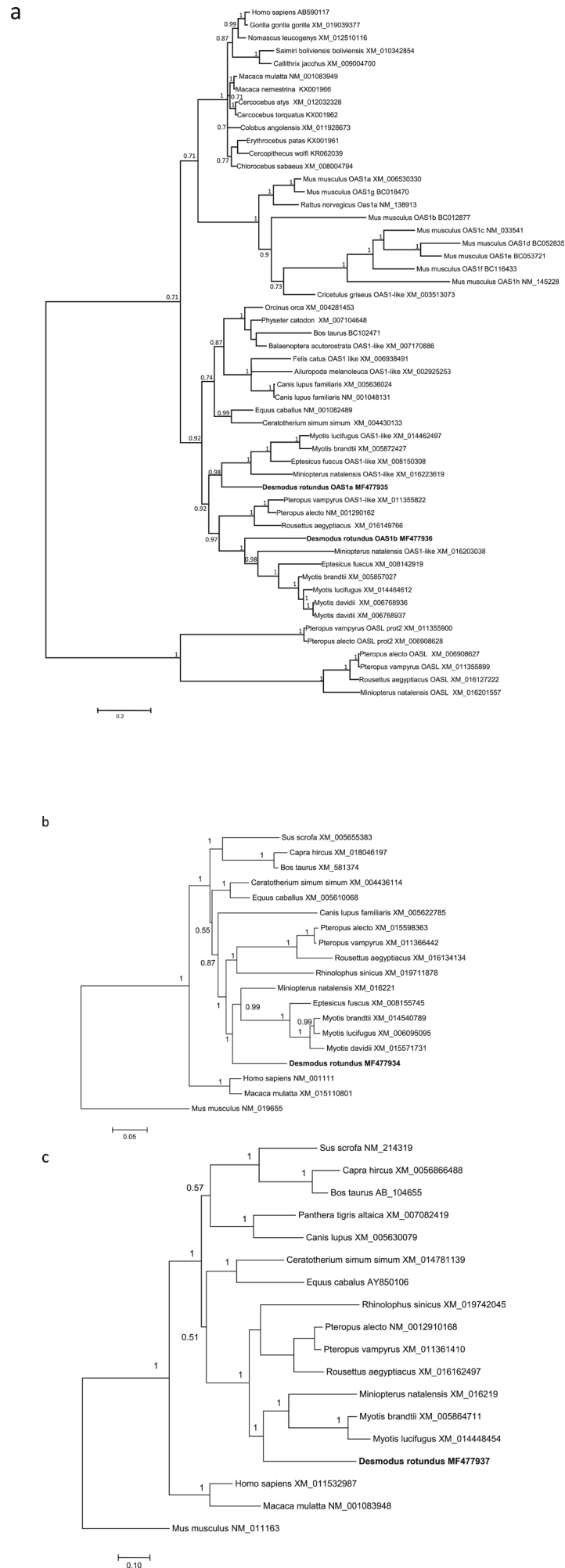


Figure 3.

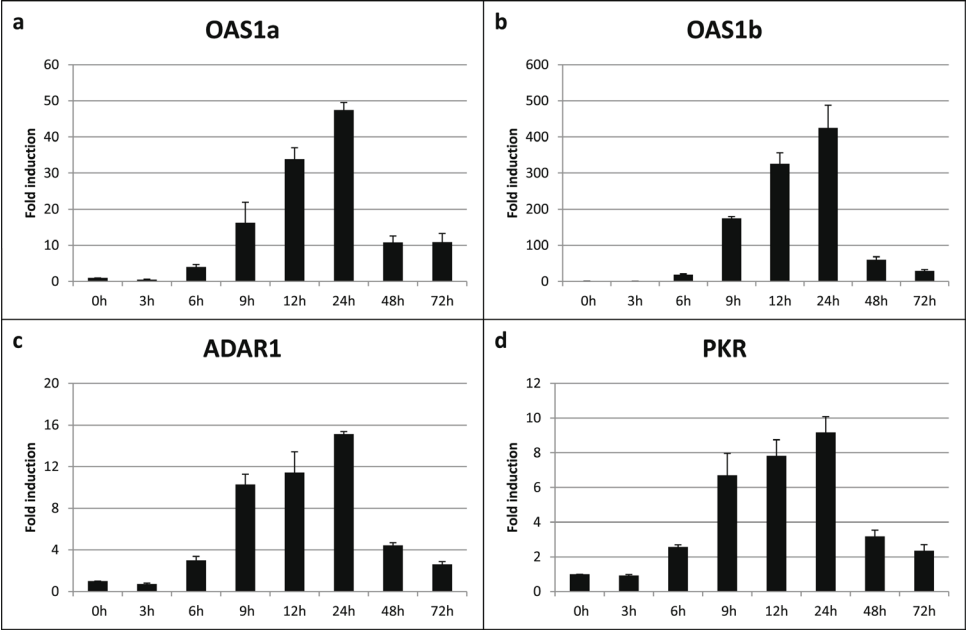


Figure 4.

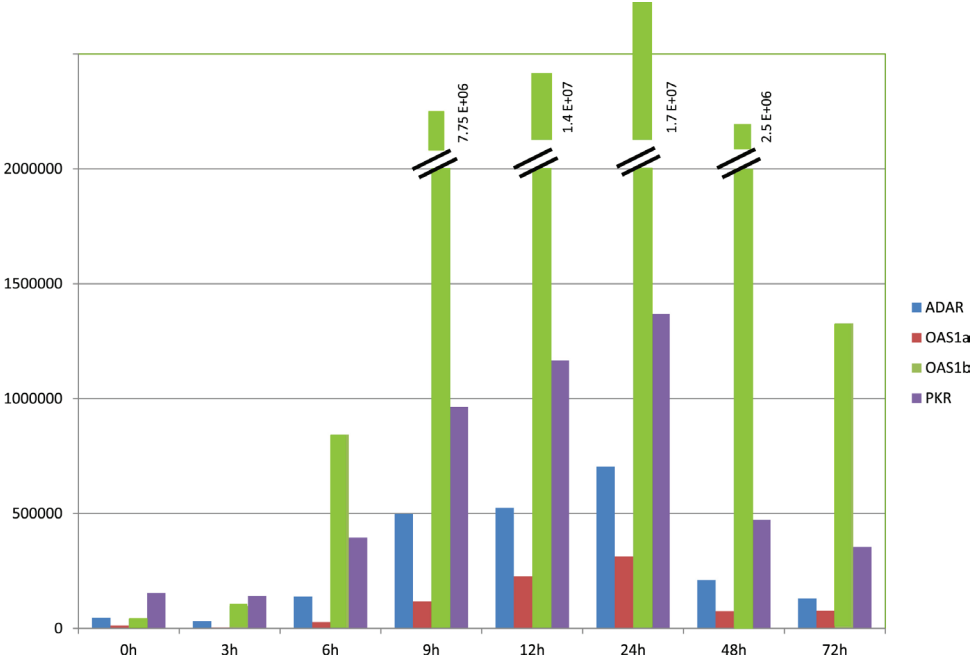


Figure 5.