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Molecular characterisation of RIG-I-like helicases in the black flying fox, *Pteropus alecto*

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ABSTRACT

The RIG-I like helicases, RIG-I, mda5 and LGP2 are an evolutionarily conserved family of cytosolic pattern recognition receptors important in the recognition of viral RNA, and responsible for the innate induction of interferons and proinflammatory cytokines upon viral infection. Bats are natural reservoir hosts to a variety of RNA viruses that cause significant morbidity and mortality in other species; however the mechanisms responsible for the control of viral replication in bats are not understood. This report describes the molecular cloning and expression analysis of RIG-I, mda5 and LGP2 genes in the fruit bat *Pteropus alecto*, and is the first description of RIG-I like helicases from any species of bat. Our results demonstrate that *P. alecto* RIG-I, mda5 and LGP2 have similar primary structures and tissue expression patterns to their counterparts in humans and other mammals. Stimulation of bat kidney cells with synthetic dsRNA (poly I:C) induced high levels of interferon β and rapid upregulation of all three helicases. These findings reveal that the cytoplasmic virus sensing machinery is present and intact in *P. alecto*. This study provides the foundation for further investigations into the interactions between bat RIG-I-like helicases and viruses to elucidate the mechanisms responsible for the asymptomatic nature of viral infections in bats.

1. Introduction

Bats are poorly understood, yet have attracted increasing attention since being recognized as the source of numerous high-profile emerging viral diseases (Calisher et al., 2006). Fruit bats are known reservoir hosts of Hendra virus (Murray et al., 1995; Halpin et al., 2000), Nipah virus (Chua et al., 2000), Ebola virus (Leroy et al., 2005), Marburg virus (Towner et al., 2009), Melaka virus (Chua et al., 2007), and Australian bat lyssavirus (van der Poel et al., 2006; Speare et al., 1997), whereas microbats are known reservoir hosts of Rabies, and likely reservoir hosts of Severe acute respiratory syndrome coronavirus (SARS CoV) (Lau et al., 2005; Li et al., 2005). While these viruses are highly pathogenic in other mammals, experimental studies and field observations have shown that bats rarely display clinical signs upon viral infection (Williamson et al., 1998; Calisher et al., 2006; Middleton et al., 2007; Towner et al., 2009; van den Hurk et al., 2009). One hypothesis under consideration is that bats possess qualitative differences in their innate immune system. In order to address this hypothesis, we are seeking to identify and characterise elements of the bat innate immune system.

In higher organisms, cellular recognition of infection begins with the engagement of pattern recognition receptors (PRRs) which function by recognising conserved pathogen-associated molecular patterns (PAMPs), thereby initiating signalling cascades that lead to inflammatory responses. The two major classes of virus-sensing PRRs are the transmembrane Toll-like receptors (TLRs) expressed on the cell surface or within endosomes, and the retinoic acid-inducible gene I (RIG-I)-like RNA helicases (RLHs) which are expressed cytoplasmically. The RLH gene family is evolutionarily ancient and consists of three genes; RIG-I (also known as DDX58), melanoma differentiation associated protein 5 (mda5/IFIH1), and laboratory of genetics and physiology 2 (LGP2/DHX58). We previously showed that fruit bats transcribe intact TLRs 1–10 and a TLR13-like pseudogene, and thus possess all of the TLRs believed to be involved in antiviral immunity in other mammals (Cowled et al., 2011); however this is the first study to determine whether bats also possess an intact set of RLHs.

RIG-I, mda5 and LGP2 each contain an ATP-binding DExD/H-box helicase domain and a C-terminal regulatory domain, both of which are required for ligand recognition (Fuller-Pace, 2006; Lu et al., 2011). RIG-I and mda5 (but not LGP2) each contain two N-terminal caspase activation and recruitment domains (CARDs) required for downstream signalling (Johnson and Gale, 2006). RIG-I and mda5 recognise short and long dsRNAs respectively and RIG-

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I detects dsRNA with uncapped 5′-triphosphate RNA (Hornung et al., 2006; Kato et al., 2008). Ligand binding produces a conformational change in RIG-I and mda5 that exposes the CARD domains, thereby permitting interaction with mitochondria-located adaptor molecule (MAVS/IPS1). This event initiates a signalling pathway that culminates in activation of transcription factors including IRFs and NF-κB, which translocate to the nucleus to induce transcription of type I IFNs, proinflammatory cytokines and chemokines (Yoneyama and Fujita, 2010).

Knockout studies in mice have demonstrated critical roles for the RLHs in initiating antiviral immune responses *in vivo*, and have shown that rather than being redundant to each other, the different RLHs provide protection from specific classes of viruses. RIG-I is implicated in the response to negative sense RNA viruses including flaviviruses, orthomyxoviruses, paramyxoviruses and rhabdoviruses, whereas mda5 is important in the response to positive sense RNA viruses such as picornaviruses, and is also a potent cytoplasmic receptor for poly I:C (Kato et al., 2006; Gitlin et al., 2006). When initially identified, overexpression of LGP2 *in vitro* resulted in apparent negative regulation of RIG-I and mda5 (Yoneyama et al., 2005; Rothenfusser et al. 2005), however knockout studies have since shown that LGP2 can positively regulate both RIG-I and mda5 signalling in some instances (Venkataraman et al., 2007, Satoh et al., 2010). The exact role of LGP2 requires further clarification.

In the absence of commercial reagents or prototype sequence data, there is a strong need to identify and define components of the bat immune system. RLHs are important components of the innate antiviral immune system in other species but to date have not been described in bats. To address the hypothesis that bats may have atypical RLH genes, we performed molecular cloning and transcriptional analysis of the RLH gene family in the fruit bat, *Pteropus alecto*.

2. Materials and methods

2.1. Tissue collection and cell culture

P. alecto bats were trapped in Southern Queensland, Australia, and transported alive by air to the Australian Animal Health Laboratory (AAHL) in Victoria, where they were euthanised for dissection using methods approved by the AAHL animal ethics committee. Tissues were stored at −80 °C in RNA*later* (Ambion). PaKiT03 cells are an immortalised kidney cell line derived from *P. alecto* primary tissue as described in Crameri et al. (2009). They were maintained in DMEM supplemented with 10% FCS and antibiotic/antifungal mixture.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from frozen *P. alecto* tissues using a Precellys 24 tissue homogeniser (Bertin technologies) and an RNeasy mini kit (Qiagen) with on-column DNase-I treatment (Qiagen) to remove traces of genomic DNA. Total RNA was extracted from peripheral blood mononuclear cells (PBMC) harvested from *P. alecto* blood by density centrifugation using lymphoprep (Axis-Shield) and QIAshredders (Qiagen) for homogenisation, followed by RNA extraction using an RNeasy mini kit with on-column DNase-I treatment. RNA was quantified by absorption spectrometry, and then reverse transcribed into cDNA using Omniscript reverse transcriptase (Qiagen) according to the manufacturer's instructions.

2.3. Sequence identification and molecular cloning

Sequences were identified in the genome of the Malaysian flying fox *Pteropus vampyrus* located in the Ensembl database (assembly pteVam1, 2.63x coverage, July 2008) and the corresponding GenBank trace file archive. Primers for Rapid Amplification of cDNA ends (RACE) and PCR were designed using Clone Manager 9.0 (Sci-Ed Software). Full-length amplification was performed using LongAmp Taq DNA polymerase (New England BioLabs Inc). 5′ and 3′ RACE were performed using a GeneRACER kit (Invitrogen). PCR and RACE products were cloned into the pCR4-TOPO vector using the TOPO TA Cloning Kit for sequencing (Invitrogen). M13 forward and M13 reverse universal sequencing primers were employed for DNA sequencing using a BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems) and an Applied Biosystems 3130 XL Genetic Analyser.

2.4. Sequence and phylogenetic analysis

Sequence data were assembled with Seqman PRO (Lasergene) and analysed with Clone Manager 9.0 (Sci-Ed Software). Intron–exon maps were drawn using Fancy Gene v1.4 (Rambaldi and Ciccarelli, 2009). Protein domains were identified using the NCBI Conserved Domains search tool (Marchler-Bauer et al., 2011). Sequence alignments and phylogenetic trees were generated in MEGA 5 (Tamura et al., 2011) using the neighbour joining method with 1000 bootstrap replicates. Evolutionary distances were computed using Poisson correction.

2.5. Quantitative reverse transcription PCR (qRT-PCR)

qRT-PCR was performed on cDNA derived from tissues collected from three apparently healthy wild-caught *P. alecto* bats. Total RNA was prepared from peripheral blood mononuclear cells (PBMC), lymph node, spleen, liver, lung, heart, kidney, small intestine, brain and salivary glands following the procedure described above, including on-column DNaseI treatment to control for residual genomic DNA (gDNA) contamination. Quantitect reverse transcriptase for RT-PCR (Qiagen) was then used to perform a second round of gDNA digestion followed by cDNA synthesis. For each sample, 0.5–1 μg total RNA was used as template in a 20 μl cDNA synthesis reaction. qRT-PCR primers were designed using Primer Express 3.0 (Applied Biosystems) with default settings (listed in Table 2). Reactions were carried out using EXPRESS SYBR® GreenER™ qPCR Supermix Universal (Invitrogen) and an Applied Biosystems 7500 Fast Real-Time qPCR instrument. Final qPCR reaction volumes were 10 μl and carried out in 96 well plates. Each reaction contained a final concentration of 200 nmol each primer and 2 μl of 1:5 diluted cDNA. Thermocycling consisted of 50 °C/2 min, 95 °C/2 min, 40× cycles of 90 °C/15s followed by 60 °C/1 min, and was followed by melt curve analysis. Copy numbers of target sequences were calculated using standard curves and normalised to 18s ribosomal RNA (for baseline tissue expression) or analysed without normalisation (poly I:C stimulation experiments).

2.6. In vitro stimulation with polyinosinic-polycytidylic acid

PaKiT03 cells (an immortal cell line derived from *P. alecto* primary kidney cells) were challenged *in vitro* with 0.001–10 μg/ml of the dsRNA homologue polyinosinic-polycytidylic acid (poly I:C, Sigma) either by direct addition to the culture media or in complex with Lipofectamine 2000 (Invitrogen) to mimic viral infection. Three hours post challenge cells were collected into buffer RLT (Qiagen) for total RNA extraction and qRT-PCR as described above.

3. Results and discussion

3.1. Cloning and sequence analysis of P. alecto RLH's

Although no whole genome sequence is currently available for *P. alecto*, a low coverage genome sequence is publicly available in