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Species-Specific Variation in RELA Underlies Differences in NF-κB Activity: a Potential Role in African Swine Fever Pathogenesis

Christopher J. Palgrave,¹,²,³ Linzi Gilmour,² ³ C. Stewart Lowden,³ Simon G. Lillico,² Martha A. Mellencamp,⁴§ and C. Bruce A. Whitelaw² ⁴

Veterinary Pathology Unit, Division of Veterinary Clinical Sciences, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush Veterinary Centre, Roslin, Midlothian EH25 9RG, United Kingdom;² Division of Developmental Biology, The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush Campus, Roslin, Midlothian EH25 9RG, United Kingdom;† Veterinary Health Research Pty Ltd., Trevenna Rd., West Armidale, NSW 2350, Australia; and Genus/PIC, 100 Bluegrass Commons Blvd., Hendersonville, Tennessee 37075

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African swine fever virus (ASFV) is a highly infectious disease of domestic pigs, with virulent isolates causing a rapidly fatal hemorrhagic fever. In contrast, the porcine species endogenous to Africa tolerate infection. The ability of the virus to persist in one host while killing another genetically related host implies that disease severity may be, in part, modulated by host genetic variation. To complement transcription profiling approaches to identify the underlying genetic variation in the host response to ASFV, we have taken a candidate gene approach based on known signaling pathways that interact with the virus-encoded immunomodulatory protein A238L. We report the sequencing of these genes from different pig species and the identification and initial in vitro characterization of polymorphic variation in RELA (p65; v-rel reticuloendotheliosis viral oncogene homolog A), the major component of the NF-κB transcription factor. Warthog RELA and domestic pig RELA differ at three amino acids. Transient cell transfection assays indicate that this variation is reflected in reduced NF-κB activity in vitro for warthog RELA but not for domestic pig RELA. Induction assays indicate that warthog RELA and domestic pig RELA are elevated essentially to the same extent. Finally, mutational studies indicate that the S531P site conveys the majority of the functional variation between warthog RELA and domestic pig RELA. We propose that the variation in RELA identified between the warthog and domestic pig has the potential to underlie the difference between tolerance and rapid death upon ASFV infection.

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studies identified numerous upregulated host genes, but to date, all are limited to analysis in domestic pig cells. In this study, we take a complementary approach to this question by the testing of variation in targeted candidate genes. Clearly A238L represents a novel and versatile immunoregulatory mechanism by which ASFV can inhibit both the NF-κB and NFAT signaling pathways (11, 28, 29, 36, 49). We therefore consider the three A238L target proteins, RELA, PPP3CB, and PPIA, and the two proteins it mimics, NFKBIA and NFATC1, as candidates for the genetic variation between pig species which may contribute to species-specific responses to ASFV infection. We now report the sequencing of these genes from different pig species and identification and initial in vitro characterization of polymorphic variation in one of them.

MATERIALS AND METHODS

mRNA isolation, cDNA synthesis, and DNA sequencing. Whole blood (5 ml) was collected into EDTA from a domestic pig (Sus scrofa, commercial pig; United Kingdom), common warthog (Phacochoerus africanus; Rotterdam Zoo, Holland), and babirusa (Babyrousa babyrussa; Marwell Zoo, United Kingdom) and transferred immediately into DNA/RNA stabilization reagent for blood/ bone marrow (Roche Diagnostics). This was processed using an mRNA isolation kit for white blood cells (Roche Diagnostics). Initially, cDNA libraries were synthesized using the SMART RACE (rapid amplification of cDNA ends) cDNA amplification kit (Clontech) to enable partial sequencing and design of PCR products, the products were ligated into the open plasmid. These constructs allowed constitutive expression of warthog RELA and domestic pig RELA driven by the cytomegalovirus (CMV) promoter. In addition, an N-terminal eight-amino-acid FLAG sequence is incorporated into the protein, which is recognized by an anti-FLAG monoclonal antibody. Site-directed mutagenesis was performed using a QuikChange II site-directed mutagenesis kit (Stratagene).

Plasmid transient transfection was performed using Lipofectamine2000 (Invitrogen) in ~1 × 10⁶ cells/well of a 12-well plate in culture medium at 37°C and 5% CO₂. COS-7 cells were cultured in Glasgow minimal essential medium (GMEM; Sigma), containing 10% fetal bovine serum (Invitrogen), 1% l-glutamine (Invitrogen), 1% sodium pyruvate (Invitrogen), 1% nonessential amino acids (Invitrogen), and 0.2% β-mercaptoethanol (Invitrogen). Mouse embryonic fibroblasts (MEFs) and RELA−/− MEFs were cultured in Dulbecco’s modified Eagle medium (DMEM; Sigma), containing 10% fetal bovine serum (Invitrogen). Luciferase activity was determined in triplicate using the dual reporter assay system (Promega) and analyzed with Excel (Microsoft), with total protein measured with the bicinchoninic acid (BCA) protein assay kit ( Pierce).

The data are presented as the means ± standard deviations; statistical significance was evaluated using the unpaired Student t test, with a difference between groups being considered statistically significant if the P value of the comparison was <0.05.

Western blotting. For Western blot analysis, denatured protein (10 µg) was run in Tris-glycine SDS running buffer (National Diagnostics) on precast NuPage 12% Tris-glycine gels (Invitrogen) before transfer to a nitrocellulose membrane (National Diagnostics). Proteins were visualized using anti-FLAG M2-peroxidase (horseradish peroxidase [HRP]) conjugated primary antibody and mouse β-actin primary antibody with goat anti-mouse IgG HRP (Sigma)-conjugated secondary antibody and detected with Immobilon Western (Millipore) chemiluminescent HRP substrate.

RESULTS

Limited sequence variation in candidate porcine genes. cDNAs were produced for PPIA, NFKBIA, NFATC1 (regulatory domain), PPP3CB, and RELA. These were obtained from the following three pig species: the domestic pig (Sus scrofa), common warthog (Phacochoerus africanus), and babirusa (Babyrousa babyrussa). The domestic pig and warthog represent ASFV-susceptible and ASFV-tolerant species, respectively. The “outspecies” is the babirusa, which is considered to be the most ancient extant species of pig; its range is restricted to the island of Sulawesi in the Indonesian archipelago. It has no common ancestor with the domestic pig more recently than approximately 10 to 19 million years ago (38). It is not known how this pig species would respond regarding ASFV infection.

We generated sequence data (deposited in the EMBL database) (Table 2) and aligned all sequences to those of the human homologues which we used as reference sequences. Limited sequence variation was observed in this study (data not shown), with the following genes displaying complete homology at the translated protein level between the domestic pig and warthog: PPIA, NFATC1 regulatory domain, and...
NFKBIA. In the warthog, PPP3CB contained two insertions with respect to the domestic pig. However, these were located outwith the known functional domains. Furthermore, these correlate with splice variants described in human PPP3CB (22, 27). Only in the RELA subunit of NF-κB were potentially significant coding differences between the domestic pig and warthog identified (Fig. 1).

Sequence of the porcine RELA gene. The RELA open reading frame (ORF) is 1,662 nucleotides in the domestic pig and warthog and is slightly smaller at 1,556 nucleotides in the babirusa. These encode proteins which are 554 and 552 amino acids in length, respectively. Human RELA ORF and protein are the same lengths as the babirusa sequences. Due to insufficient primer binding sites in the 5' untranslated region (UTR), we sequenced all but the first 6 nucleotides for all three pig species (Fig. 1).

Although the babirusa and human sequences are both 6 nucleotides shorter than the other pig sequences, the nucleotide deletions occur at different locations. The babirusa sequence has a single 6-nucleotide deletion, in relation to those of the other pig species, located between the Rel homology domain and the transactivation 2 domain. Human RELA has two 3-nucleotide deletions, one 20 nucleotides upstream from the babirusa deletion and the other in the transactivation 2 domain.

Of the 13 nucleotide differences between the domestic pig and warthog, only three are nonsynonymous and result in codon changes, T448A, S485P, and S531P. The threonine at 448 in the domestic pig occurs as an alanine in the warthog and babirusa and is absent in human RELA. The serine at 485 in the domestic pig sequence is a proline in the other porcine sequences and in human RELA. The serine at 531 in the domestic pig is also found in babirusa and human but is a proline in warthog RELA. All three amino acid differences occur outside the Rel homology domain, with amino acids 448 and 485 located within transactivation domain 2 and amino acid 531 within transactivation domain 1. To confirm these sequence differences, a 268-nucleotide region was amplified directly from genomic DNA and sequenced for an additional 5

<table>
<thead>
<tr>
<th>Gene</th>
<th>Domestic pig</th>
<th>Warthog</th>
<th>Babirusa</th>
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<tr>
<td>PPLA</td>
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<td>FN401369</td>
<td>FN401370</td>
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<td>RELA</td>
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**TABLE 2. Sequences deposited into the EMBL database**

**FIG. 1.** RELA primary protein sequence minus the first two amino acids of the porcine sequences. The Rel homology domain is underlined, the transactivation 2 domain is dashed underlined, and the transactivation 1 domain is double underlined. The nuclear localization signal (KRKR) is dotted underlined. The T448A, S485P, and S531P variations between the domestic pig and warthog are highlighted. SS, *Sus scrofa*; PA, *Phacochoerus africanus*; BB, *Babyrousa babyrussa*; HS, *Homo sapiens* (M62399).
domestic pigs and 11 warthogs (domestic pig sequences, EMBL accession numbers FN424224 to FN424228; warthog sequences, EMBL accession numbers FN424229 to FN424239). All domestic pig sequences were identical to each other over this region, and similarly, all warthog sequences were identical to each other over this region.

**Predicted structural changes as a consequence of RELA sequence variation.** Given that even single amino acid changes can have significant effects on protein structure, we performed *in silico* analyses on the identified changes in RELA in an attempt to determine the structural consequences of the amino acid substitutions. At the three sites, the greater hydrophobicity was conferred by the threonine at 448 in the domestic pig sequence compared to the prolines at 385 and 531 in the warthog sequence. No difference in structural disorder was evident (Phyre analysis) (25), and the only site predicted to be highly conserved (Phospho.ELM database) (4) was in the nuclear localization signal (NLS) at 408 in both species.

**Basal activity of porcine RELA variants.** To determine whether the identified sequence variation between domestic pig RELA and warthog RELA affects NF-κB activity, we established a cell transfection assay. Cells were transiently transfected in duplicate using a triple plasmid cotransfection strategy involving the following: (i) 1 μg expression vector for FLAG-tagged domestic pig or warthog RELA or an empty vector control (pFLAG-CMV4; Sigma Aldrich); (ii) 1 μg NF-κB reporter plasmid comprising 4 copies of the NF-κB consensus binding sequence, driving expression of firefly luciferase (pNFκB-Luc; BD Biosciences, Clontech); and (iii) 1 μg transfection control vector expressing Renilla luciferase driven off the herpes simplex virus (HSV) thymidine kinase (TK) promoter (pRL-TK; Promega). Cells were harvested at 24 h posttransfection, and a dual luciferase assay (firefly luciferase activity relative to Renilla luciferase activity) was performed on MEFs after 24 h of transient transfection with warthog (WH) and domestic pig (DP) RELA genes. Activity is presented as the fold difference between RELA-induced NF-κB-luciferase activity in MEFs that lacked endogenous RELA activity (derived from RELA^{-/-} mouse embryonic fibroblasts (MEFs) that lacked endogenous RELA activity) and the activity in RELA^{-/-} murine embryos using the same transfection regime (n = 3) (Fig. 2C).

We attempted to produce stably transfected RELA^{-/-} MEFs for the domestic pig and warthog RELA genes. Although we could generate numerous colonies for warthog RELA, we were reproducibly unsuccessful in generating those for domestic pig RELA. It is possible that the higher activity level of domestic pig RELA may not be compatible with survival in these cells; a similar scenario may have occurred during a study investigating phosphorylation at the equivalent site (S529) in human RELA (54).

**Effect of RELA sequence variation on induced NF-κB activity.** To determine if the ability to induce NF-κB activity was altered by the different pig RELA proteins, we stimulated transiently transfected RELA^{-/-} MEFs with inducers of NF-κB signaling (9, 14, 15, 16, 42–44, 56). Induction with tumour necrosis factor alpha (TNF-α) nearly doubled (90% increase) warthog RELA activity, whereas domestic pig RELA showed a 70% increase in activity (Fig. 3). We tested a further three known NF-κB inducing agents, lipopolysaccharide, phorbol-12-myristate-13-acetate, and hydrogen peroxide. For all three additional agents, the fold induction observed for warthog RELA was always greater than that of domestic pig (Fig. 3). FLAG immunostaining of COS-7 cells transfected with either domestic pig or warthog RELA and treated with phorbol-12-myristate-13-acetate did not identify any gross differences in cytoplasm-nuclear transit time (RELAs of both species was in the nucleus within 1 min and back in the cytoplasm after 20 min) (data not shown).

**Identification of functional mutations in porcine RELA.** In our sequence data, we identified three amino acid differences between domestic pig RELA and warthog pig RELA. To determine if the *in vitro* difference in domestic pig and warthog
RELAX activities was a cumulative effect of these three differences, or due to one or other individual mutations, we generated versions of RELAX carrying the following single-base changes: T448A, S485P, and S531P. Transfection of these RELAX variants into RELAX$^{-/-}$ MEFs demonstrated that the majority of the reduced basal activity observed for warthog RELAX compared to that observed for domestic pig RELAX was attributed to the S531P mutation (Fig. 4).

**DISCUSSION**

In this study, we have taken a candidate approach, identifying genes which may affect the severity of the host response to ASFV infection in the highly susceptible domestic pig and ASFV-tolerant warthog. The ASFV immunomodulatory factor A238L is known to interact with components of both NF-kB and NFAT host signaling pathways (11, 28, 29, 36, 49). We have sequenced five key factors in these pathways, including three A238L-targeted proteins, RELAX, PPP3CB, and PPIA, and the two proteins it mimics, NFKBIA and NFATC1 (regulatory domain). Modest sequence differences have been identified at the cDNA (mRNA) level between the domestic pig and warthog; however, the majority of these are synonymous (silent) and do not alter the resulting amino acid sequences. We also sequenced the same genes from the ancient babirusa and observed a small number of amino acid differences between this species and the other two species of pig. These differences likely reflect the long evolutionary distance that exists between these species (38).

Despite the high degree of conservation observed between warthog and domestic pig PPP3CB, PPIA, NFKBIA, and NFATC1-translated protein sequences, significant variation was detected in RELAX. RELAX is the predominant member of the heterodimeric transcription factor NF-kB (47). Moreover, the sequence variation includes a phosphorylation site in transcription activation domain 1 (position 531), which is highly conserved across mammals and has been demonstrated to modulate the activity of human RELAX (equivalent to S529) (54, 55). The function of approximately one-third of all eukaryote proteins is controlled by phosphorylation; thus, the observed S531P sequence variation represents an intriguing candidate regulator for the reduced pathology observed in African pigs infected with ASFV. We demonstrate that the genetic variation in the RELAX sequence between the domestic pig and warthog is reflected in NF-kB activity in vitro, with warthog RELAX displaying significantly reduced basal and induced NF-kB activity. We discuss three (not mutually exclusive) scenarios of how the genetic variation we have identified between the domestic pig and warthog may underlie the dramatic phenotypic difference in how these two pig species respond to ASFV.

First, as suggested by our in vitro assays, warthog RELAX is inherently less active than the domestic pig RELAX, exhibiting lower basal and induced levels. In a study of macrophage transcription profiles following ASFV infection in vitro, several factors within the NF-kB signaling pathway displayed elevated expression (NFKB1, NFKBIA); however, RELAX expression was not observed to be altered (56). The impact of ASFV infection on the warthog RELAX expression level is not known, since this study used only domestic pig macrophages. Therefore, although we have not determined whether the porcine RELAX variants are differentially phosphorylated, it would appear that variation in domestic pig RELAX activity may not be determined by altered expression levels. Alternatively, the warthog will have to function with reduced basal NF-kB activity, which would presumably indicate an adapted NF-kB-dependent transcriptome between the two species.

In the second scenario, phosphorylation of domestic pig RELAX at S531 activates a set(s) of genes which are not activated by warthog RELAX, as it lacks this phosphorylation site. Much is known about activation of the NF-kB pathway, with data coming primarily from studies of human RELAX (47). NF-kB activity is regulated by two different mechanisms. The classical canonical pathway involves inhibitors (e.g., NFKBIA) that sequester this transcription factor in the cytoplasm until they are proteolytically degraded by the ubiquitin pathway (24). The alternative pathway revolves around posttranscriptional modifications, predominantly phosphorylation of RELAX (46). At least 8 inducible phosphorylation sites have been identified in human RELAX, which enable transcription of subsets of NF-kB-dependent genes (26, 45). One such site is S529, which is equivalent to domestic pig S531, suggesting that this site may perform a similar role. Indeed, differential expression of subsets of immune and inflammatory proteins as a result of S531
phosphorylation could play a role in determining ASFV pathogenesis in the domestic pig. Furthermore, phosphorylation not only controls specific transcription profiles but also can underlie the developmental timing in gene activation (13). It is tempting to speculate that if a similar mechanism is applied through the domestic pig S531 site, then it could also play a role in the gross physical differences that characterize the various pig species.

In the third scenario, we consider whether the S531P variation in RELA results in different outcomes of interaction with A238L during ASFV infection. Phosphorylation of human S529 (equivalent to domestic pig S531) is inhibited by the interaction of RELA with NFKBIA; only upon activation and degradation of NFKBIA can S529 phosphorylation occur (55). During ASFV infection, NFKBIA is degraded and replaced by A238L, which mimics NFKBIA but is not susceptible to proteolytic degradation (36, 49). As a result, in domestic pig cells infected with ASFV, S531 may not be exposed for phosphorylation. In comparison, warthogs lack this phosphorylation site; infected with ASFV, S531 may not be exposed for phosphorylation. A238L, which mimics NFKBIA but is not susceptible to phosphorylation, could play a role in determining ASFV pathogenesis in the domestic pig. Furthermore, phosphorylation not affect nuclear translocation (54). Furthermore, studies using recombinant ASFV lacking A238L indicate that neither nuclear import nor export of RELA is affected by this immune modulator (48). This suggests that the immunomodulatory functions of A238L are not the result of preventing nuclear translocation in a manner similar to that used by NFKBIA. Instead, A238L may inhibit NF-κB-mediated transcription by other mechanisms, for example, by preventing phosphorylation of RELA in domestic pigs, as discussed above. Whether such differences in how domestic pig RELA and warthog RELA interact with A238L exist will require further investigation. Likewise, elucidating how different mechanisms governing RELA activity and NF-κB-mediated transcription have evolved in these species, and determining the full extent of their functional implications on the immune system and wider transcriptome, will require additional study.

ASFV is highly infectious, with virulent isolates causing an acute, rapidly fatal hemorrhagic fever in domestic pigs (10, 34). In contrast, the porcine species endogenous to Africa tolerate the virus. Outbreaks have significant economic repercussions in addition to welfare concerns. In Africa, ASFV limits the use of the genetically improved breeding pig stock involved in meeting insatiable host defense demands. In domestic pigs, A238L, which mimics NFKBIA but is not susceptible to proteolytic degradation, is not exposed for phosphorylation, and nuclear import nor export of RELA is affected by this immune modulator. This suggests that the immunomodulatory functions of A238L are not the result of preventing nuclear translocation in a manner similar to that used by NFKBIA. Instead, A238L may inhibit NF-κB-mediated transcription by other mechanisms, for example, by preventing phosphorylation of RELA in domestic pigs, as discussed above. Whether such differences in how domestic pig RELA and warthog RELA interact with A238L exist will require further investigation. Likewise, elucidating how different mechanisms governing RELA activity and NF-κB-mediated transcription have evolved in these species, and determining the full extent of their functional implications on the immune system and wider transcriptome, will require additional study.

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