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Characterization of porcine P58^{IPK} gene and its up-regulation after H1N1 or H3N2 influenza virus infection

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ABSTRACT

Background: The 58-kDa inhibitor of the interferon-induced double-stranded RNA-activated protein kinase (P58^{IPK}) is a cellular protein that is activated during influenza virus infection. Although the function of human P58^{IPK} has been studied for a long time, porcine P58^{IPK} (pP58^{IPK}) has little been studied except for its cloning.
Objective: In this study, we aimed to investigate the characteristics of the pP58^{IPK} gene, determine its subcellular localization, and find its expression change during H1N1 or H3N2 infection.
Study design: First, the sequence and structure of pP58^{IPK} were analyzed. Second, pP58^{IPK} gene was cloned into pEGFP-N1 and pEGFP-C1 vectors, respectively, which were transfected into cells to determine its subcellular localization. Third, Lung tissues of piglets from H1N1 infected, H3N2 infected and control groups were analyzed using histopathology, real-time PCR, and immunohistochemistry.
Results: The sequence and structure of pP58^{IPK} was highly similar to the counterpart of human. pP58^{IPK} protein distributed only in the cytoplasm. Lung tissues of piglets infected by H1N1 or H3N2 appeared obvious pathological changes, and the expression of pP58^{IPK} in both mRNA and protein level was up-regulated by approximate 1.5-fold in piglets infected by H1N1 or H3N2 comparing with control piglets.
Conclusions: We analyzed the characteristics of the pP58^{IPK} gene, constructed a phylogenetic tree, determined its subcellular localization, and investigated its expression changes during H1N1 or H3N2 infection. The fundamental data accumulated in this study provides a potential medical model for investigating the function of P58^{IPK} during influenza A viruses infection.

1. Background

Influenza viruses are enveloped, negative-strand RNA viruses which are transmitted through contact with infected individuals or contaminated items, and through inhalation of aerosols, resulting in seasonal outbreaks of human acute respiratory tract infection, for which yearly vaccination can provide some measure of protection. However, rapid mutation can yield emerging variants of influenza with the potential to cause pandemic infections.[1,2] Interactions between viral proteins and host factors are often crucial for successful replication of the virus in host cells.[3] Many of these interactions are aimed at overcoming the early innate immune response of infected cells against the virus.[4] Mammalian cells respond to viral infections through some innate immune mechanisms.[5] One such crucial antiviral mechanism is activation of dsRNA dependent protein kinase (PKR) which is phosphorylated upon

Abbreviations: P58^{IPK}, the 58-kDa inhibitor of the interferon-induced double-stranded RNA-activated protein kinase; pP58^{IPK}, porcine P58IPK; PKR, dsRNA dependent protein kinase; eIF2a, the eukaryotic translation initiation factor 2 alpha subunit; ER, endoplasmic reticulum; SUVEC, swine umbilical vein endothelial cell line; ORF, Open Reading Frame; p_I, isoelectric point; M_w, molecular weight; C_T, threshold cycle; HE, hematoxylin–eosin; TPR, tetratricopeptide repeat.

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encountering viral dsRNA.[6] Activated PKR can phosphorylate the eukaryotic translation initiation factor 2 alpha subunit (eIF2a), which leads to translation arrest and inhibition of protein synthesis from viral mRNAs.[7,8] However, PKR can be inhibited by the 58-kDa inhibitor of the interferon-induced double-stranded RNA-activated protein kinase ($P58^{IPK}$) that is activated by influenza virus infection at the post-translational level,[9–11] which promotes influenza viral replication.[12,13] Influenza nucleoprotein is the viral factor that facilitates the inhibition of PKR activation by releasing $P58^{IPK}$ from Hsp40-$P58^{IPK}$ complex.[14]

Not only is $P58^{IPK}$ activated by influenza virus infection, but it is also activated at the transcriptional level in response to endoplasmic reticulum (ER) stress. During ER stress, $P58^{IPK}$ inhibits another eIF2a kinase, PERK, which functions to regulate protein synthesis during the unfolded protein response.[15,16] Further evidence has also shown that $P58^{IPK}$ plays a larger role in the protein processing efficiency of the ER by binding to misfolded proteins and acting as a co-chaperone.[17–19]

Pigs serve as a suitable model for studying respiratory tract and influenza disease because they have a close phylogenetic relationship with humans, share many physiological similarities with humans, and offer several breeding and handling advantages.[20] Although the function of human $P58^{IPK}$ has been studied for a long time, porcine $P58^{IPK}$ ($pP58^{IPK}$) has little been studied except for its cloning.[21]

2. Objectives

The purpose of the present study is to characterize the features of the $pP58^{IPK}$ gene and investigate its subcellular localization and the changes of its expression during H1N1 or H3N2 infection in order to determine whether $pP58^{IPK}$ have similar structures and play a role similar to its homolog in human.

3. Study design

3.1. Cloning and storage of $pP58^{IPK}$ gene

The full length cDNA of $pP58^{IPK}$ with 1608 bp was cloned from porcine spleen by reverse transcription PCR and deposited in GenBank (GenBank ID: HQ287801.1), and its polyclonal antibody was also prepared as previously described.[21] All of the products were stored in the Research Laboratory of Virology, Immunology and Bioinformatics, College of Veterinary Medicine, Northwest A&F University.

3.2. Experimental animals and virus infection

Nine healthy 30-day-old Yorkshire piglets were randomly divided into H1N1 infected (G1), H3N2 infected (G2) and control (G3) groups according to complete randomization criteria. These piglets were free of influenza A virus, classical swine fever virus, PRRSV, porcine circovirus type 2, porcine parvovirus and pseudorabies virus, confirmed by both serology and PCR at the arrival of the piglets. The influenza viruses used described below were propagated in MDCK cells and titrated to determine the 50% tissue culture infective dose ($TCID_{50}$) for use in the studies. Three piglets in G1 were inoculated with 10^5 $TCID_{50}$/ml H1N1 (A/Swine/Guangdong/LM/2004(H1N1))[22] by tracheal injection (3 ml). Another three piglets in G2 were inoculated with 10^5 $TCID_{50}$/ml H3N2 (A/Swine/Guangdong/164/06(H3N2))[23] by tracheal injection (3 ml). The rest three control piglets in G3 received the same treatment with DMEM. Piglets were kept in separate rooms and monitored 7 days after inoculation. Clinical signs including coughing, dyspnea, anorexia, lameness and shivering were recorded daily.

3.3. Bioinformatic analysis of sequence and structure

Initially, the $pP58^{IPK}$ cDNA sequence was blasted with pig genomic database Sscrofa10.2 in NCBI to analyze the exon–intron organization. Then, the mRNA sequence was entered into Open Reading Frame (ORF) Finder software (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) to determine its ORF and acquire amino acid sequences. The theoretical isoelectric point (p_I) and molecular weight (M_w) of $pP58^{IPK}$ protein were computed using the Compute p_I/M_w tool (http://web.expasy.org/compute_pi/). The protein sequence was compared with the homologues from other 15 species using the DNAman program to determine its conservatism. The subcellular localization of $pP58^{IPK}$ protein was predicted using the protein localization software (http://www.bioinfo.tsinghua.edu.cn/SubLoc/). The conservative domains were analyzed using the PROSITE tools (http://www.expasy.org/prosite/). The tertiary structure was predicted using the SWISS-MODEL Workspace.

3.4. Phylogenetic and molecular evolutionary analyses

Phylogenetic and molecular evolutionary analyses were conducted using MEGA4 software.[24] $P58^{IPK}$ protein sequences from 16 different species, i.e., *Dictyostelium discoideum* AX4, western clawed frog, *Nicotiana benthamiana*, *Solanum lycopersicum*, honey bee, thale cress, *Schistosoma japonicum*, cattle, zebrafish, fruit fly, chicken, human, mouse, rat, African clawed frog, and pig were used for the unrooted phylogenetic tree constructed. These sequences were deposited in GenBank (GenBank Nos. NP_001008437.1, NP_001080099.1, NP_955904.1, NP_777181.1, NP_006251.1, NP_032955.2, NP_071568.1, XP_637810.1, ADO20319.1, AAF54411.1, NP_001011485.1, AAP41819.1, AAP41818.1, XP_396885.3, NP_195936.1, and CAX69829.1). The evolutionary history of $P58^{IPK}$ was inferred using the neighbor-joining method.[25] The optimal tree with the sum of branch length = 4.19792160 was shown. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method[26] and were in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset. There were a total of 384 positions in the final dataset.

3.5. Intracellular distribution of porcine $pP58^{IPK}$ protein

cDNAs containing the ORF of $pP58^{IPK}$ without additional flanking sequences were generated by PCR with the following primer pairs: P1, 5′-TCACCGCCCGCGTCCGGTACCATGGT-3′ and P2, 5′-CCGGATCCCTGGGAAAAACGGAAGGAAGAGG-3′; P3, 5′-GCCCGCGTCCAAGCTTATGGTGGC-3′ and P4, 5′-GAGACAGGGATCCGGATTGAAGTGGAATT-3′. To avoid the interference of GFP-tag on the localization of $pP58^{IPK}$, the PCR products were digested with restriction endonucleases and subcloned into the *Kpn* I and *Bam*H I sites of pEGFP-C1 vector (BD Biosciences Clontech, CA, USA) and the *Hind* III and *BamH* I sites of pEGFP-N1 vector (BD Biosciences Clontech, CA, USA). The recombinant vectors were named pEGFP-$pP58^{IPK}$ and $pP58^{IPK}$-pEGFP. The established swine umbilical vein endothelial cell line (SUVEC) was cultured first in 6-well plates as previously described[27] for 24 h and then in medium without serum for 1 h. Cells were transfected with 4 μg of the pEGFP-$pP58^{IPK}$ or $pP58^{IPK}$-pEGFP vectors combined with Lipofectamine 2000