The viral ankyrin repeat protein (ORF124L) from infectious spleen and kidney necrosis virus attenuates nuclear factor-κB activation and interacts with IκB kinase β

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The ankyrin (ANK) repeat is one of the most common protein–protein interaction motifs, found predominantly in eukaryotes and bacteria, but the functions of the ANK repeat are rarely researched in animal viruses, with the exception of poxviruses. Infectious spleen and kidney necrosis virus (ISKNV) is a typical member of the genus Megalocytivirus in the family Iridoviridae and is a causative agent of epizootics in fish. The genome of ISKNV contains four putative viral ANK (vANK) repeat proteins and their functions remain largely unknown. In the present study, it was found that ORF124L, a vANK repeat protein in ISKNV, encodes a protein of 274 aa with three ANK repeats. Transcription of ORF124L was detected at 12 h post-infection (p.i.) and reached a peak at 40 h p.i. ORF124L was found to localize to both the nucleus and the cytoplasm in mandarin fish fry cells. ISKNV ORF124L interacted with the mandarin fish IκB kinase β protein (scIKKβ), and attenuated tumour necrosis factor alpha (TNF-α)- or phorbol myristate acetate (PMA)-induced activity of a nuclear factor κB (NF-κB)–luciferase reporter but did not interfere with the activity of an activator protein 1 (AP-1)–luciferase reporter. Phosphorylation of IκBα and nuclear translocation of NF-κB were also impaired by ISKNV ORF124L. In summary, ORF124L was identified as a vANK repeat protein and its role in inhibition of TNF-α-induced NF-κB signalling was investigated through interaction with the mandarin fish IKKβ. This work may help to improve our understanding of the function of fish iridovirus ANK repeat proteins.

INTRODUCTION

Iridoviruses are icosahedral cytoplasmic DNA viruses that infect insects and cold-blooded vertebrates including fish, amphibians and reptiles (Williams, 1996). The genome of the virus consists of linear dsDNA and has been found to be circularly permuted and terminally redundant, which is unique for eukaryotic viruses (Tidona & Darai, 2000). The family Iridoviridae has been subdivided into five genera: Iridovirus, Chloriridovirus, Ranavirus, Lymphocystivirus and Megalocytivirus (Chinchar et al., 2005). Megalocytiviruses naturally infect about 100 species of marine and freshwater fish, and cause significant mortality in aquaculture facilities in China, Japan and South-east Asia (Wang et al., 2007). Infectious spleen and kidney necrosis virus (ISKNV) is a type of megalocytivirus and has been reported as the major pathogen responsible for mass mortalities in mandarin fish (He et al., 1998). The complete 110 kb genome of the ISKNV has been sequenced and the organization of its ORFs has been analysed and described previously (He et al., 2001). The genome of ISKNV contains 124 putative ORFs, including four putative ankyrin (ANK) repeat proteins (ORF77R, ORF102R, ORF119L and ORF124L), five RING finger proteins and a viral vascular endothelial growth factor (Wang et al., 2008; Xie et al., 2007).

Nuclear factor κB (NF-κB) comprises a family of key transcriptional regulators of inducible factors needed by the immune system, which in mammals comprise RelA (p65),

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Details of the sequence and secondary structure analysis of ISKNV ORF124L and of the primers used in this study are available with the online version of this paper.
ReLB, c-Rel, NF-κB1 (p50) and NF-κB2 (p52) (Hayden & Ghosh, 2008). In unstimulated cells, NF-κB dimers are sequestered in the cytoplasm through their Rel homology domains by NF-κB inhibitory proteins, called IκBs (Ghosh et al., 1998). NF-κB1 and NF-κB2 are first expressed as the precursor proteins p105 and p100, which share structural homology in the C-terminal region with IκB (Qing et al., 2005). The canonical NF-κB signalling pathway can be triggered by various inducers such as tumour necrosis factor alpha (TNF-α) and requires the degradation of IκB by the ubiquitin–proteasome pathway (Schmid & Adler, 2000). NF-κB signalling events rapidly lead to activation of the IκB kinase (IKK) complex, which is composed of three subunits, IKKα, IKKβ and IKKγ (NEMO) (Shambharkar et al., 2007). Activated IKK phosphorylates IκB, predominantly through the action of IKKβ, triggering its polyubiquitination and proteasomal degradation and inducing the nuclear translocation of associated NF-κB subunits (Mohamed & McFadden, 2009).

The ANK repeat, a 33-residue domain, is one of the most common protein–protein interaction motifs found in nature (Mosavi et al., 2004). The function of ANK repeat proteins has been studied extensively in eukaryotes and bacteria. Proteins containing ANK repeats, such as IκB, p100 and p105, have been implicated in the control of many cellular processes, including cytoskeleton integrity, cell–cell signalling, transcription, cell-cycle regulation, the inflammatory response and development (Werden & McFadden, 2008). Numerous genes encoding ANK-containing proteins have been identified in eukaryotic viruses, such as poxviruses and iridoviruses (Bork, 1993; Lua et al., 2005). To date, only the viral ANK (vANK) repeat proteins found in poxviruses have been studied in detail and they have been found to be involved in modulating intracellular signalling networks during viral infection. For example, the rabbit-specific poxvirus, myxoma virus, encodes four unique vANK repeat proteins, termed M-T5, myxoma nuclear factor (MNF), M148R and M149R, all of which have been described as virulence factors (Blanie et al., 2009; Mossman et al., 1996). M-T5 has the ability to bind and activate cellular Akt, leading to permisive myxoma virus replication in a variety of human cancer cell lines (Wang et al., 2006). MNF has been shown to co-localize with the transcription factor NF-κB in the nucleus of TNF-α-stimulated cells (Camus-Bouclainville et al., 2004). However, the functional roles of vANK proteins in other vertebrate viruses such as fish iridoviruses remain largely unknown.

In the present study, we identified ISKNV ORF124L as a vANK repeat protein and investigated its function in inhibition of the NF-κB signalling transduction pathway through interaction with the mandarin fish IKKβ.

RESULTS

Sequence analysis of ISKNV ORF124L

A 495 bp fragment (see Supplementary Fig. S1a, available in JGV Online) was obtained by 5′-RACE amplification from the cDNA of mandarin fish fry (MFF-1) cells infected with ISKNV and subcloned into a pMD-19T vector. 5′-RACE showed that the transcription start site of ORF124L was at the ISKNV genomic coordinate nt 484, and the full-length cDNA of ORF124L contained 1182 bp with an ORF of 825 bp. As reported previously, computer-assisted analysis showed that ISKNV ORF124L (GenBank accession no. AF371960; note that the ORF in the published paper was defined as ORF124 but in GenBank was named ORF125L) is an ORF of 687 bp at nt 110665–111351 in the genomic sequence (He et al., 2001). ORF124L encoded a protein of 274 aa with a molecular mass of 29.7 kDa and an isoelectric point of 5.78. Domain prediction by SMART (http://smart.embl-heidelberg.de/) revealed that ORF124L had three vANK repeats at aa 95–113, 131–159 and 172–192 (see Supplementary Fig. S1b). The deduced protein was 46 aa longer at the N terminus than that predicted from the ISKNV genome by bioinformatic analysis. None of the conserved motifs was found in the region of aa 1–94 at the N terminus of ORF124L and no signal peptide was predicted by the SignalP program. A BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis revealed that both orange-spotted grouper iridovirus (OSGIV) ORF121L and rock bream iridovirus ORF118L are homologues of ISKNV ORF124L.

ISKNV ORF124L expression in ISKNV-infected MFF-1 cells

We produced an anti-ORF124L antibody by immunizing New Zealand white rabbits with a recombinant maltose-binding protein (MBP)–ORF124L protein. The antisera were used for detection of the expression of ORF124L at the protein level by Western blotting. As shown in Fig. 1(a), a specific band between 25 and 32 kDa was detected in the sample from ISKNV-infected MFF-1 cells, and no band was observed in the sample from uninfected cells. MBP fusion proteins of the other three ISKNV ANK repeat proteins (ORF77R, ORF102R and ORF119L) could not be detected using anti-ORF124L serum (data not shown). The size of the specific band was consistent with the predicted size of ORF124L.

Real-time PCR was performed to detect transcription of ORF124L in ISKNV-infected MFF-1 cells at various time intervals post-infection (p.i.). The major capsid protein gene (mcp) of ISKNV was used as a control. As shown in Fig. 1(b), transcription of the mcp gene was first detected at 32 h p.i., whilst transcription of ORF124L was first detected at 10 h p.i. and its level had increased significantly (P<0.01) at 32 h after infection with ISKNV.

Subcellular localization of ORF124L

As the function of a protein is closely correlated with its subcellular localization, we investigated the subcellular distribution of ORF124L. We transfected MFF-1 cells with the expression vector pEGFP-ORF124L and observed the
localization of the recombinant protein by confocal microscopy at 48 h post-transfection. As shown in Fig. 2(a), recombinant GFP–ORF124L localized to both the nucleus and cytoplasm of MFF-1 cells. In fact, a larger proportion of GFP–ORF124L was observed to localize to the nucleus than to the cytoplasm.

To investigate any possible roles of the vANK repeat domains of ORF124L in its subcellular localization, we created the ORF124L deletion mutants GFP–ΔANK1, GFP–ΔANK2 and GFP–ΔANK3, which lacked individual vANK repeat domains in an otherwise intact GFP-tagged ORF124L protein. As shown in Fig. 2(b), GFP–ΔANK1, GFP–ΔANK2 and GFP–ΔANK3 were all observed to localize to the cytoplasm only, in contrast to the nuclear and cytoplasmic distribution of GFP–ORF124L. We investigated this further by Western blotting using anti-GFP antibody. As shown in Fig. 2(c), GFP–ORF124L was detected in the nuclear and cytoplasmic fragments, whilst GFP–ΔANK1, GFP–ΔANK2 and GFP–ΔANK3 were detected only in the cytoplasmic fragment. These observations indicated that ORF124L localizes to both the nucleus and the cytoplasm and that its vANK repeat domains are necessary for nuclear localization.

**Interaction of ISKNV ORF124L with IKKβ**

In order to determine whether ORF124L interacts with IKKβ, we cloned scIKKβ, the IKKβ gene from mandarin fish (*Siniperca chuatsi*) (Chen et al., 2011). pc-Myc-ORF124L and pFLAG-scIKKβ expression vectors were co-transfected into human embryonic kidney 293T (HEK293T) cells, and a co-immunoprecipitation assay was performed using anti-FLAG antibody. As shown in Fig. 3(a), scIKKβ was indeed present in the cells co-transfected with pFLAG-scIKKβ and pc-Myc-ORF124L (Fig. 3a, lane 5). When control lysates from cells transfected with pcDNA3.0 alone (Fig. 3a, lane 1), or co-transfected with pcDNA3.0 and pFLAG-scIKKβ (Fig. 3a, lane 2) or pcDNA3.0 and pc-Myc-ORF124L (Fig. 3a, lane 3) were precipitated with anti-FLAG antibody, no c-Myc-tagged ORF124L was detected in the precipitated proteins. However, when the lysates from cells co-transfected with pFLAG-scIKKβ and pc-Myc-ORF124L were precipitated with anti-FLAG antibody, c-Myc-tagged ORF124L was detected by anti-c-Myc antibody (Fig. 3a, lane 4), indicating that c-Myc-tagged ISKNV ORF124L interacted with FLAG-tagged scIKKβ.

To confirm that ISKNV ORF124L can indeed interact with scIKKβ, the reciprocal co-immunoprecipitation was performed using anti-c-Myc antibody. When the control lysate from cells co-transfected with pcDNA3.0 and pFLAG-scIKKβ was precipitated with anti-c-Myc antibody, FLAG-tagged scIKKβ was not detected by anti-FLAG antibody (Fig. 3b, lane 6). However, when the lysate from cells co-transfected with pFLAG-scIKKβ and pc-Myc-ORF124L was precipitated with anti-c-Myc antibody, FLAG-tagged scIKKβ was detected by anti-FLAG antibody (Fig. 3b, lane 7). As a positive control, lysate from cells co-transfected with pFLAG-scIKKβ and pc-Myc-ORF124L was precipitated with anti-FLAG antibody, and FLAG-tagged scIKKβ was detected by anti-FLAG antibody (Fig. 3b, lane 8). Together, these results indicated that Myc-tagged ISKNV ORF124L can interact with FLAG-tagged scIKKβ protein.
ISKNV ORF124L attenuates the NF-κB signalling pathway

IKKβ is a member of the IKK complex and serves as the master regulator for activation of NF-κB by various stimuli (Shambharkar et al., 2007). To examine whether ISKNV ORF124L interferes with NF-κB activity, HEK293T and HeLa cells were transfected with the pc-Myc-ORF124L plasmid with an NF-κB–luciferase (NF-κB–Luc) reporter vector or a control vector pRL-TK in the presence or absence of TNF-α or phorbol myristate acetate (PMA). Firefly luciferase activity was normalized to Renilla luciferase activity. A luciferase vector that does not contain a cis-acting element responsive to a particular pathway was used as a negative control (pTAL-Luc; Clontech). The relative luciferase activity (RLA) levels of cells transfected with the empty plasmid without stimulation (control sample) were arbitrarily set as 1. Other RLA levels are presented as the fold increase compared with that of the control sample. After TNF-α stimulation, RLA levels in HEK293T cells were significantly increased (10–12-fold). However, RLA levels in cells transfected with pc-Myc-ORF124L remained at a low level (one- to twofold increase) with or without TNF-α stimulation. Similar experiments using PMA instead of TNF-α produced a similar result in HEK293T cells. However, AP-1 activity was not inhibited by ORF124L when HEK293T cells were stimulated by TNF-α (Fig. 4d). These results suggested that ISKNV ORF124L specifically attenuates NF-κB activity.

To confirm that activation of the NF-κB–Luc reporter by TNF-α was indeed inhibited by ISKNV ORF124L, a dose-dependent assay was performed. Cells were transfected with increasing amounts of pc-Myc-ORF124L and stimulated with TNF-α (100 ng ml⁻¹) for 6 h, following which RLA levels were measured. As shown in Fig. 4(e), RLA levels were reduced by 50–70% in cells transfected with 20 and 100 ng pc-Myc-ORF124L compared with the RLA levels of untransfected cells. These results indicated that ISKNV ORF124L can inhibit TNF-α-induced stimulation of NF-κB activity in a dose-dependent manner.

ISKNV ORF124L impairs IκBα phosphorylation and NF-κB nuclear translocation

TNF-α recruits TNF receptor-associated factor 2 (TRAF2) through TNF receptor 1 to activate the IKK complex, and the active IKK complex phosphorylates IκBs, which allows NF-κB to translocate into the nucleus and regulate transcription of its target genes. In order to determine the mechanism by which ORF124L inhibits NF-κB activity,
we compared the subcellular distribution of p65 in control and ORF124L-transfected cells. Cytoplasmic and nuclear fractions of cells were extracted at different time points after the cells were treated with TNF-α and analysed by Western blotting using anti-p65 antibody. As shown in Fig. 5(a), in the control group, the ratio of nuclear to cytoplasmic p65 increased 15 min after the cells were treated with TNF-α and reached a 3.9-fold increase at 30 min after treatment compared with the ratio before TNF-α treatment (0 h). Thereafter, the ratio of nuclear to cytoplasmic p65 gradually decreased between 60 and 360 min after TNF-α treatment. In contrast, overexpression of ORF124L decreased the ratio of nuclear to cytoplasmic p65 after stimulation with TNF-α at all time points. These results indicated that ORF124L prevents endogenous NF-κB from translocating into the nucleus. NF-κB has been shown to be sequestered in the cytoplasm by a group of ANK repeat-containing protein IkB (Glasgow et al., 2001). To get an insight into how ORF124L interfered with NF-κB in the cytoplasm, we examined the effect of ORF124L on phosphorylation of IkB. Overexpression of ORF124L resulted in a relatively low level of phosphorylation of IxBz following TNF-α treatment at 15 min compared with the control group (Fig. 5b). These findings raise the possibility that ISKNV ORF124L may inhibit TNF-α-induced nuclear translocation of NF-κB by interfering with phosphorylation of IkB.

**DISCUSSION**

Successful viral pathogens have evolved efficient strategies to overcome or manipulate innate and adaptive immune mechanisms, and one common target for manipulation by viral pathogens is NF-κB. Many viruses are known to encode multiple proteins that can interfere with NF-κB function either directly by inhibiting the immediate signalling members of the NF-κB family or indirectly by targeting upstream events that trigger the activation of NF-κB (Werden & McFadden, 2008). In poxviruses, viral cytokine receptors such as the viral TNF receptor encoded by the myxoma virus directly block activation of the NF-κB signalling pathway (Upton et al., 1991). Similarly, the cowpox virus CrmA protein can inhibit caspase-1-mediated processing of pro-interleukin-6β (pro-IL-6β) and pro-IL-8β to their mature forms, leading essentially to the inhibition of NF-κB activation (Ray et al., 1992). However, the mechanisms by which iridoviruses escape the immune response and interfere with NF-κB signalling pathway are relatively less known. Here, we have reported that ISKNV ORF124L can inhibit TNF-α-induced NF-κB activation in HEK293T cells and interact with IKKβ.

As is well known, activation of the IKK complex is necessary for the NF-κB signalling pathway. Many viral proteins are known to interfere with the activity of the IKK complex to disrupt the NF-κB signalling pathway. The vaccinia virus B14R gene encodes a 17 kDa cytosolic protein (B14) that contributes to viral virulence and inhibits the IKK complex (Chen et al., 2006, 2008). The interaction of B14 with the IKK complex depends on the presence of IKKβ, and B14 binding to the IKK complex prevents phosphorylation of the IKKβ activation loop. Furthermore, the ability of some vANK repeat-containing proteins to inhibit NF-κB activity through the IKK complex has also been reported (Shisler & Jin, 2004). For example, vaccinia virus K1, a vANK repeat protein, has been shown to inhibit NF-κB activation by preventing IxB degradation. CP77, a cowpox virus vANK repeat protein, is able to block TNF-α-induced NF-κB activation at a step downstream of IKK kinase activation (Chang et al., 2009). In order to find out whether ISKNV ORF124L interacted with IKKβ, we subcloned the mandarin fish scIKKβ (Chen et al., 2011), and showed by co-immunoprecipitation assays that ORF124L interacts with scIKKβ (Fig. 3). This result indicated that an event downstream of the IKK complex might be affected by ISKNV ORF124L.

The NF-κB system is an ancient signalling pathway found in both insects and vertebrates (Friedman & Hughes, 2002). Components of the NF-κB signalling pathway are
ISKNV ORF124L attenuates the activity of the NF-κB–Luc reporter but not the AP-1–Luc reporter. (a) ISKNV ORF124L inhibited TNF-α-induced NF-κB–Luc activity in HEK293T cells. HEK293T cells were transfected with pc-Myc-ORF124L (0.3 μg) together with the NF-κB–Luc reporter vector and control vector pRL-TK in the presence or absence of TNF-α (100 ng ml⁻¹). Firefly luciferase activity was normalized to Renilla luciferase activity. The RLA levels of cells transfected with the empty plasmid (pcDNA3.0) without stimulation (control sample) were arbitrarily set as 1. All other RLA levels are presented as the fold increase over that of the control sample. (b–d) Cells were treated as described in (a) using the NF-κB–Luc reporter vector (b, c) or AP-1–Luc reporter vector (d). ISKNV ORF124L inhibited TNF-α-induced NF-κB–Luc activity in HeLa cells (b) and PMA-induced NF-κB–Luc activity in HEK293T cells (c) but did not inhibit TNF-α-induced AP-1–Luc activity in HEK293T cells (d). (e) NF-κB–Luc reporter activity in cells transfected with increasing amounts of pc-Myc-ORF124L plasmid. Cells were transfected with different amounts of pc-Myc-ORF124L (1–100 ng) and treated with TNF-α (100 ng ml⁻¹) for 6 h, following which NF-κB–Luc reporter activity was analysed. RLA levels in the cells transfected with the control plasmid (pcDNA 3.0), pTAL–Luc control plasmid or pRL-TK without TNF-α stimulation. Firefly luciferase activity was normalized to Renilla luciferase activity. The RLA levels of cells transfected with the empty plasmid without stimulation (control sample) were arbitrarily set as 1. One out of three representative experiments is shown. Results are shown as means ± SD (n=4).
Fig. 5. ISKNV ORF124L impairs nuclear translocation of NF-κB and phosphorylation of IκBα. (a) Western blot analysis of the levels of NF-κB protein in the nucleus and cytoplasm. HEK293T cells were transiently transfected with pc-Myc-ORF124L or pcDNA3.0 (control) and serum starved for 12 h at 24 h post-transfection, followed by treatment with TNF-α (100 ng ml⁻¹) for 0, 15, 30, 60, 120 and 360 min. Cytoplasmic and nuclear fractions were extracted from cell lysates and assayed by Western blotting using anti-p65 antibody. Densitometric measurements of p65 protein in the nucleus were normalized to their detected levels in the cytoplasm. (b) Western blot analysis of the levels of IκBα phosphorylation. Cells were transiently transfected with pc-Myc-ORF124L or pcDNA3.0 (control) and serum starved for 12 h at 24 h post-transfection, followed by treatment with TNF-α (100 ng ml⁻¹) for 0, 5, 10, 15 and 30 min. The cells lysates were then assayed by Western blotting using anti-IκBα-p antibody.

highly conserved from fish to humans (Wang et al., 2009; Chen et al., 2011). Due to limitations imposed by the low efficiency of transfection and the difficulty in studying protein phosphorylation in fish cells, the effect of ISKNV ORF124L on the NF-κB signalling pathway was investigated further in mammalian cells. As shown in Fig. 4, TNF-α- and PMA-induced activity of an NF-κB–Luc reporter was inhibited by ISKNV ORF124L. To find out whether this inhibition occurred via interaction with IKKβ, an AP-1–Luc reporter gene was used to monitor the c-Jun N-terminal kinase (JNK) signalling pathway induced by TNF-α independently of the IKK complex. Our results showed that TNF-α-mediated stimulation of JNK signalling was not blocked by ISKNV ORF124L (Fig. 3d). These results are consistent with the idea that interaction between ORF124L and IKKβ may underlie the inhibition of NF-κB activation by ORF124L. Furthermore, we found that ISKNV ORF124L impairs the TNF-α-induced phosphorylation of IκB and the nuclear translocation of NF-κB (Fig. 5). These results suggest that ISKNV utilizes the vANK repeat-containing protein ORF124L to inhibit the NF-κB signalling pathway and thus escape the immune response of host cells.

vANK repeat proteins have been identified in many viruses, such as poxviruses, iridoviruses and ichnoviruses. The majority of poxvirus vANK repeat proteins, including MNF and M-T5, contain several vANK repeat domains within the N terminus and a highly conserved F-box motif located at their C terminus (Mercer et al., 2005). It is well known that the F-box motif mediates protein–protein interactions and is necessary for the interaction of proteins with Skp1–Cullin-1–F-box (SCF1) complexes (Blanie et al., 2010). The SCF1 complex facilitates the ubiquitination of substrates destined for degradation by 26S proteasome and plays key roles in the selective degradation of regulatory proteins that mediate various cellular functions, such as signal transduction and regulation of the cell cycle (Werden & McFadden, 2008). It has been reported that the vANK protein M-T5 binds with the SCF1 complex of Skp1 and cullin-1 proteins, resulting in promotion of cell-cycle progression beyond the G0/G1 checkpoint during viral infection (Johnston et al., 2005). It therefore appears that poxviruses acquired the F-box motif early in evolutionary history from ancestrally infected hosts (Werden & McFadden, 2008). In contrast, ISKNV ORF124 has three vANK repeat domains (see Supplementary Fig. 1B) but no F-box motif, which is also the case in other iridovirus genomes, such as OSGIV (Lü et al., 2005), turbot reddish body iridovirus (Shi et al., 2010) and Banggai cardinalfish iridovirus (Weber et al., 2009).

We also studied the subcellular localization of ISKNV ORF124L in mandarin fish cells. Recombinant GFP–ORF124L protein was observed to localize in both the nucleus and the cytoplasm (Fig. 2a), which is comparable to the subcellular distribution of M-T5, a vANK repeat protein in myxoma virus. M-T5 co-localizes with the cell-cycle regulatory protein cullin-1 in both nuclear and cytosolic compartments to protect infected cells from the stress of cell-cycle arrest. As the F-box motif is necessary for the interaction of ANK repeat proteins with SCF1, absence of the F-box motif in ORF124L suggests that, despite the similarity in subcellular distribution, the function of ORF124L may not be identical to that of M-T5. ANK repeat regions are known to function as protein–protein interaction domains. Deletion of any of the vANK repeat domains affected the nuclear translocation of ORF124L (Fig. 2b). The observations suggested that the function of ORF124L in the nucleus might be associated with the
vANK repeat domains, although this remains unclear. A nuclear localization of vANK repeat proteins has also been observed in other viruses. Myxoma virus MNF has been shown to co-localize with NF-κB in the nucleus of TNF-α-stimulated cells (Camus-Bouclainville et al., 2004). CvBV805 of Cotesia vestalis polydnavirus has also been reported to localize within the nuclear region in haemocytes (Shi et al., 2008). However, the roles of vANK repeat proteins in the nucleus in infected cells are still poorly understood. More work needs to be done to understand better the functions of ORF124L in the nucleus.

In summary, we identified a role for ORF124L, a vANK repeat protein from ISKNV, in NF-κB activation. ISKNV ORF124L was observed to interact with IKKβ, attenuate TNF-α- and PMA-induced activity of an NF-κB–Luc reporter and impair nuclear translocation of NF-κB, possibly via inhibition of the phosphorylation of IκB. Our findings may help further improve our understanding of the function of fish iridovirus ANK repeat proteins.

METHODS

Cell lines and virus. MFF-1 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 % FBS at 27 °C in a humidified atmosphere containing 5 % CO2. HEK293T and HeLa cells were a kind gift from Professor Ying-Qiu Li (Sun Yat-sen University, Guangzhou, PR China) and were cultured in DMEM supplemented with 10 % FBS at 37 °C. The ISKNV used in this study was originally isolated from diseased mandarin fish and maintained by our laboratory.

Antibodies and reagents. Rabbit polyclonal serum against ISKNV ORF124L (anti-ORF124L) was prepared according to a method described previously (Guo et al., 2011). Mouse anti-GFP mAb was purchased from Clontech. Rabbit anti-p65 polyclonal antibody, rabbit anti-β-actin polyclonal antibody, mouse anti-FLAG M2 mAb, and mouse anti-c-Myc mAb were purchased from Sigma-Aldrich. The rabbit anti-histone 2A mAb was purchased from Epitomics. Anti-phosphorylated IκBα (pSer32/36) mAb (anti-IκBα-p) was purchased from Calbiochem. Goat anti-mouse IgG and goat anti-rabbit IgG conjugated to HRP were purchased from Promega. TNF-α and PMA were purchased from Peprotech.

5′-RACE. 5′-RACE was performed to confirm the full-length transcripts of ISKNV ORF124L using a BD SMART RACE DNA Amplification kit (Clontech) according to the user’s manual. The RACE Ready cDNA was synthesized from total RNA of MFF-1 cells infected with ISKNV. A gene-specific primer (RP1) and a universal primer mix (UPM) (see Supplementary Table S1, available in JGV Online) were used for the first round of 5′-RACE. The product obtained in the first round of PCR was used directly as the template for nested PCR. The second gene-specific primer (RP2) and nested universal primer (NUP) (Supplementary Table S1) were used for the second round of PCR. The PCR product obtained was cloned into a pMD19-T vector (Takara) and sequenced.

Plasmid construction. Full-length cDNA of ISKNV ORF124L was PCR amplified from cDNA of ISKNV-infected MFF-1 cells using the corresponding primer pairs (Supplementary Table S1) and cloned into a pcDNA3.0 or pEIGHT-N1 vector to generate pc-Myc-ORF124L or pEIGHT-ORF124L, expressing a c-Myc-tagged ORF124L protein or a GFP fusion protein (GFP–ORF124L), respectively. The pcDNA-siKKβ expression vector with a FLAG tag (pFLAG-siKKβ) was constructed by amplifying the full-length sequence of siKKβ using its corresponding primer pair (Supplementary Table S1) and inserting the PCR product into pcDNA3.0. To construct the ANK repeat domain deletions of ISKNV ORF124L, a two-step recombinant PCR was performed using appropriate primer pairs, as reported previously (Higuchi, 1990). The PCR products were then cloned into a pEGFP-N1 plasmid to generate pEIGHT-ΔANIK1, pEIGHT-ΔANIK2 and pEIGHT-ΔANIK3, expressing the GFP fusion proteins GFP–ΔANIK1, GFP–ΔANIK2 and GFP–ΔANIK3, respectively. All recombinant plasmids were confirmed by sequencing.

Virus infection and detection of ORF124L. MFF-1 cells cultured in six-well plates were infected with ISKNV (m.o.i. ~10). After 1 h incubation at 27 °C, the virus inocula was aspirated and replaced with fresh medium, and incubation of the cultures was continued. At 96 h p.i., cells were harvested and lysed with modified RIPA cell lysis buffer [50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 1 % sodium orthovanadate, 1 % Triton X-100, 0.5 % deoxycholate, 0.1 % SDS] containing Protease Inhibitor Cocktail (Calbiochem). After centrifugation, the supernatant was subjected to Western blotting using anti-ORF124L antibody.

Real-time quantitative PCR. MFF-1 cells were harvested by centrifugation at various times (2, 4, 6, 8, 12, 16, 24, 48, 72, 96 and 120 h) after infection with ISKNV (m.o.i. ~10). Total RNAs were extracted and reverse transcribed to cDNA. Expression of the ISKNV ORF124L and mcp genes was investigated by quantitative RT-PCR on a LightCycler (Roche Diagnostics), as reported previously (Guo et al., 2009). Primers for real-time PCR were designed using Primer Express software (Applied Biosystems) (Supplementary Table S1). β-Actin, a housekeeping gene, was used as a control. The expression level of each transcript was normalized to β-actin expression. Real-time quantitative PCR data of target genes were analysed using Q-gene statistics (Mulder et al., 2002). All data are expressed as means ± SD.

Transfection and luciferase reporter assay. Transient transfections were conducted with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The cells were transfected with the appropriate plasmids in serum-free culture medium. After 4 h, the medium was replaced by complete medium with 10 % FBS. Transfections were normalized with appropriate empty vector plasmids if necessary. For the luciferase assay, cells were cultured in 48-well plates and co-transfected with luciferase reporter plasmids (NF-κB–Luc or AP–1–Luc), pRl-TK and the ORF124L expression vector pcDNA-ORF124L. At 36 h post-transfection, transfected cells were treated with TNF-α (100 ng ml−1) or PMA (100 nM) for 6 h. The luciferase activity of total cell lysates was measured using a Dual-Glo Luciferase Assay System (Promega). NF-κB-mediated luciferase activity was normalized with Renilla luciferase activity. The luciferase assay was carried out in triplicate.

Subcellular localization and confocal microscopy analysis. To investigate the subcellular localization of ISKNV ORF124L, MFF-1 cells were transfected with the pEGFP-ORF124L, pEIGHT-ΔANIK1, pEIGHT-ΔANIK2 or pEIGHT-ΔANIK3 vector as described above. At 36 h post-transfection, cells were subjected to confoal microscopy or Western blotting analysis using an anti-GFP antibody. For confocal microscopy, cells were washed three times with PBS and fixed with 4 % paraformaldehyde for 15 min. Cell nuclei were then stained with propidium iodide (PI) or Hoechst 33342. The cells transfected with fluorescent vectors were observed directly using a laser-scanning confocal microscope.

Co-immunoprecipitation. HEK293T cells were co-transfected with plasmids pc-Myc-ORF124L and pcDNA-siKKβ, pcDNA 3.0 and
pc-Myc-ORF124L, or pcDNA 3.0 and pcDNA-scIKK/β. At 48 h post-transfection, the cells were harvested and lysed using RIPA cell lysis buffer supplemented with PMSF (1 mM), aprotonin (2 g ml⁻¹), leupeptin (2 g ml⁻¹) and soybean trypsin inhibitor (37.5 g ml⁻¹). For co-immunoprecipitation, 1 μg of the indicated antibody was added to pre-cleared cell lysate and incubated overnight at 4 °C. Immune complexes were then captured by 10 μl protein A–Sepharose, washed six times with RIPA buffer and subjected to Western blotting using anti-FLAG M2 or anti-c-Myc antibody.

Isolation of nuclear and cyttoplasmic fragments. Cells were transfected with pc-Myc-ORF124L and, at 24 h post-transfection, the cells were serum-starved for 12 h, followed by treatment with TNF-α (100 ng ml⁻¹) for up to 360 min. At different time intervals, the cells were lysed with RIPA buffer and collected for preparation of cyttoplasmic and nuclear fractions using an NE-PER Nuclear and Cyttoplasmic Extraction Reagent kit (Pierce) according to the manufacturer’s instructions.

Western blotting. Total protein from all samples was quantified using a DC Protein Assay kit (Bio-Rad) to ensure equal loading and then subjected to SDS-PAGE. Proteins were transferred to PVDF membranes and non-specific binding was inhibited by blocking the membranes for 2 h at room temperature with 5% (w/v) BSA in TBS containing 0.05% (v/v) Tween 20. The membranes were incubated overnight at 4 °C in primary antibodies. Anti-p65, anti-ORF124L, anti-c-Myc, anti-FLAG M2, anti-ΔNp75 or anti-p-IκBα antibodies were used as primary antibodies. Signals were developed by incubating the membranes with the corresponding HRP-conjugated secondary antibodies for 1.5 h and detected using 3,3′-diaminobenzidine tetrachloride, or enhanced chemiluminescence reagent (Pierce) and developed by incubating the membranes for 2 h at room temperature with 5% (w/v) BSA in TBS containing 0.05% (v/v) Tween 20. The membranes were incubated overnight at 4 °C in primary antibodies. Anti-p65, anti-ORF124L, anti-c-Myc, anti-FLAG M2, anti-ΔNp75 or anti-p-IκBα antibodies were used as primary antibodies. Signals were developed by incubating the membranes with the corresponding HRP-conjugated secondary antibodies for 1.5 h and detected using 3,3′-diaminobenzidine tetrachloride, or enhanced chemiluminescence reagent (Pierce) and exposing the PVDF membranes to Hyperfilm (Amersham Biosciences).

Statistical analysis. All data were analysed using one-way analysis of variance in sss version 11.5. P<0.01 was considered highly significant.

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