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Short communication: Molecular characterization of dog and cat p65 subunits of NF-kappaB

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ABSTRACT

Nuclear factor kappa B (NF-κB) plays an important role in the immune system. The p65 subunit is an important part of NF-κB unit, and studies of dog and cat p65 subunits of NF-κB (dp65 and cp65) are important in understanding their immune function. In this study, we described the molecular characterization of dp65 and cp65. The dp65 and cp65 complementary DNA encoded 542 and 555 amino acids, respectively, showing a high sequence homology with the mammalian p65 subunit (>87.5%). Quantitative polymerase chain reaction revealed that the p65 messenger RNA is highly expressed in the dog stomach and cat heart and adipose tissue. Functional NF-κB promoter-luciferase reporter vectors revealed that our isolated dp65 and cp65 cDNA encodes a functionally active protein. Transiently expressed dp65 and cp65 up-regulated pro-inflammatory cytokine expression levels in dog and cat, respectively. These findings suggest that dp65 and cp65 play important roles in regulating immune function.

Nuclear factor-kappa B (NF-κB) plays an important role in the immune system and cell survival, differentiation, and proliferation (Courtois and Gilmore, 2006; Li and Verma, 2002). In mammals, there are five members in the NF-κB family, namely: RelA (p65), RelB, c-Rel, p50 (NF-κB1), and p52 (NF-κB2; Caamaño and Hunter, 2002). All members share an N-terminal Rel homology domain (RHD), which is required for homo- and hetero-dimerization, nuclear translocation, association with inhibitory proteins, and DNA binding (Ghosh and Karin, 2002). p65, c-Rel, and RelB subunits have a transcriptional activation domain (TAD; 1 and 2) and can activate target gene transcription (Hayden and Ghosh, 2008).

The p50/p65 heterodimer is considered to be the most abundant and ubiquitously expressed among the NF-κB dimers, and the p65 subunit is the only ubiquitously expressed mammalian NF-κB protein containing TAD (Chen et al., 1999). The vital importance of p65 subunit is revealed by the finding that its absence results in hepatocyte apoptosis and embryonic lethality (Beg et al., 1995), although the lack of other subunits only results in immunodeficiency (Li and Verma, 2002). In addition, post-translational modification is required for the p65 subunit *in vivo* or *in vitro*. Multiple p65 subunit phosphorylation sites have been described, and these phosphorylations have modulatory roles in the NF-κB transcriptional activity (Perkins, 2006). In addition, the p65 subunit is inducibly acetylated at various sites, which has different effects on its activity (Chen et al., 2001; Perkins, 2006).

Activated NF-κB turns on many downstream target genes to mediate immune responses, especially pro-inflammatory cytokines induce NF-κB signaling, resulting in the transcriptional regulation of pro-inflammatory genes (Lawrence, 2009). IL-1β, IL-6, and TNF-α are key mediators in forming the inflammatory milieu that is deeply involved with the development of diseases such as cancer (Multhoff et al., 2012) and diabetes (Tilg and Moschen, 2008).

Immune-mediated diseases, particularly autoimmune diseases, are of major clinical significance in dogs and cats (Whitley and Day, 2011). Moreover, extension of their life span has led to changes and the failure of immune function and increased cancer and metabolic diseases morbidities (Day, 2010). As described above, studies on the dog and cat p65 subunits of NF-κB (dp65 and cp65, respectively) would be important in understanding the immune function and immune related diseases. Recently, clinical pathology research targeting dp65 and cp65 has been reported (Ito et al., 2013; Tamamoto et al., 2013). However, very little information is available on the molecular characterization of dp65 and cp65. Therefore, this study aimed to clone and characterize dp65 and cp65 and examine their regulation of immune function through pro-inflammatory cytokine gene expression in dog and cat fibroblast cells.

For cloning of dp65 and cp65 cDNA, we performed 5' and 3'-end rapid amplification of cDNA ends (RACE). A cDNA library was prepared from the dog and cat duodenal total RNA (Zyagen Co., San Diego, CA) using the SMARTer RACE cDNA Amplification Kit

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(Clontech, Mountain View, CA). We designed specific primers for dp65 and cp65 with reference to the predicted sequence in the p65 subunit mRNA (GenBank Accession numbers XM_005631473 for dog and XM_006937605 for cat). dp65 and cp65 partial cDNA sequences were obtained using primers 1 and 2 (Supplementary Table S1). Primer 3 was used for the amplification of the 3'-ends, and primer 4 was used for the 5'-end rapid amplification of cDNA ends of dp65 and cp65. The full-ORF region of dp65 and cp65 cDNA fragment was amplified with pair of sense (primer 5) and antisense (primer 6). The amplified fragment was cloned using pMD-19T vector cloning kit (Takara, Shiga, Japan). The dp65 cDNA consisted of an 89-bp 5'-untranslated region (UTR), a 1626-bp open reading frame (ORF), which encoded 542 amino acid residues, and a 788-bp 3'-UTR. The cp65 cDNA consisted of a 115-bp 5'-UTR, a 1665-bp ORF, which encoded 555 amino acid residues, and a 793-bp 3'-UTR. The predicted dog and cat p65 full-length amino acid sequences were compared with those of other animals, revealing high sequence similarity across mammals (Supplementary Fig. S1; dog vs cat [94.2%], vs pig [91.5%] vs cattle [91.1%], vs human [90.3%], vs mouse [87.5%]). The comparison identified the expected domains of dp65 and cp65 as RHD, TAD1 and TAD2. RHD is conserved at 97.4–99.2%, and TADs are conserved at 89.2–96.6% among mammals. In addition, the serine and threonine residues, which take part in the phosphorylation, and the lysine residues, which take part in the acetylation, were completely conserved in dp65 and cp65 compared with those in other mammals (Chen et al., 2001; Perkins, 2006). These conserved phosphorylation and acetylation sites may be important for the dp65 and cp65 function, although it is not evident whether these sites are actually phosphorylated or acetylated.

To determine the expression levels of dp65 and cp65 mRNAs in dog and cat tissues, we performed quantitative real-time polymerase chain reaction (q-PCR) with pair of sense (primer 7) and antisense (primer 8) primer for dp65 and cp65. The total RNA of dog and cat adipose tissue, bone marrow, cerebral cortex, duodenum, heart, kidney, liver, pancreas, skeletal muscle, skin, spinal cord, spleen and stomach were obtained from Zyagen. After reverse transcription reaction, the cDNA product was subjected to real-time PCR using SYBR premix ExTaq II (Takara). Quantitative measurement was performed by establishing a linear amplification curve from serial 10-fold dilutions ($10^{2–6}$ copy number) of a plasmid containing dp65, cp65 and 18S ribosomal RNA (primer 9 and 10) cDNA fragments. As shown in Fig. 1, the dp65 and cp65 mRNAs were expressed in all examined tissues, similar to other animals (Li et al., 2011; Nolan et al., 1991). In particular, high expression levels of dp65 were observed in the stomach, and of cp65 in the heart and adipose tissue. Because the mRNA expression pattern of the p65 subunit is different in each animal, the p65 subunit may play a part in the development of different diseases in each animal species. Adipose tissue with high expression of cp65 in cats releases a greater amount of inflammatory factors in obese subjects and causes chronic inflammation (Hotamisligil, 2006; Laflamme, 2012) and the development of type 2 diabetes in cats (O'Brien, 2002; Tilg and Moschen, 2008). In contrast, almost all diabetic dogs have type 1 diabetes because of the immune destruction of pancreatic beta-cells, and very few dogs have type 2 diabetes (Rand et al., 2004). Further investigation is required to understand the correlation between p65 subunits and the pathogenesis of diabetes in dogs and cats.

To clarify whether the obtained cDNAs encode functional proteins, we performed the luciferase assay in Chinese hamster ovary cells (CHO-K1), human embryonic kidney epithelial cells (HEK293), and dog and cat uterine fibroblast cells. Uterine tissues were obtained from 5 to 8 month old healthy client-owned dogs and cats that were spayed (written informed consent for the experiment was obtained from the owners) and culture as previously

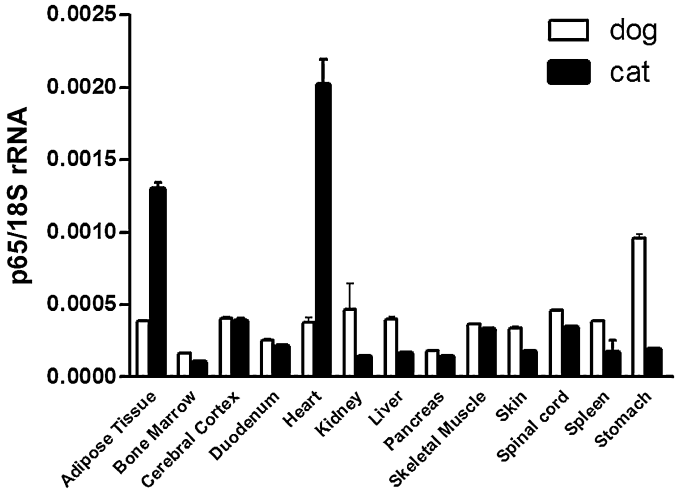

Fig. 1. mRNA expression of the p65 subunit in dog and cat tissues. These were determined by quantitative real-time PCR (q-PCR). Each value was normalized to that of 18S ribosomal RNA and the mean ± standard error of the mean of triplicate experiments.

described (Fukuyama et al., 2012). The full-ORF regions were cloned into mammalian based expression vectors (pcDNA3.1 V5-His B, Invitrogen, Carlsbad, California). Cloned dp65 and cp65 expression vectors (pcDNA3.1-dp65 and pcDNA3.1-cp65) were sequenced, and they confirmed the mRNA expression in cultured cell lines. CHO-K1 cells, HEK293 cells, and the dog and cat fibroblast cells were plated in a 96-well plates at a density of 3×10^4 cells/well (for CHO-K1 and HEK293) or 2×10^4 cells/well (for dog and cat fibroblast cells), and each vector was transfected using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were transfected with 40 ng/well of expression vectors and 40 ng/well of pGL4.32 [Luc2P/NF-κB-RE/Hygro] reporter vector (Promega). The pGL4.73 [*hRluc*/SV40] vector (Promega) was included in all transfections at 20 ng/well to allow normalization for transfection efficiency. Luciferase activities of the pGL4.32 [Luc2P/NF-κB-RE/Hygro] vector, with the luciferase coding sequence under the control of a minimal promoter containing five NF-κB-binding sites, were significantly up-regulated ($P < 0.01$) in all cell lines by transiently expressing dp65 and cp65 compared with those of the mock plasmid (Fig. 2A). Similar to other mammals (Doleschall et al., 2007; Li et al., 2011), transiently expressed dp65 and cp65 caused increased NF-κB transactivation in various mammalian cells, and these data suggest that our isolated dp65 and cp65 cDNA sequence encodes a functionally active dp65 and cp65 protein.

To investigate the effect of dp65 and cp65 on inflammatory reaction, we determined the mRNA expression levels of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) mRNA in fibroblast cells that were transiently expressing dp65 and cp65 by q-PCR (Fig. 2B). Dog and cat fibroblast cells were plated in 60-mm dishes at densities of 5×10^5 cells and transfected with 4 μg/well of expression vectors using lipofectamine 2000. The dog and cat IL-1β (primer 11–14), IL-6 (primer 15–18), and TNF-α (primer 19–22) amplification primers are listed in Supplementary Table S1. By transiently expressing dp65 and cp65, all cytokine expression levels were significantly up-regulated ($P < 0.05$) in dog and cat fibroblast cells, respectively. Fibroblast cells have the ability to function both as a structural element and as a vital immunoregulatory cell (Smith et al., 1997). Furthermore, the cat SAA stimulated transcription of TNF-α and cp65 translocated to the nucleus at monocytes (Tamamoto et al., 2013). Therefore, dp65 and cp65 may have important roles in regulating inflammation.