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Identification of Molecules Involved in the Vulture Immune Sensing of Pathogens by Molecular Cloning

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1. Introduction

Vultures may have one of the strongest immune systems of all vertebrates (Apanius et al., 1983; Ohishi et al., 1979). Vultures are unique vertebrates able to efficiently utilize carcass from other animals as a food resource. These carrion birds are in permanent contact with numerous pathogens and toxins found in its food. In addition, vultures tend to feed in large groups, because carcasses are patchy in space and time, and feeding often incurs fighting and wounding, exposing vultures to the penetration of microorganisms present in the carrion (Houston & Cooper, 1975). Therefore, vultures were predicted to have evolved immune mechanisms to cope with a high risk of infection with virulent parasites.

Despite the potential interest in carrion bird immune system, little is known about the molecular mechanisms involved in the regulation of this process in vultures. The aim of this chapter is to describe the molecular cloning and characterization of two key molecules involved in the immune sensing of pathogens: a griffon vulture (Gyps fulvus) orthologue of TLR1 (CD281) and an orthologue of the alpha inhibitor of NF-κB (IκBα).

The toll-like receptor (TLR) family is an ancient pattern recognition receptor family, conserved from insects to mammals. Members of the TLR family are vital to immune function through the sensing of pathogenic agents and initiation of an appropriate immune response (Takeda & Akira, 2005). The rapid identification of Toll orthologues in invertebrates and mammals suggests that these genes must be present in other vertebrates (Takeda, 2005). During the recent years, members of the multigene family of Toll-like receptors (TLRs) have been recognised as key players in the recognition of microbes during host defence (Hopkinsn & Sriskandan, 2005). Recognition of pathogens by immune receptors leads to activation of macrophages, dendritic cells, and lymphocytes. Signals are then communicated to enhance expression of target molecules such as cytokines and adhesion molecules, depending on activation of various inducible transcription factors, among which the family NF-kappaB transcription factors plays a critical role. The involvement of NF-κB in the expression of numerous cytokines and adhesion molecules has supported its role as an evolutionarily conserved coordinating element in organism's response to situations of infection, stress, and

injury. In many species, pathogen recognition, whether mediated via the Toll-like receptors or via the antigen-specific T- and B-cell receptors, initiates the activation of distinct signal transduction pathways that activate nuclear factor-kappa B (NF-κB) (Ghosh et al., 1998). TLR-mediated NF-κB activation is also an evolutionarily conserved event that occurs in phylogenetically distinct species ranging from insects to mammals.
The identification of orthologues of TLRs in other species, particularly in those showing a strong immune system, together with the elucidation of their TLR-mediated signal transduction pathways, would contribute to our understanding of how these receptors have evolved and the importance of different orthologues to resistance to different pathogens.

2. Strategy for cloning of vulture TLR1 and IκBα

In order to identify key components of the vulture system for sensing of pathogens, we constructed and screened a cDNA library from vulture peripheral blood monuclear cells (PBMC) using specific probes for TLR1 and IκBα. Since the majority of toll-like receptors are expressed in leukocytes and lymphoid tissues in human and other vertebrates, we decided to use vulture PBMC as the source of RNA to obtain a specific probes for TLR1 and IκBα and to construct a cDNA library. Using this strategy we cloned cDNAs encoding for griffon vulture (Gyps fulvus) orthologues of mammalian TLR1 (CD281) and for the alpha inhibitor of NF-κB (IκBα). The tissue and cell expression pattern of vulture TLR1 and IκBα were analyzed by real-time RT-PCR and correlated with the ability to respond to various pathogenic challenges.

2.1 Design of specific probes for vulture TLR1 and IκBα

To obtain specific probes for vulture TLR1 and IκBα, total RNA was isolated from vulture PBMC and from cells and tissues using the Ultraspec isolation reagent (Biotecx Laboratories, Houston TX, USA). Ten micrograms of total RNA was heated at 65 ºC for 5 min, quenched on ice for 5 min and subjected to first strand cDNA synthesis. The RNA was reverse transcribed using an oligo dT12 primer by incubation with 200 U RNase H- reverse transcriptase (Invitrogen, Barcelona, Spain) at 25ºC for 10 min, then at 42ºC for 90 min in the presence of 50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl2, 10 mM DTT, 30 U RNase-inhibitor and 1mM dNTPs, in a total volume of 20 µl.
For the vulture TLR probe, a partial fragment of 567 bp showing sequence similarity to human TLR-1 was amplified by PCR from vulture PBMC cDNA using two oligonucleotide primers TLR1/2Fw (5′-GAT TTC TTC CAG AGC TG–3′) and TLR1/3Rv (5′-CAA AGA TGG ACT TGT AAC TCT TCT CAA TG -3′), which were designed based on regions of high homology among the sequences of human and mouse TLR1 (GenBank, accession numbers NM_003263 and NM_030682, respectively). Cycling conditions were 94ºC for 30 s, 52ºC for 30 s and 72ºC for 1.5 min, for 30 cycles.
For the vulture IκBα probe, a partial fragment of 336 bp showing sequence similarity to human and chicken IκBα was amplified by PCR from vulture PBMC cDNA using two oligonucleotide primers IκBα-Fw (5′-CCT GAA CTT CCA GAA CAA C-3′) and IκBα-Rv (5′-GAT GTA AAT GCT CAG GAG CCA TG-3′), which were designed based on regions of high homology among the sequences of human and chicken IκBα (GenBank, accession numbers

M69043 and S55765, respectively). Cycling conditions were 94ºC for 30 s, 52ºC for 30 s and 72ºC for 1.5 min, for 30 cycles.
The obtained PCR products were cloned into pGEM-T easy vector using a TA cloning kit (Promega, Barcelona, Spain) and sequenced bidirectionally to confirm their respective specificities. These fragments were DIG-labelled following the recommendation of the manufacturer (Roche, Barcelona, Spain) and used as probes to screen 500 000 plaque colonies of the vulture-PBMC cDNA library.

2.2 cDNA library construction and screening

Total RNA (500 µg) was extracted from PBMC (pooled from 6 birds) using the Ultraspec isolation reagent (Biotecx). mRNA (20 µg) was extracted by Dynabeads (Dynal biotech-Invitrogen, Barcelona, Spain) and used in the construction of a cDNA library in Lambda ZAP vector (Stratagene, La Jolla, CA, USA) by directional cloning into EcoRI and XhoI sites. The cDNA library was plated by standard protocols at 50 000 plaque forming units (pfu) per plate and grown on a lawn of XL1-Blue E. coli for 6-8 h. Screening of the library was performed with DIG labelled probes. Plaques were transferred onto Hybond-N+ membranes (Amersham, Barcelona, Spain) denatured in 1.5 M NaCl/0.5M NaOH, neutralised in 1.5 M NaCl/0.5 Tris (pH 8.0) and fixed using a cross-linker oven (Stratagene). The filters were then pre-incubated with hybridisation buffer (5XSSC [1XSSC is 150 mM NaCl, 15 mM trisodium citrate, pH 7.7], 0.1% N-laurylsarcosine, 0.02% SDS and 1% blocking reagent (Roche)) at 65 °C for 1 h and then hybridised with hybridisation buffer containing the DIG-labelled probe, overnight at 65 °C. The membranes were washed at high stringency (2XSSC, 0.1% SDS; 2x5 min at ambient temperature followed by 0.5XSSC, 0.1% SDS; 2x15 min at 65 °C). DiG-labelled probes were detected using phosphatase-labelled anti-digoxygenin antibodies (Roche) according to the manufacturer's instructions. Positive plaques on membranes were identified, isolated in agar plugs, eluted in 1 ml of SM buffer (0.1M NaCl, 10 mM MgSO4, 0.01% gelatin, 50 mM Tris-Hcl, pH 7.5) for 24 h at 4ºC and replated. The above screening protocol was then repeated. Individual positive plaques from the secondary screening were isolated in agar plugs and eluted in SM buffer. The cDNA inserts were recovered using the Exassist/SOLR system (Stratagene). Individual bacterial colonies containing phagemid were grown up in LB broth (1% NaCl, 1% trytone, 0.5% yeast extract, pH 7.0) containing 50 µg/ml ampicillin. Phagemid DNA was purified using a Bio-Rad plasmid mini-prep kit and sequenced.

3. Structural analysis of vulture TLR1 and IκBα sequences

Sequences were analyzed using the analysis software LaserGene (DNAstar, London, UK) and the analysis tools provided at the expasy web site (http://www.expasy.org). PEST regions are sequences rich in Pro, Glu, Asp, Ser and Thr, which have been proposed to constitute protein instability determinants. The analysis of the PEST region for the putative protein was made using the webtool PESTfind at http://www.at.embnet.org/toolbox/pestfind. The potential phosphorylation sites were calculated using the NetPhos 2.0 prediction server at http://www.cbs.dtu.dk/services/NetPhos. The prediction of the potential attachment of small ubiquitin-related modifier (SUMO) was made using the webtool SUMOplot™.

The alignment of vulture TIR domain sequences with TLR-1 from other species and of the vulture IκBα sequences with IκBα from other species was done using the program ClustalW v1.83 with Blosum62 as the scoring matrix and gap opening penalty of 1.53. Griffon vulture TLR-1 and IκBα sequences were deposited in the Genbank under accession numbers DQ480086 and EU161944, respectively.

3.1 Vulture TLR1

The screening of the vulture PBMC cDNA library for TLR1 yielded seven clones with identical open reading frame (ORF) sequences. The fact that the screening of 500,000 vulture cDNA clones resulted in 7 identical sequences suggested that this TLR receptor is broadly represented in PBMC, possibly illustrating its important role in pathogen recognition during vulture innate immune response. This result was consistent with the real time RT-PCR analysis of TLR1 transcripts in vulture cells.

The largest clone (2,355 bp) contained an ORF that encoded a 650 amino acid putative vulture orthologue to TLR1, flanked by 319 bp 5'UTR and a 83 bp 3'UTR that contained a potential polyadenylation signal, AATAAA, 21 bp upstream of the poly (A) tail (Fig. 1). The predicted molecular weight of the putative vulture TLR1 was of 74.6 KDa. The predicted protein sequence had a signal peptide, an extracellular portion, a short transmembrane region and a cytoplasmic segment (Fig. 1). In assigning names to the vulture TLR, we looked at the closest orthologue in chicken and followed the nomenclature that was proposed for this species (Yilmaz et al., 2005). Therefore, the discovered sequence was identified as vulture TLR1.

3.1.1 Amino acid sequence comparison of vulture TLR1 with other species

The comparison of the deduced amino acid sequence of vulture TLR1 with the sequence of chicken, pig, cattle, human and mouse TLR1 indicated that the deduced protein had a higher degree of similarity to chicken (64% of amino acid similarity) than to pig (51%), cattle (51%), human (51%) and mouse (48%) sequences (Fig. 2). Protein sequence similarity was different on different TLR domains (Fig. 2).

Amino acid sequence of vulture TLR1 was aligned with the orthologous sequence of chicken (Gallus gallus), pig (Sus scrofa), cattle (Bos taurus), human (Homo sapiens) and mouse (Mus musculus) based on amino acid identity and structural similarity. Identical amino acid residues to vulture TLR1 from the aligned sequences are shaded. Gaps were introduced for optimal alignment of the sequences and are indicated by dashes (-). GenBank or Swiss protein accession numbers are: DQ480086, Q5WA51, Q59HI9, Q706D2, Q5FWG5 and Q6A0E8, respectively.

For the TLRs, it is assumed that the structure of the ectodomain has evolved more quickly than the structure of the TIR (Johnson et al., 2003). Similarly to other TLR receptors, the degree of homology of vulture TLR1 was higher in the transmembrane and cytoplasmic domains than in the extracellular domain.

The vulture TLR1 with 650 amino acids is probably the TLR with the shortest length and the smallest predicted MW (74.6 kDa). Recently, a chicken isoform of TLR1 (Ch-TLR1 type 2) was identified *in silico* and predicted to have a similar number of residues than vulture TLR1 (Yilmaz et al., 2005). However, this receptor also contains an additional transmembrane region in its N-terminal end, and the pattern of expression in tissues is also different from that ChTLR1 type 1 (Yilmaz et al., 2005).

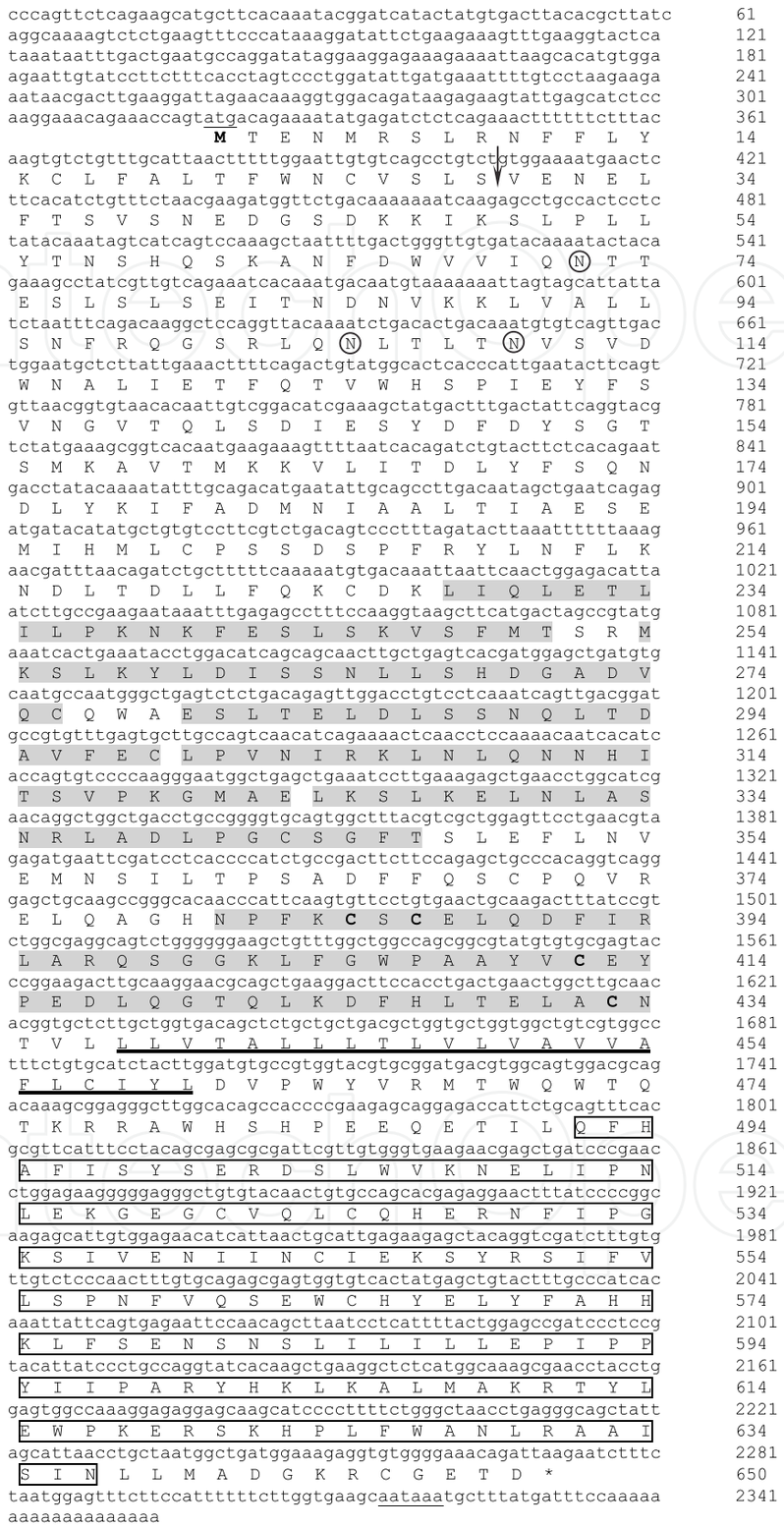

Fig. 1. Nucleotide and deduced amino acid sequence of vulture TLR1. Complete sequence of the full-length Vulture TLR obtained from the cDNA library (GenBank accession number:

DQ480086). Translated amino acid sequence is also shown under nucleotide sequence. Numbers to the right of each row refer to nucleotide or amino acid position. The cleavage site for the putative signal peptide is indicated by an arrow. LRRs domains are shaded. Potential N-glycosylation sites are circled. The predicted transmembrane segment is underlined. The initiation codon (atg) and the polyadenilation site are underlined. The translational stop site is indicated by an asterisk. The cysteines critical for the maintenance of the structure of LRR-CT are in bold.

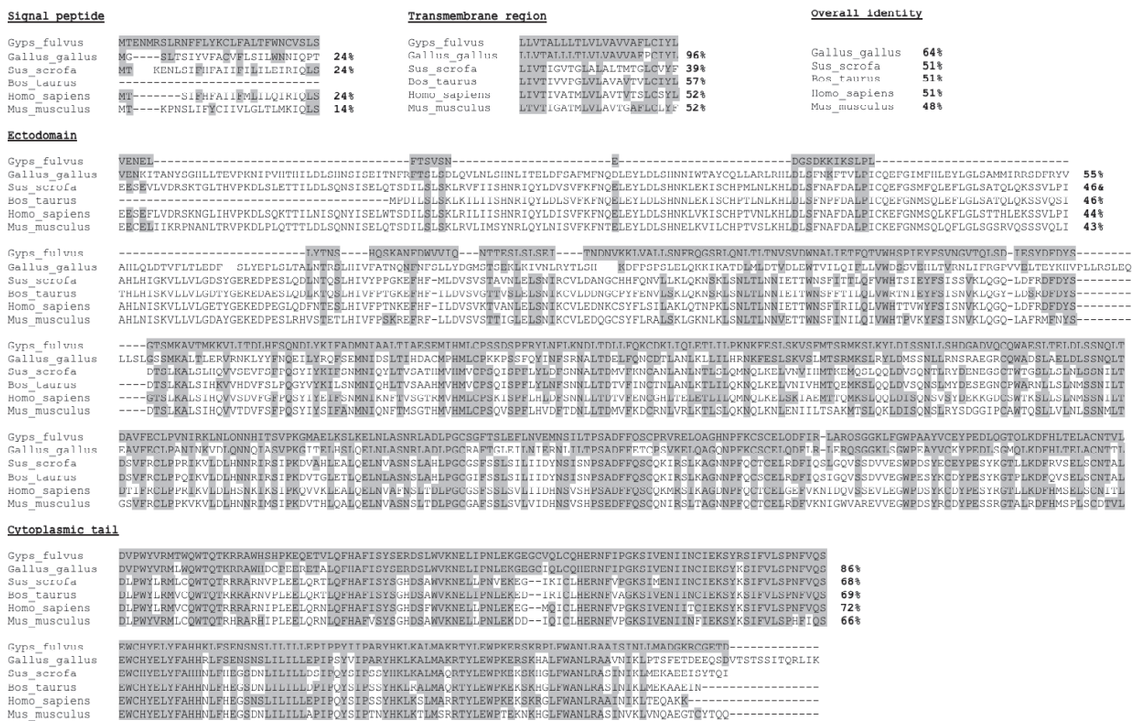

Fig. 2. Alignment of amino acid sequences of TLR1 from different species.

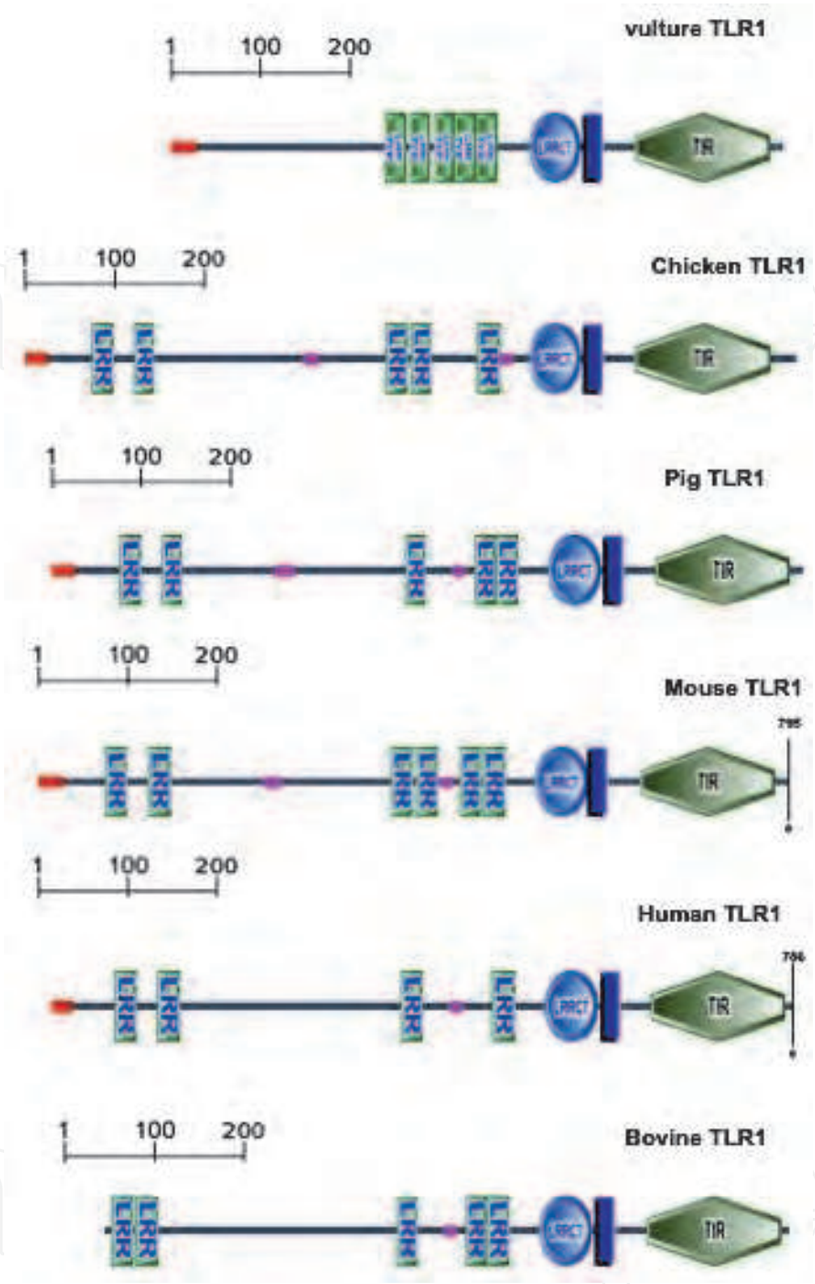

Fig. 3. Schematic structure of TLR1 from various species

Comparison of the structure obtained from the SMART analysis (at expasy web server) of the amino acid sequence from human, bovine, pig, mouse, chicken and vulture TLR1. Each diagram shows a typical structure of a member of the toll-like receptor family. Vulture TLR1 consists of an ectodomain containing five leucine rich repeats (LRRs) followed by an additional leucine rich repeat C terminal (LRR-CT) motif. The Vulture TLR has a transmembrane segment and a cytoplasmic tail which contains the TIR domain. Genbank or swiss accession number for proteins are DQ480086 (vulture), Q5WA51 (chicken), Q59HI9 (pig), Q706D2 (bovine), Q5FWG5 (human) and Q6A0E8 (mouse).

In general, the structure of vulture TLR1 shows similarity to chicken and mammalian TLR1 (Table 1). However, vulture TLR1 exhibits some structural features that could influence its functional role as pathogen receptor (Fig. 3). For example, it is possible that the smaller size of vulture TLR1, the lower number of N-glycosylation sites and the grouping of its LRRs in the proximal half of its ectodomain have functional implications.

Structural feature	*G fulvus*	*G gallus*	*S scrofa*	*B taurus*	*H sapiens*	*M musculus*
Amino acid residues	650	818	796	727	786	795
Number of LRRs	5	5	5	5	4	6
N-glycosylation sites	3	5	4	6	7	8
Predicted MW(KDa)	74.60	94.46	90.94	83.04	90.29	90.67
Length of ectodomain	409	569	560	521	560	558

Table 1. Structural features of TLR1 receptor from Griffon vulture (G fulvus), Chicken (G gallus), pig (S scrofa), cattle (B Taurus) human (H sapiens) and mouse (M musculus) amino acid sequences. The theoretical molecular weight, number of LRRs, and of glycosilation sites was calculated using the software available at the expasy web server (http://www.expasy.org). Genbank or Swiss accession number for proteins are DQ480086 (G fulvus), Q5WA51 (G gallus), Q59HI9 (S scrofa), Q706D2 (B Taurus), Q5FWG5 (H sapiens) and Q6A0E8 (M musculus).

The set of Toll proteins for humans and insects each contain widely divergent LRR regions, and this is viewed as providing the potential to discriminate between different ligands. Perhaps these features provide vulture TLR1 some advantages on pathogen recognition. TLR glycosylation is also likely to influence receptor surface representation, trafficking and pattern recognition (Weber et al., 2004).

3.2 Vulture IκBα

The screening of the vulture PBMC cDNA library yielded one clone that contained an ORF that encoded a 313 amino acid putative vulture orthologue to IκBα, flanked by 15 bp 5'UTR and a 596 bp 3'UTR (Fig. 4).

The predicted molecular weight of the putative vulture IκBα was of 35170 Da. Structurally, the vulture I kappa B alpha molecule could be divided into three sections: a 70-amino-acid N terminus with no known function, a 205-residue midsection composed of five ankyrin-like repeats, and a very acidic 42-amino-acid C terminus that resembles a PEST sequence. Examination of the Griffon vulture sequence revealed the features characteristic of an IκB molecule (Fig. 4) The putative vulture IκBα protein was composed of a N-terminal regulatory domain, a central ankyrin repeat domain (ARD), required for its interaction with NF-κB, and a putative PEST-like sequence in the C-terminus (Fig. 5), which is similar to IκBα proteins from other organisms (Jaffray et al., 1995). Together with the N-terminal regulatory domain and the central ARD domain, the presence of an acidic C-terminal PEST region rich in the amino acids proline (P), glutamic acid (E), serine (S) and threonine (T) is characteristic of IκBα inhibitors (Luque & Gelinas, 1998). PEST regions have been found in the C-terminus of avian IκBα (Krishnan et al., 1995) and mammalian IκBα and it was also present in the vulture IκBα sequence (Fig. 5). Particularly, the PEST sequence of IκBα seems to be critical for its calpain-dependent degradation (Shumway et al., 1999).

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cggagccctgccgctatgatcagcgcccgccgcctcgtcgagccgccggttatggagggc   60
               M  I  S  A  R  R  L  V  E  P  P  V  M  E  G    15
tacgagcaagcgaagaaagagcgccagggcggcttcccgctcgacgaccgccacgacagc   120
 Y  E  Q  A  K  K  E  R  Q  G  G  F  P  L  D  D  R  H  D  S    35
ggcttggactccatgaaggaggaagagtaccggcagctggtgaaggagctggaggacata   180
 G  L  D  S  M  K  E  E  E  Y  R  Q  L  V  K  E  L  E  D  I    55
cgcctgcagccccgcgagccgcccgcctgggcgcagcagctgacggaggacggagacact   240
 R  L  Q  P  R  E  P  P  A  W  A  Q  Q  L  T  E  D  G  D  T    75
tttctccacttggcgattattcacgaggaaaaagccctgagcctggaggtgatccggcag   300
 F  L  H  L  A  I  I  H  E  E  K  A  L  S  L  E  V  I  R  Q    95
gcggccggggaccgtgctttcctgaacttccagaacaacctcagccagactcctcttcac   360
 A  A  G  D  R  A  F  L  N  F  Q  N  N  L  S  Q  T  P  L  H   115
ctggcagtgatcaccgatcagcctgaaattgccgagcatcttctgaaggccggatgcgac   420
 L  A  V  I  T  D  Q  P  E  I  A  E  H  L  L  K  A  G  C  D   135
ctggaactcagggacttccgaggaaacacccccctgcatattgcctgccagcagggctcc   480
 L  E  L  R  D  F  R  G  N  T  P  L  H  I  A  C  Q  Q  G  S   155
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 L  R  S  V  S  V  L  T  Q  Y  C  Q  P  H  H  L  L  A  V  L   175
caggcaaccaactacaacgggcatacatgtctccatttggcatctattcaaggatacctg   600
 Q  A  T  N  Y  N  G  H  T  C  L  H  L  A  S  I  Q  G  Y  L   195
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gtgaaacatggggcggacgtgaacaaagtgacctaccaaggctattccccctatcagctc   780
 V  K  H  G  A  D  V  N  K  V  T  Y  Q  G  Y  S  P  Y  Q  L   255
acatggggaagagacaactccagcatacaggaacagctgaagcagctgaccacagccgac   840
 T  W  G  R  D  N  S  S  I  Q  E  Q  L  K  Q  L  T  T  A  D   275
ctgcagatgttgccagaaagtgaggacgaggagagcagtgaatcggagcctgaattcaca   900
 L  Q  M  L  P  E  S  E  D  E  E  S  S  E  S  E  P  E  F  T   295
gaggatgaacttatatacgatgactgccttattggaggacgacagctggcatttaaagc   960
 E  D  E  L  I  Y  D  D  C  L  I  G  G  R  Q  L  A  F  *      313
agagctatctgtgaaaagaagtgactgtgtacatatgtatagaaaaaggactgacttcat  1020
ttaaaaagaaagtcgcaatgcaaagggaaaaaccaggagggaaatactacactgcccagc  1080
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ttactgaccggcctcagctgctcttggtggggtgtcccaggtggaggagtcaaaccaagg  1440
gactggtgacctcctgactgttagaagaaagtagcaataatgttaactgtgggcattgga  1500
aactgtgtgtttcacaccatgtgtgtcataattgctacacttttagcaattg          1553
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Fig. 4. Nucleotide and deduced amino acid sequence of vulture IκBα. Complete sequence of the full-length vulture IκBα obtained from the cDNA library (GenBank accession number: EU161944). Translated amino acid sequence is also shown under nucleotide sequence. Numbers to the right of each row refer to nucleotide or amino acid position. Ankyrin domains are shaded. The PEST region is underlined. The ATTTA domain is in bold. Phosphorylation sites Ser-35 and Ser-39 are circled. The translational stop site is indicated by an asterisk.

Fig. 5. Schematic structure of vulture IκBα.

Structure obtained from the SMART analysis (at expasy web server) of the amino acid sequence from vulture IκBα. Each box shows a typical structure of a member of the IκBα inhibitor. Vulture IκBα consists of an N-terminus regulatory domain, a central ankyrin domain containing five ankyrin repeats followed by an additional PEST-like motif. Number shows the amino acid flanking the relevant domains.

Classical activation of NF-kappaB involves phosphorylation, polyubiquitination and subsequent degradation of IκB. Several residues are known to be important in the N-terminal regulatory domain (Luque & Gelinas, 1998, Luque et al., 2000). In nonstimulated cells, NF-kappa B dimers are maintained in the cytoplasm through interaction with inhibitory proteins, the IκBs. In response to cell stimulation, mainly by proinflammatory cytokines, a multisubunit protein kinase, the I kappa B kinase (IKK), is rapidly activated and phosphorylates two critical serines in the N-terminal regulatory domain of the I kappa Bs. Phosphorylated IκBs are recognized by a specific E3 ubiquitin ligase complex on neighboring lysine residues, which targets them for rapid degradation by the 26S proteasome, which frees NF-kappaB and leads to its translocation to the nucleus, where it regulates gene transcription (Karin & Ben-Neriah, 2000). It has been demonstrated that phosphorylation of the N-terminus residues Ser-32 and Ser-36 is the signal that leads to inducer-mediated degradation of IκBα in mammals (Brown et al., 1997; Good et al., 1996). As can be observed in the alignment of Figure 6, the griffon vulture equivalent residues seem to be Ser-35 and Ser-39, which are part of the conserved sequence DSGLDS (Luque et al., 2000; Pons et al., 2007). This observation suggests that the phosphorylation of these serine residues could trigger the IκBα inducer-mediated degradation in vulture in a similar manner to that in mammals. Unlike ubiquitin modification, which requires phosphorylation of S32 and S36, the small ubiquitin-like modifier (SUMO) modification of IκBα is inhibited by phosphorylation. Thus, while ubiquitination targets proteins for rapid degradation, SUMO modification acts antagonistically to generate proteins resistant to degradation (Desterro et al., 1998; Mabb & Miyamoto, 2007). This SUMO modification occurs primarily on K21 (Mabb & Miyamoto, 2007). This residue was also conserved in the IκBα sequence from human, mouse, pig, rat and vulture, but not from chicken (Fig. 6).

Amino acid sequence of vulture IκBα was aligned with the orthologous sequence of chicken (Gallus gallus), pig (Sus scrofa), cattle (Bos taurus), human (Homo sapiens) and mouse (Mus musculus) based on amino acid identity and structural similarity. Identical amino acid residues to vulture IκBα from the aligned sequences are shaded. Gaps were introduced for optimal alignment of the sequences and are indicated by dashes (-). SUMOlation sites are squared and phosphorylation sites are circled. GenBank or Swiss protein accession numbers are: DQ480086, Q5WA51, Q59HI9, Q706D2, Q5FWG5 and Q6A0E8, respectively. Griffon vulture IκBα sequence was deposited in the Genbank under accession number EU161944.

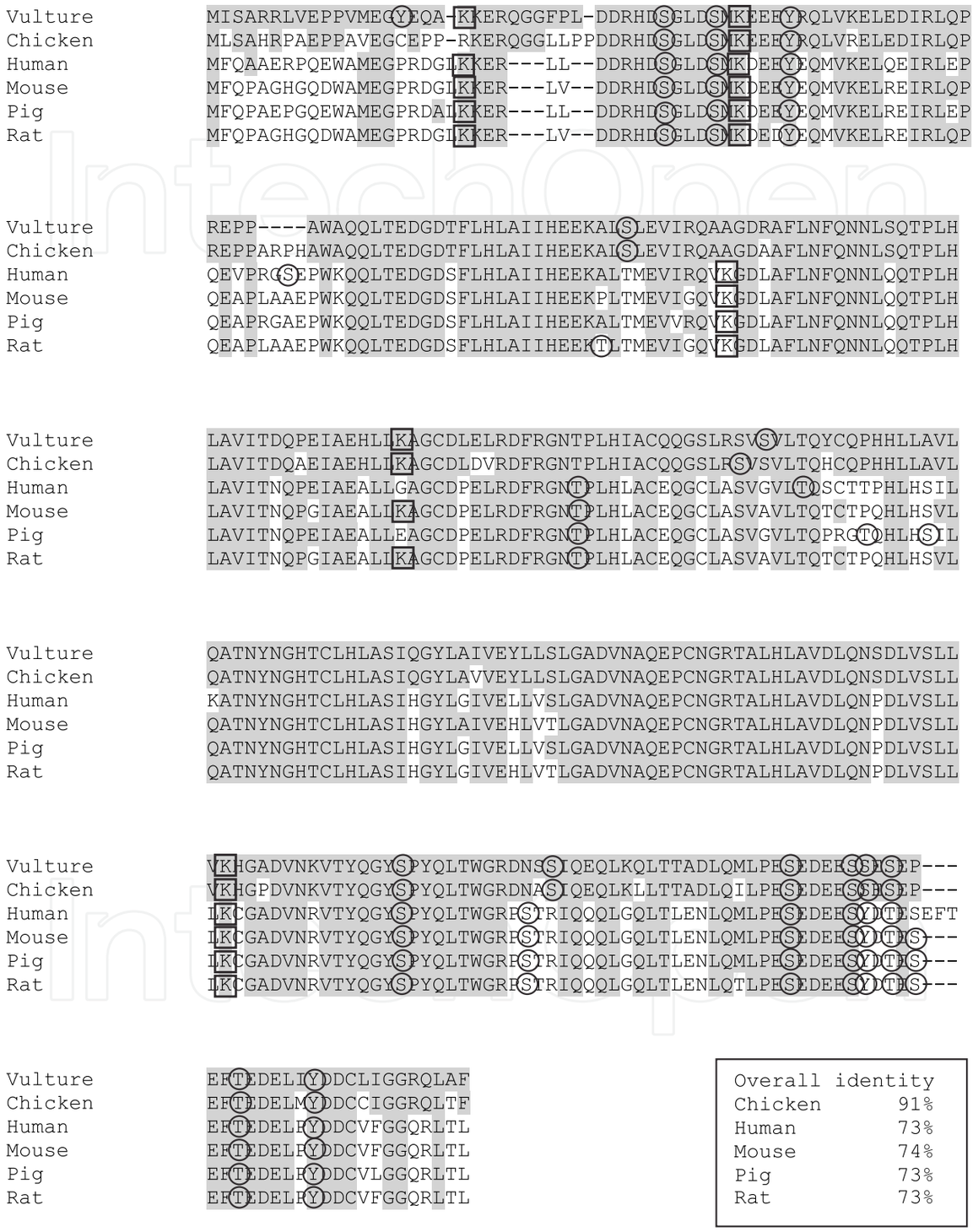

Fig. 6. Alignment of amino acid sequences of IκBα from different species.

A common characteristic of the IκB proteins is the presence of ankyrin repeats, which interact with the Rel-homology domain of NF-κB (Aoki et al., 1996; Luque & Gelinas, 1998). In the vulture sequence, five ankyrin repeats were detected using the Simple Modular Architecture Research Tool (SMART) at EMBL (Table 2). Five ankyrin repeats also exist in human and other vertebrates IκBα (Jaffray et al., 1995). It is possible that individual repeats have remained conserved because of their important structural and functional roles in regulating NF-κB.

Compared with other species, vulture IκBα exhibited the lowest number of predicted SUMOlation sites (Table 2).

Structural feature	*G fulvus*	*G gallus*	*H sapiens*	*S scrofa*	*R norvegicus*	*M musculus*
Amino acid residues	313	318	317	314	314	314
Number of ankyrin repeats	5	5	5	5	5	5
Phosphorylation sites	14	13	14	15	14	13
Predicted MW(KDa)	35,17	35,40	35,61	35,23	35,02	35,02
SUMOlation sites	2	3	4	4	5	5

Table 2. Structural features of IκBα from Griffon vulture (G fulvus), Chicken (G gallus), human (H sapiens), pig (S scrofa), rat (R norvegicus) and mouse (M musculus) amino acid sequences. The theoretical molecular weight, number of ankyrin repeats, SUMOlation and of phosphorylation sites was calculated using the software available at the expasy web server (http://www.expasy.org). Genbank or Swiss accession number for proteins are EU161944 (G fulvus), Q91974 (G gallus), P25963 (H sapiens), Q08353 (S scrofa), Q63746 (R norvegicus), and Q9Z1E3 (M musculus).

3.2.1 Amino acid sequence comparison of vulture IκBα with other species

The comparison of the deduced amino acid sequence of vulture IκBα with the sequence of chicken, human, mouse, pig, and rat IκBα indicated that the deduced protein had a higher degree of similarity to chicken (91% of amino acid similarity) than to human (73%), mouse (74%), pig (73%) and rat (73%) sequences (Fig. 6). The analysis of the vulture IκBα sequence using the software NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos) revealed 14 potential phosphorylation sites: 10 Ser (S35, S39, S89, S160, S251, S263, S282, S287, S288, and S290), 1 Thr (T295) and 3 Tyr (Y16, Y45, and Y301). Although many of these residues were conserved in the aligned sequences from chicken, human, mouse, pig and rat IκBα, two phosphorylation sites (Y16 and S160) were distinctive to the vulture sequence (Fig. 6).

4. Detection of vulture TLR1 and IκBα expression in tissues

In order to better understand the biological roles of TLR1 and IκBα, we analyzed their tissue expression pattern. The presence of transcripts encoding vulture TLR1 and IκBα in tissues was determined by real time RT-PCR. Biological samples were collected from vultures (about 8-10 months old) that were provisionally captive at the Centre for Wild Life Protection, "El Chaparrillo", Ciudad Real, Spain. Blood was obtained by puncture of the branquial vein, located in the internal face of the wing, and collected in 10 ml tubes with EDTA as anti-coagulant. Blood (10 ml) was diluted 1:1 (vol:vol) with PBS (Sigma) and the

mononuclear fraction containing PBMC was obtained by density gradient centrifugation on Lymphoprep (Axis-Shield, Oslo, Norway). All vulture tissues used for cDNA preparation were obtained fresh from euthanised birds that were impossible to recover.
RT-PCR was performed on a SmartCycler® II thermal cycler (Cepheid, Sunnyvale, CA, USA) using the QuantiTect® SYBR® Green RT-PCR Kit (Quiagen, Valencia, CA, USA), following the recommendations of the manufacturer. We used primers GfTLR-Fw (5′-GCT TGC CAG TCA ACA TCA GA-3′) and GfTLR-Rv (5′-GAA CTC CAG CGA CGT AAA GC-3′), which amplify a fragment of 158 bp of vulture TLR1 and primers IκBα -L (5′- CTG CAG GCA ACC AAC TAC AA -3′) and IκBα –R (5′- TGA ATT CTG CAG GTC GAC AG-3′), which amplify a fragment of 165 b of vulture IκBα. Cycling conditions were: 94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min, for 40 cycles. As an internal control, RT-PCR was performed on the same RNAs using the primers BA-Fw (5′-CTA TCC AGG CTG TGC TGT CC-3′) and BA-Rv (5′-TGA GGT AGT CTG TCA GGT CAG G-3′), which amplify a fragment of 165 bp from the conserved housekeeping gene beta-actin. Control reactions were done using the same procedures, but without RT to control for DNA contamination in the RNA preparations, and without RNA added to control contamination of the PCR reaction. Amplification efficiencies were validated and normalized against vulture beta actin, (GenBank accession number DQ507221) using the comparative Ct method. Experiments were repeated for at least three times with similar results. Tissues used for the study were artery, liver, lung, bursa cloacalis, heart, small intestine, peripheral blood mononuclear cells (PBMC), large intestine and kidney.
The level of TLR1 mRNA was higher in kidney, small intestine and PBMC (Fig. 7).

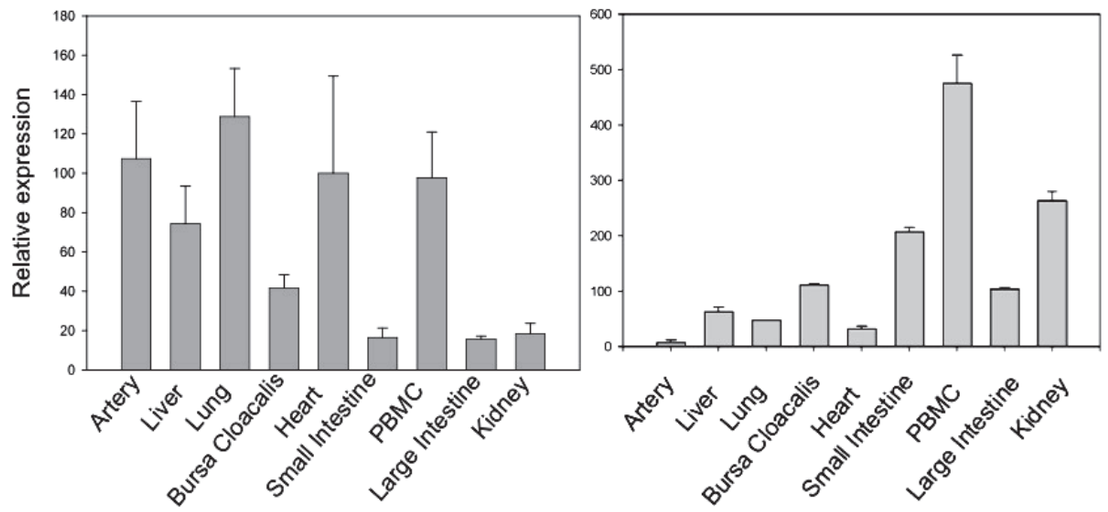

Fig. 7. Relative expression of TLR1 and IκBα mRNA transcripts in vulture cells and tissues.

Real time RT-PCR was used to examine the relative amount of TLR1 (right) and IκBα (left) transcripts in vulture cells and tissues. The data were normalised using the beta-actin gene and calculated by the delta Ct method.
Moderate vulture TLR1 mRNA levels were observed in Bursa cloacalis and large intestine, whereas the lowest TLR1 mRNA levels were found in liver, heart and artery (Fig. 6). It has

been reported that the patterns of TLR tissue expression are variable, even among closely related species (Zarember & Godowski 2002). Likewise, the intensity and the anatomic location of the innate immune response may vary considerably among species (Rehli, 2002). Consistent with its role in pathogen recognition and host defense, the tissue and cell expression pattern of vulture TLR1, as revealed by real time RT-PCR, correlated with vulture ability to respond to various pathogenic challenges. The expression of vulture TLR1 was higher in cells such as circulating PBMC and intestinal epithelial cells that are immediately accessible to microorganisms upon infection.

The analysis of the relative expression of IκBα mRNA transcripts, using real-time RT-PCR, demonstrated that vulture IκBα mRNAs were higher in lung, artery, heart, and in PBMC cells (Fig. 7), which was consistent with its role in numerous physiological processes. Interestingly, the expression of vulture IκBα mRNA was observed in tissues at which the lowest expression of vulture Toll-like receptor was found. This is consistent with the role of IκBα as inhibitor of the TLR-signalling pathway.

5. Analysis of the evolutionary relationship of vulture TLR and IκBα

The dendrogram of sequences was calculated based on the distance matrix that was generated from the pairwise scores and the phylogenetic trees were constructed based on the multiple alignment of the sequences using the PHYLIP (Phylogeny Inference Package) available at the expasy.org web page. All ClustalW phylogenetic calculations were based around the neighbor-joining method of Saitou and Nei (Saitou & Nei, 1987).

For the analysis of the evolutionary relationship of vulture and other vertebrate TLR and IκBα, a phylogenetic tree was constructed with the TIR-domain sequences of human, macaque, bovine, pig, mouse, Japanese pufferfish and chicken TLR1. GenBank or swiss protein accessions numbers Q5WA51, Q706D2, Q6A0E8, Q59HI9, Q5H727 and Q5FWG5, respectively. The phylogenetic analysis of the TIR domain of vulture TLR1 revealed separate clustering of TLR1 from birds, fish, mouse and other mammals (Fig. 8B)

For the TLRs, it is assumed that the structure of the ectodomain has evolved more quickly than the structure of the TIR (Johnson, 2003). Similarly to other TLR receptors, the degree of homology of vulture TLR1 was higher in the transmembrane and cytoplasmic domains than in the extracellular domain. As expected, phylogenetic analysis of the TIR domains revealed separate clustering of TLR1 from birds, fish and mammals (Fig. 8B), suggesting independent evolution of the Toll family of proteins and of innate immunity (Beutler & Rehli, 2002; Roach et al., 2005).

The unrooted trees were constructed by neighbor-joining analysis of an alignment of the ankirin repeats of IκBα sequences from vulture and other species (A) or the alignment of the TIR domains of TLR1 from vulture and other species (B). The branch lengths are proportional to the number of amino acid differences. GenBank or swiss protein accessions numbers of chicken (Gallus gallus), human (Homo sapiens), mouse (Mus musculus), rat (Rattus norvegicus), African frog (Xenopus laevis), cattle (Bos taurus), zebrafish (Danio rerio), Mongolian gerbil (Meriones unguiculatus), Rainbow trout (Oncorhynchus mykiss) and pig (Sus scrofa) sequences used for the phylogenetic tree were Q91974, P25963, Q08353, Q63746, Q1ET75, Q6DCW3, Q8WNW7, Q6K196, Q1ET75, Q8QFQ0 and Q9Z1E3, respectively.

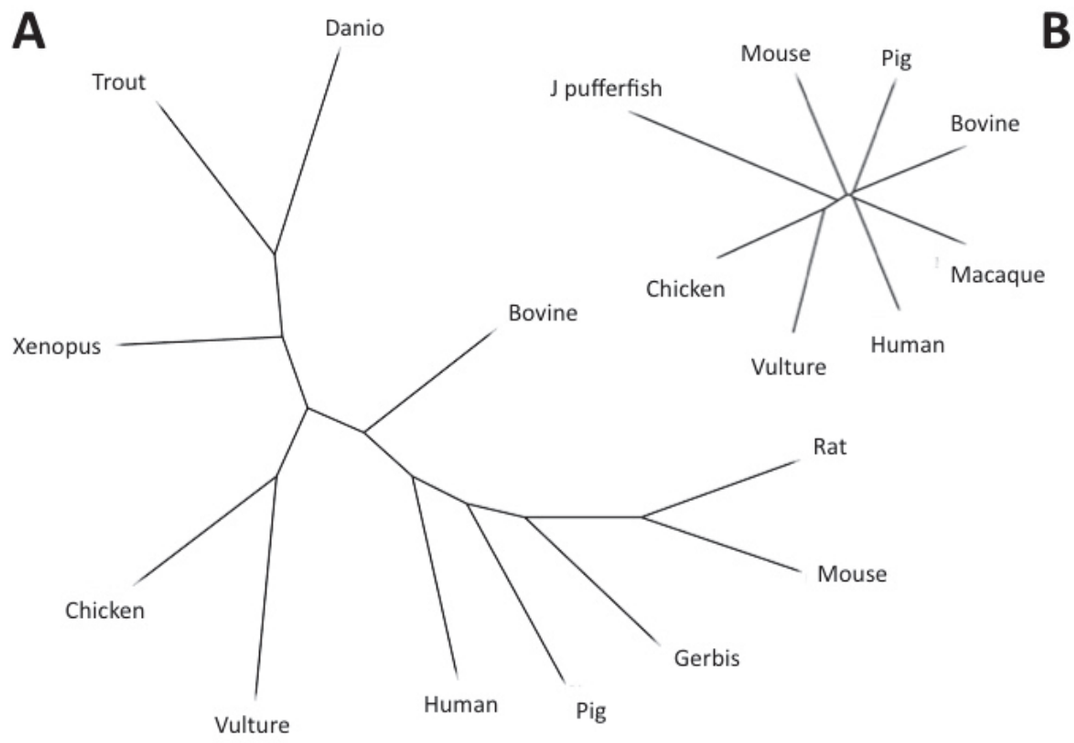

Fig. 8. Phylogenetic trees illustrating the relationship between TLR and IκBα sequences from vulture and other species.

For the analysis of the evolutionary relationship of vulture and other vertebrate IκBα, a phylogenetic tree was constructed with the sequences of chicken, human, mouse, rat, African frog, cattle, zebrafish, Mongolian gerbil, Rainbow trout and pig IκBα. The phylogenetic analysis of the ankyrin domain of vulture IκBα revealed separate clustering of IκBα from rodents, fish and other species and the sequence of vulture IκBα clustered together with that of chicken IκBα (Fig 8A). The IκB family includes IκBα, IκBβ, IκBγ, IκBε, IκBζ , Bcl-3, the precursors of NFκB1 (p105), and NF-κB2 (p100), and the Drosophila protein Cactus (Hayden et al., 2006; Karin & Ben-Neriah, 2000; Totzke et al., 2006; Gilmore, 2006). Why multiple IκB proteins now exist in vertebrates has been a subject of great interest, and much effort has been expended on establishing the roles of individual members of this protein family in the regulation of NF-κB. The recent identification of a novel member of IκB family (IκBζ) indicates that there might exist species-specific differences in the regulation of NF-κB (Totzke et al., 2006).

Evolutionarily, the IκB protein family is quite old, as members have been found in insects, birds and mammals (Ghosh & Kopp, 1998). However, the finding that individual ankyrin repeats within each IκB molecule are more similar to corresponding ankyrin repeats in other IκB family members, rather than to other ankyrin repeats within the same IκB, suggests that all IκB family members evolved from an ancestral IκB molecule (Huguet, et al., 1997).

Consistent with the hypothesis that all these factors evolved from a common ancestral RHD-ankyrin structure within a unique superfamily, explaining the specificities of interaction

between the different Rel/NF-kappa B dimers and the various I kappa B inhibitors (Huguet, et al., 1997).
Recently, the presence of two IkappaB-like genes in Nematostella encoded by loci distinct from nf-kb suggested that a gene fusion event created the nfkb genes in insects and vertebrates (Sullivan et al., 2007). This is consistent with the hypothesis that interactions between transcription factors of the Rel members and members of the IκB gene family evolved to regulate genes mainly involved in immune inflammatory responses (Bonizzi & Karin, 2004).
NF-kappaB represents an ancient, generalized signaling system that has been co-opted for immune system roles independently in vertebrate and insect lineages (Friedman & Hughes, 2002). Therefore, while these proteins share a basic three-dimensional structure as predicted by their shared ankyrin repeat pattern and sequence, a possible evolutionary scenario based on this phylogenetic tree could be that subtle differences in the amino acid substitutions in the ankyrin repeats and flanking sequences occurred throughout evolution, which contributed to their specificity of interaction with various members of the Rel family.

6. Conclusions

In summary, the molecular cloning methods reported herein identified and characterized the vulture orthologues to TLR1 (CD281) and to IκBα, the first NF-κB pathway element from the griffon vulture G. fulvus. These results have implications for the understanding of the evolution of pathogen-host interactions. Particularly, these studies help to highlight a potentially important regulatory pathway for the study of the related functions in vulture immune system (Perez de la Lastra & de la Fuente, 2007; 2008). Despite the overall structure of vulture TLR1 and expression pattern was similar to that of chicken, pig, cattle, human and mouse TLR, vulture TLR1 had differences in the length of the ectodomain, number and position of LRRs and N-glycosylation sites that makes vulture TLR1 structurally unique with possible functional implications.
Strong selective pressure for recognition of and response to pathogen-associated molecular patterns (PAMPs) has probably maintained a largely unchanged TLR signalling pathways in all vertebrates. The IκBα gene reported here expands our understanding of the immune regulatory pathways present in carrion birds that are in permanent contact with pathogens. Current investigations should focus on the cloning and characterization of other members of NF-κB signalling cascade and genes controlled by this signalling pathway. At this point it is difficult to understand the implications of the structural differences between vulture TLR1, chicken TLR1 and TLR1 in different mammalian species. A greater understanding of the functional capacity of non-mammalian TLRs and, particularly in carrion birds that are in permanent contact with pathogens, has implications for the understanding of the evolutionary pressures that defined the TLR repertoires in present day animals.

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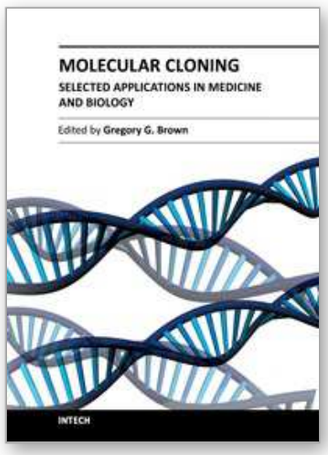

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The development of molecular cloning technology in the early 1970s created a revolution in the biological and biomedical sciences that extends to this day. The contributions in this book provide the reader with a perspective on how pervasive the applications of molecular cloning have become. The contributions are organized in sections based on application, and range from cancer biology and immunology to plant and evolutionary biology. The chapters also cover a wide range of technical approaches, such as positional cloning and cutting edge tools for recombinant protein expression. This book should appeal to many researchers, who should find its information useful for advancing their fields.

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