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Identification of a fourth ancient member of the IL-3/IL-5/GM-CSF cytokine family, KK34, in many mammals

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ABSTRACT

The related cytokine genes *IL-3*, *IL-5* and *GM-CSF* map to the (extended) T_H2 cytokine locus of the mammalian genome. For chicken an additional related cytokine gene, *KK34*, was reported downstream of the *IL-3* plus *GM-CSF* cluster, but hitherto it was believed that mammalian genomes lack this gene. However, the present study identifies an intact orthologue of chicken *KK34* gene in many mammals like cattle and pig, while remnants of *KK34* can be found in human and mouse. Bovine *KK34* was found to be transcribed, and its recombinant protein could induce STAT5 phosphorylation and proliferation of lymphocytes upon incubation with bovine PBMCs. This concludes that KK34 is a fourth functional cytokine of the IL-3/IL-5/GM-CSF/KK34-family (alias IL-5 family) in mammals.

While analyzing *KK34*, the present study also made new identifications of cytokine genes in the extended T_H2 cytokine loci for reptiles, birds and marsupials. This includes a hitherto unknown cytokine gene in birds and reptiles which we designated "*IL-5famE*". Other newly identified genes are *KK34*, *GM-CSF*(-like), *IL-5*, and *IL-13* in reptiles, and *IL-3* in marsupials.

1. Introduction

The human cytokines interleukin 3 (IL-3), IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF alias CSF2) and their homologues in other species form one of the sub-families of the large family of four α-helix bundle cytokines. Despite their minimal sequence similarities (Yamaguchi et al., 2015), IL-3, IL-5 and GM-CSF are thought to be related because their genes map to the same genomic region and they functionally interact with heterodimer(-based) receptor complexes which include the IL-3Rβ chain (aliases beta common chain, βc, or CD131) (Martinez-Moczygemba and Huston, 2003; Broughton et al., 2015). IL-3, IL-5 and GM-CSF all have the capacity to induce phosphorylation and thereby activation of transcription factor STAT5 (Leonard, 2013). Their functional differences are importantly determined by which cells express the cytokine-specific chains of the receptor complexes: IL-3Rα (alias CD123), IL-5Rα (alias CD125), or GM-CSF-Rα (alias CD116) (Broughton et al., 2012). As common for many cytokines, IL-3, IL-5 and GM-CSF can be expressed by a variety of cells and have pleiotropic and overlapping functions. The pleiotropic functions of IL-5 are reflected in some of its synonyms like BCGFII (B cell growth factor II), IgA-enhancing factor, IL-2Rα-inducing factor, killer helper factor, EDF (eosinophil differentiation factor), and eosinophil colony stimulating factor (reviewed by Takatsu, 2011). IL-5 is best known for its expression by T_H2 lymphocytes and its stimulation of eosinophils (Cherwinski et al., 1987; Yamaguchi et al., 1988a; 1988b; Coffman et al., 1989), and a pronounced feature of IL-5 knockout mice is that they are severely deficient in generating eosinophilic responses following helminth infection (Kopf et al., 1996). IL-3 and GM-CSF are quite general factors for growth, survival and differentiation of both immature and mature hematopoietic cells, which show a considerable level of redundancy and are of special importance for development of cells of myeloid lineages (Sideras and Palacios, 1987; Metcalf, 1993; Martinez-Moczygemba and Huston, 2003; Selleri et al., 2008; Broughton et al., 2015). IL-3 knockout mice are characterized by decreased basophil and mast cell responses upon nematode infection (Lantz et al., 1998), while the lungs of GM-CSF knockout mice develop surfactant accumulation and extensive lymphoid hyperplasia implying a role of GM-CSF in pulmonary homeostasis

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(Dranoff et al., 1994; Stanley et al., 1994). Besides that effects like the above can be observed, it is of importance for understanding the nature of this cytokine family and its involvement in redundant functions that despite the multiple functions of all of the three cytokines, their single or combined functional knockout in mice leave most aspects of steady-state hematopoiesis unchanged (Metcalf, 1993; Dranoff et al., 1994; Stanley et al., 1994; Kopf et al., 1996; Nishinakamura et al., 1996; Lantz et al., 1998; Gillessen et al., 2001).

A difference between the IL-3/IL-5/GM-CSF systems of human and mouse is the presence of two different IL-3Rβ molecules in mice, one of which functionally interacts with all three cytokines as known in humans, while the other only does so with IL-3 (Hara and Miyajima, 1992; Mirza et al., 2010). A difference between human and chicken was reported by the presence of an additional cytokine gene, designated "*KK34*" (after a sequence clone number), which maps head-to-tail directly downstream of the *GM-CSF* plus *IL-3* gene cluster (Koskela et al., 2004; Avery et al., 2004). Chicken *KK34* was found to be expressed by TCRγδ cells, and the deduced protein was shown to have some sequence similarity with IL-5 while recombinant chicken KK34 protein did not interact with chicken IL-5Rα (Koskela et al., 2004; Avery et al., 2004; Fukushima et al., 2012). Hitherto it was reported that mammals do not have an orthologue of *KK34* (e.g. Kaiser and Stäheli, 2014).

At the sequence identity level, the members of the IL-3/IL-5/GM-CSF/KK34 family are notoriously poorly conserved (e.g. Huising et al., 2006), and human and murine IL-3 only share 29% amino acid identity (Yang et al., 1986). Because of these low sequences similarities, only very recently we managed to convincingly detect IL-3/IL-5/GM-CSF/KK34 family genes in cartilaginous and bony fishes at the genomic location expected for *IL-5* (Dijkstra, 2014; Yamaguchi et al., 2015), which we designated "*IL-5fam*" followed by capital letters for numbering because we cannot be sure about their identity beyond being members of the IL-3/IL-5/GM-CSF/KK34-family. For convenience, in the present study we designate the IL-3/IL-5/GM-CSF/KK34-family as "IL-5 family", because IL-5 may be the member with the most conservative evolution (e.g. see sequence comparisons in the present study), including mapping to the probable locus of family origin (Ohtani et al., 2008). During analyses of the fish *IL-5fam* genes and using their deduced protein sequences for database similarity searches, we found indications for an extra family member in mammals (Yamaguchi et al., 2015) to which we then addressed the investigation reported in the present study. The additional gene is maintained intact and expressed in some animals like cattle, while it acquired pseudogene features in other mammals like human and mouse. Based on its genomic position, conservation of motifs and phylogenetic analyses, we conclude that it should be considered the orthologue of the chicken *KK34* gene, and hence we follow the "*KK34*" designation. Recombinant expressed bovine KK34 was able to induce STAT5 phosphorylation and proliferation of lymphocytes upon incubation with bovine PBMCs, proving the capacity of at least some of the intact *KK34* genes in mammals to encode a functional cytokine.

2. Materials and methods

2.1. Database searching and genetic software analysis for predictions of genes and molecule structures

Sequence databases at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) and ENSEMBL (http://www.ensembl.org/index.html) were used for retrieving of IL-5 family member sequences in various species using searches based on names and/or sequence similarities (various types of blast analysis). Gene predictions were made based on using the specialized software GENSCAN, http://genes.mit.edu/GENSCAN.html, and FGENESH, http://www.softberry.com/berry.phtml?topic=fgenesh&group=programs&subgroup=gfind, as well as on similarity searches focusing on the expected genomic regions and on knowledge of the cytokine family-specific features. Leader peptides were predicted by SignalP software (http://www.cbs.dtu.dk/services/SignalP/), with in case of platypus IL-5 the discrimination score being slightly under 0.5. Protein secondary structures were predicted using Phyre^2 software (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index).

2.2. cDNA synthesis, PCR amplification, and sequencing

Ready-to-Use Total RNA samples from kidney of cattle and pig were purchased from Zyagen Laboratories, and cDNA was synthesized using this RNA and random hexamer primers based on the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen). Then cDNA solution was used for PCR using ExTaq polymerase (Takara), with amplification schedule: 94 °C for 5 min, 40x (94 °C for 30 s, 60 °C for 30 s, 72 °C for 40 s), 72 °C for 1 min. For amplification of bovine *KK34* the primer combinations *Cow-KK34-F1*, 5′-TAGAGGTACATCTCCTCACAGAAATGC, plus *Cow-KK34-R2*, 5′-GTCTCAGAGGATGCCCTTGATCCT, and *Cow-KK34-F3*, 5′-CAGGACAATCACACGGCAGTCT, plus *Cow-KK34-R1*, 5′-ATCAGGACGTGAGAGGACGAGGTC, were used. For amplification of porcine *KK34* the primer combination *Pig-KK34-F*, 5′-CTTGGAGCCATCCTCAAGTCACAC, plus *Pig-KK34-R*, 5′-CCCAGGATGTGAGAGGTTGAGGTC, was used. PCR fragments were eluted from gel, cloned into plasmid vector pGEM-T (Promega), and sequenced by using an automated sequencer 'ABI Prism 3130xl genetic analyzer' (Applied Biosystems). PCR or sequencing errors were excluded by analysis of multiple clones and comparison with published genomic sequences. The (assembled) bovine and porcine cDNA sequences are available as GenBank accessions KX108886–KX108887.

2.3. Construction of pRcCMV2-Cow-KK34, pRcCMV2-Cow-KK34-FLAG and pRcCMV2-Cow-IL-2-FLAG expression vectors

For construction of expression vectors, bovine *KK34* was amplified from an intact *KK34* ORF containing pGEM-T clone using the primer combinations *Cow-KK34-NotI-F*, 5′-GATATGCGGCCGCACCATGTTCCTGTACGTGCTCCTGGCT, plus *Cow-KK34-XbaI-R*, 5′-GTAGTTCTAGATCAGAGGATGCCCTTGATCCTT, and *Cow-KK34-NotI-F* plus *Cow-KK34-FLAG-XbaI-R*, 5′-GTAGTTCTAGATTACTTATCGTCGTCATCCTTATAATCGAGGATGCCCTTGATCCTT. The first primer combination amplifies an unaltered *KK34* ORF whereas the latter results in coding a FLAG-tag at the KK34 C-terminus. After elution from gel and digestion with *Not*I and *Xba*I restriction enzymes, the sequences were cloned into *Not*I/*Xba*I digested eukaryotic expression vector pRc/CMV2 (Invitrogen) behind a CMV promoter. The resulting plasmids were checked by sequencing, and designated *pRcCMV2-Cow-KK34* and *pRcCMV2-Cow-KK34-FLAG*.

IL-2 was amplified from bovine cDNA (data not shown), after which from a clone with the ORF sequence identical to GenBank accession NM_180997 a gene for a C-terminal FLAG-tagged IL-2 was created using the primers *Cow-IL-2-Hind3-F*, 5′-TACCAAGCTTACCATGTACAAGATACAACTCTTGTC, and *Cow-IL-2-FLAG-ApaI-R*, 5′-GTAGGGGCCCTTACTTATCGTCGTCATCCTTATAATCAGTCATTGTTGAGTAGATGC. This sequence was cloned using *Hin*dIII and *Apa*I double digestion into expression vector pRc/CMV2, resulting in *pRcCMV2-Cow-IL-2-FLAG*.