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Short Paper

Identification of a cDNA sequence coding for kruppel-like factor 2b (*Klf2b*) from the skin mucosa of common carp (*Cyprinus carpio*)

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Summary

Kruppel-like factors (*Klf*s) are a highly related zinc-finger family of transcription factors implicated in the regulation of the eukaryotic cellular growth and differentiation of a diverse set of cells in mammal. Using RT-PCR technique, a 456 bp cDNA fragment encoding N-terminus part of a *Klf2b* was isolated from the skin mucosa of common carp (*Cyprinus carpio*) using two degenerative oligonucleotide primers. Use of this fragment as a probe allowed the isolation of a larger cDNA clone through the searching of the GenBank expressed sequence tag database. The size of the amplified product is 1157 bp, which encodes a polypeptide of 274 amino acid residues with a predicted molecular mass of 30.359 kDa and theoretical pI of 4.88. The deduced amino acid sequence exhibited 79, 54, and 53% identity to the homologous *Klf2b* identified from zebrafish *Danio rerio*, Spotted Green Pufferfish *Tetraodon nigroviridis* and Atlantic salmon *Salmo salar*, respectively. The common carp protein is 50% similar to *Klf2* orthologues in African clawed frog *Xenopus laevis*, 44% in chicken *Gallus gallus* and is 30% similar to the mammalian Klf in house mouse *Mus musculus*.

Key words: Kruppel-like factor 2b (*Klf2b*), Common carp

Introduction

Kruppel-like factor 2 (*Klf2*) is a multigene family of transcriptional factors having a three-Cys2-His2-zincfinger domain that plays a role in the differentiation of a diverse set of cells in mammals (Philipsen and Suske, 1999; Turner and Crossley, 1999; Bieker, 2001; Kaczynski *et al*., 2003). Recent targeted-deletion experiments in mice have revealed that these proteins play a key role in various processes of cell differentiation. For example: *KLF1/EKLF* (erythroid kruppel-like factor) is essential for red blood cell maturation (Nuez *et al*., 1995; Feinberg *et al*., 2004), whereas *KLF4/GKLF* (gut kruppel-like factor) regulates the differentiation and maturation of dermal and gastrointestinal epithelial cells (Segre *et al*., 1999; Katz *et al*., 2002). The Klf family plays key roles for members of the Klf family in erythroid cell maturation (Feng *et al*., 1994; Perkins *et al*., 1995), regulation of T cell activation and monocyte proinflammatory gene expression (Das *et al*., 2006), lung development (Wani *et al*., 1999), blood vessel stability (Kuo *et al*., 1997) as well as skin permeability (Segre *et al*., 1999). Previous studies have shown that expression of *Klf2* is rapidly extinguished after T cell activation and is re-expressed in $CD8^+$ memory T cells (Schober *et al*., 1999),

indicating that *Klf2* might also play a crucial role in regulating the activation of T cells and the survival of memory T cells. However, the exact biological role of *Klf2* in T cell activation is still unknown.

Considering the various role of mammalian *Klf2* in cell growth, it will be interesting to investigate the conservation and function of the homologues proteins in other vertebrates. Five Klf genes *(Klf2a*, *Klf2b*, *Klf4*, *Klfd* and *Klf12*) have recently been isolated from the kidney of zebrafish, *Danio rerio*, and the structure of their products, their genetic map positions, and their expression during development of the zebrafish have been characterized (Oates *et al*., 2001). Developmental expression patterns suggest potential roles for these zebrafish genes in diverse processes, including hematopoiesis, blood vessel function, and fin and epidermal development. It was found that the high degree of evolutionary conservation of the zebrafish genes with their mammalian homologs suggested functional conservation (Oates *et al*., 2001).

The present study was undertaken to study the isolation of a cDNA sequence encoding a *Klf2b* from the skin mucosa of common carp (*Cyprinus carpio*).

Materials and Methods

Animals

Common carp were obtained from the Shahid Maleki Fish Culture Ponds located in the Khouzestan province of Iran and maintained in an indoor aquarium tank with running river water. Fish weighing 800-1200 g were adapted at 20±2°C for at least three weeks before they were killed.

RNA Isolation

After removing most of the epidermal mucus by blotting the fish skin with tissue paper, epidermal cells were collected by scraping the scales along the cephalo-caudal axis of the freshly killed fish with a sterile glass microscope slide. Total RNA from the skin was extracted using the RNX plus solution (CinnaGen, Iran) according to the manufacturer's instructions, except that the extraction step was repeated two additional times. RNA was quantified by absorbance at 260 nm.

RT-PCR

Briefly, 12 µl (2 µg each) of skin total RNA was incubated with 0.5 µg of Oligo (dT) 18 primer at 70°C, for 10 min followed by a brief centrifugation. The reaction was chilled on ice for a few minutes and then 1 µl RNasin (CinnaGen, Iran), 1 µl dNTP mixture (120 mM of each nucleotide), 2.5 µl of 5 × enzyme buffer and 1 µl (200 U) of Moloney Murine Leukemia Virus (M-MulV) reverse transcriptase (CinnaGen, Iran) were added. The reaction was incubated at 42°C for 1 h followed by a brief centrifugation and then inactivation of the enzyme by heating at 100°C for 10 min. Degenerative primer sets were generated based on the sequence data of zebra fish (AF392995) using Primer3 program (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). RT-PCR reactions were carried out for cDNA template using standard reaction conditions. The reaction mixture (50 µl) contained 5 µl of the reverse transcription reaction, 0.2 µM of each primer, 250 µM of each dNTP and 1 U of *Taq* DNA polymerase in a standard PCR buffer. The thermocycler was programmed as follows: initial denaturation (94°C, 3 min) followed by PCR amplification with 40 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 1 min and a final extension of 1 cycle at 72°C for 7 min. The amplification product was then electrophosed on 1% (w/v) agarose gel. DNA fragments were then extracted from the gel using the Gel Extraction Kit (CinnaGen, Iran) according to the manufacturer's instructions.

DNA sequencing and sequence analysis

Amplicons of the cDNA were sequenced from both ends using *Taq* Dye Deoxy terminator Cycle sequencing kit in an ABI Prism 3700 DNA Analyzer (Applied Biosystems). The sequence was determined by using overlapping fragments. Database nucleotide sequence homology searches were made using the BLAST program (Altschul *et al*., 1990) at the National Center for Biotechnology Information (NCBI)

website (http://www.ncbi.nlm.nih.gov). Multiple sequence alignments were done using the CLUSTAL_W program (Thompson *et al*., 1994) and edited with the BOXSHADE software (http://www.ch.embnet.org/software/BOX_form.html).

Finding open reading frame (ORF) was determined through the deduced protein sequence by analysis with the Translate tool software available at the Expasy website (http://ca.expasy.org/tools/pi_tool.html).

Results

Sequencing of common carp *Klf2b*

In order to isolate cDNAs encoding *Klf2b* from common carp, PCR amplification was carried out on aliquots of cDNA as the template using two degenerative primers (Fig. 1). Deionised distilled water was used as the negative control. DNA sequencing of the amplified product of 456 bp fragment confirmed that it encoded the C-terminus part of a putative *Klf2b* protein and was applied for searching the database using the NCBI tool. Although no matches were found in the NCBI database nr for common carp, only one sequence from the expressed sequence tag (EST) database databases matched the query sequence. After EST database searching, an 875-bp clone (EX880829) (Williams *et al*., 2008) was found revealing an open reading frame (ORF) contiguous with the previous cDNA fragment. The complete nucleotide sequence was assembled from the two overlapping cDNA clones. The resulting common carp sequences were aligned to create a contiguous sequence. The size of the amplified product was 1157 bp containing a cDNA of 822 bp with a single open reading frame of 274 amino acids residues with a predicted molecular mass of 30.359 kDa and theoretical pI of 4.88. The cDNA sequence contains a long 5'-untranslated region spanning 335 bp.

Fig. 1: Agarose gel electrophoresis analysis of 456 bp amplification product from *Klf2b* gene isolated from the skin mucosa of common carp. M: DNA size marker. Lane 1: negative control (water), and Lane 2: RT-PCR amplification products. Each lane was loaded with 8 µl of the total reaction

Identification of common carp *Klf2b*

The sequence data was aligned to determine the cDNA sequence for common carp *Klf2b*. Comparison with the sequences contained in the GenBank database revealed a strong similarity with the Klf molecules described in various organisms. The mRNA sequence of the carp *Klf2b* gene was used to search the NCBI database. In Fig. 2, the sequence is shown aligned together with eukaryotic *Klf2b* from *Danio rerio* (NM_131857) (Gardiner *et al*., 2007), an unnamed protein product from *Tetraodon nigroviridis* (CAG07181), a putative *Klf2* protein from *Salmo salar* (BT044789), and orthologues sequences from *Xenopus laevis* (NM_001086961) (Klein *et al*., 2002), *Gallus gallus* (XM_418264) (Chervenak *et al*., 2006), *Klf2* from Chestnut-eared finch *Taeniopygia guttata* (XP_002194052) and *Mus musculus* (AF129002) (Atkins *et al*., 2008). The highest level of identity was scored with a *Danio rerio* (79%) Klf, followed by *Tetraodon nigroviridis* (54%) and *Salmo salar* (53%). The deduced amino acid sequence also exhibited 50% identity to the Klf orthologues in *Xenopus laevis*, 44% in chicken *G. gallus*, 45% in Chestnut-eared finch *Taeniopygia guttata* and 30% in the mammalian Klf in *Mus musculus*.

The common carp protein is rich in proline residues which constitute 12.8% of the total amino acids and form Proline-rich repeats of one to five amino acids in a row. A stretch of five amino acids between 268[KPKRGLR]274 at the C terminal part of the protein, adjacent to the first zinc finger sequence is conserved (Fig. 2). This should be considered as part of a potential

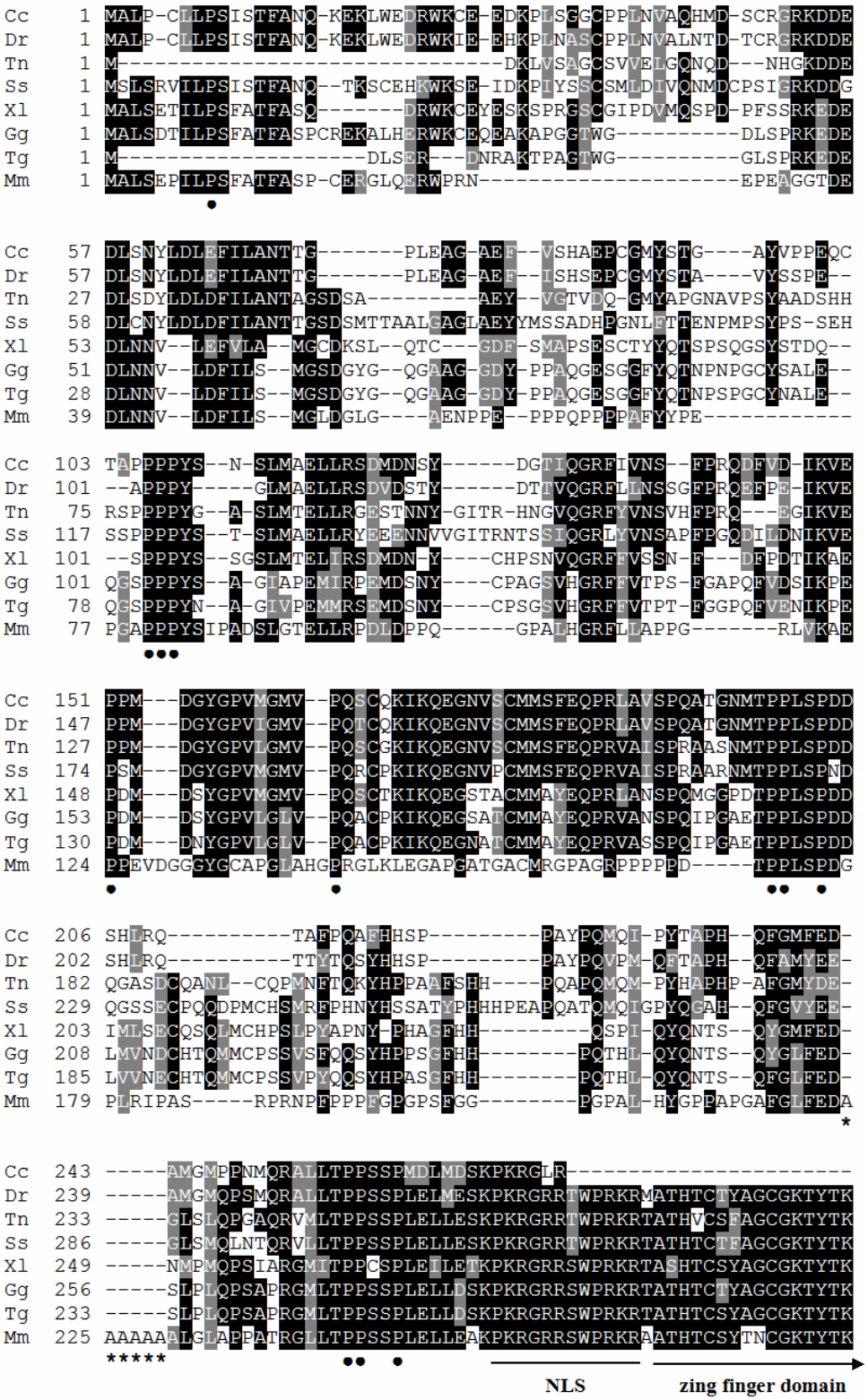

Fig. 2: Multiple sequence alignment of *Klf2b* from common carp (Cc) with other sources of KLF from *Danio rerio* (Dr; NM_131857), an unnamed protein product from *Tetraodon nigroviridis* (Tn; CAG07181), a putative *Klf2* protein from *salmo salar* (Ss; BT044789), and orthologues sequences from *Xenopus laevis* (Xl; NM_001086961), *Gallus gallus* (XM_418264), Chestnut-eared finch *Taeniopygia guttata* (Tg; XP_002194052) and *Mus musculus* (Mm; AF129002). The position of the nuclear localization signal (NLS) domain and zing finger domain are underlined. Proline residues and alanine stretch are indicated by bold circles and stars, respectively. Shading indicates identity (black) or conservative substitutions (grey) relative to common carp

nuclear localization signal (NLS) (Shields and Yang, 1997). The lack of an alanine stretch of 5 amino acids in the putative activation domain and at the border of the NLS domain is an additional feature of the common carp Klf protein. This alanine-rich region is present in mice protein.

Discussion

In this study, a cDNA coding for a Klf protein from the skin mucosa of a non-mammalian vertebrate has been described. The Klf protein has a standard kruppel-like transcription factor structure and is divided into two domains, the Pro-rich transactivating N-terminal domain and the C-terminal zinc finger containing domain (Oates *et al*., 2001). Although common carp *Klf2b* protein is similar to other fish proteins at the nucleotide level, the alignment of the amino aid sequences of mouse and common carp Klf proteins shows high similarity in the DNA-binding and nuclear localization domains, but little outside these regions. Based on amino acid sequences, common carp *Klf2b* was considered to be collectively orthologous to mammalian *Klf2*. A comparison of the sequence with homolog genes from mammalian reveals the differentiation and functional analysis of evolutionarily conserved domains.

KLF4 in mammalian is one of the most important Klf proteins in skin development since it has been shown that the systemic knockout of the *Klf4* gene results in embryonic death of mice from dehydration as a result of a damaged epidermal barrier function of the skin. While the mouse *Klf4* is highly expressed in the differentiated epithelial cells of the gastrointestinal tract and epidermis (Segre *et al*., 1999), there is no evidence of common carp or zebra fish *Klf4* expression in the epidermis, or any other epithelium. On the other hand, the expression of fish *Klf2b* gene is interesting because it appears to be expressed in the differentiating epidermis of zebra fish (Oates *et al*., 2001) and common carp, like *Klf4* in mouse. This alteration in epidermal expression of *Klf4* and *Klf2b* in fish is probably associated with the aquatic habitat of the fishes, where dehydration is not such a problem and the conditions for the barrier function of the skin are likely to be different. It is likely, therefore, that epidermal expression was an aspect of the ancestral vertebrate *Klf2/4* gene. It seems it was lost from *Klf4* and retained in *Klf2* in the zebra fish lineage, whereas in the mammalian, epidermal expression was preserved by *Klf4* and missing by *Klf2*. Our amplification of a *Klf2b* cDNA from the epidermal mucus of common carp indicated that a similar situation may also be true in the case of common carp.

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