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Identifying the activation motif in the N-terminal of rainbow trout and zebrafish melanocortin-2 receptor accessory protein 1 (MRAP1) orthologs

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ABSTRACT

The activation of mammalian melanocortin-2 receptor (MC2R) orthologs is dependent on a four-amino acid activation motif (LDYL/I) located in the N-terminal of mammalian MRAP1 (melanocortin-2 receptor accessory protein). Previous alanine substitution analysis had shown that the Y residue in this motif appears to be the most important for mediating the activation of mammalian MC2R orthologs. Similar, but not identical amino acid motifs were detected in rainbow trout MRAP1 (YDYL) and zebrafish MRAP1 (YDYV). To determine the importance of these residues in the putative activation motifs, rainbow trout and zebrafish MRAP1 orthologs were individually co-expressed in CHO cells with rainbow trout MC2R, and the activation of this receptor with either the wild-type MRAP1 ortholog or alanine-substituted analogs of the two teleost MRAP1s was analyzed. Alanine substitutions at all four amino acid positions in rainbow trout MRAP1 blocked activation of the rainbow trout MC2R. Single alanine substitutions of the D and Y residues in rainbow trout and zebrafish MRAP1 indicate that these two residues play a significant role in the activation of rainbow trout MC2R. These observations indicate that there are subtle differences in the way that teleost and mammalian MRAPs are involved in the activation of their corresponding MC2R orthologs.

1. Introduction

A key feature of chemical communication mechanisms is the match between a chemical signal and its receptor. In the case of the melanocortin peptides (i.e., ACTH, α-MSH, β-MSH, γ-MSH), polypeptides synthesized in the endocrine cells of the pituitary and neurons in the central nervous system, activation of target cells requires binding to melanocortin receptors. The melanocortin receptors are a family of five G protein-coupled receptors (i.e., MC1R, MC2R, MC3R, MC4R, MC5R) that can be activated to varying degrees by the melanocortin peptides, ACTH, α-MSH, β-MSH, γ-MSH, or δ-MSH (Cone, 2006; Dores et al., 2014). Earlier studies had shown that mammalian MC1R, MC3R, MC4R, and MC5R can be functionally expressed in heterologous non-adrenal cell lines such as CHO cells or HEK-293 cell (Noon et al., 2002; Rached et al., 2005). Conversely, mammalian MC2R orthologs could not be functional expressed in these cell lines (Kilianova et al., 2006; Roy et al., 2007) unless the cells were co-transfected with the chaperone, MRAP1 (melanocortin-2 receptor accessory protein 1; Metherell et al., 2005). Similar observations were made for MC2R orthologs from non-mammalian tetrapods, including chicken, reptile, and frog (Davis et al., 2013; Barlock et al., 2014), and teleosts (Agulleiro et al., 2010; Liang et al., 2011; Agulleiro et al., 2013).

In tetrapods and teleosts, MRAP1 is an integral protein with a single transmembrane domain (Metherell et al., 2005). At the rough endoplasmic reticulum, MRAP1 forms an antiparallel homodimer with dual topology, and three functional domains are present in this protein (see reviews by Hinkle and Sebag, 2009 and Webb and Clark, 2010; Fig. 1A). The LKANKHS motif in mouse MRAP1 is required for the formation of the dual-topology antiparallel homodimer (Fig. 1A; motif B). The trafficking of mammalian MC2R orthologs from the ER to the plasma membrane requires interactions between the transmembrane domain of MRAP1 and an as yet undetermined transmembrane domain on the MC2R ortholog (Fig. 1A; motif C). Activation of mammalian MC2R orthologs requires interaction between the LDYI motif in the N-terminal

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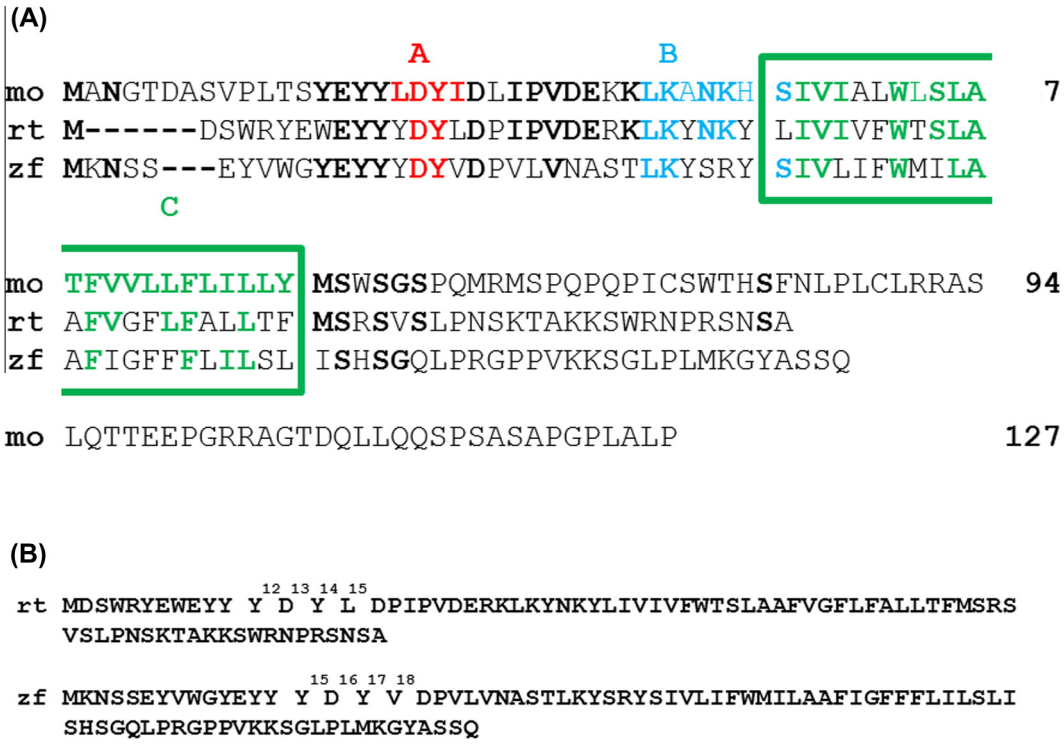

Fig. 1. Alignment of MRAP1 amino acid sequences. (A) The amino acid sequences of mouse MRAP1 (mo: accession number: NP_084120.1), rainbow trout MRAP1 (rt: accession number FR837908), and zebrafish MRAP1 (zf: Agulleiro et al., 2010) were aligned as described by Dores et al. (1996). Amino acids in moMRAP1 that were present at the same position in either rtMRAP1 or zfMRAP1 are in bold. [A] Marks the location of the activation motif in moMRAP1. Amino acid positions that are identical in this motif in moMRAP1 and present at the same position in either rtMRAP1 or zfMRAP1 are in bold red. [B] Marks the location of the duel topology motif in moMRAP1. Amino acid positions that are identical in this motif in moMRAP1 and present at the same position in either rtMRAP1 or zfMRAP1 are in bold blue. [C] Marks the location of the transmembrane domain in moMRAP1. Amino acid positions that are identical in this motif in moMRAP1 and present at the same position in either rtMRAP1 or zfMRAP1 are in bold green. The NGT amino acid motif in the N-terminal of moMRAP1 is the N-linked glycosylation site in this protein (Hinkle and Sebag, 2009), and the corresponding N-linked glycosylation motif in zfMRAP1 is NSS (Agulleiro et al., 2010). (B) The amino acid sequences of rtMRAP1 and zfMRAP1 are presented. The residues that are numbered are in the putative activation site of each MRAP1 sequence. This figure provides the reference for the alanine substituted mutants that were made for rtMRAP1 and zfMRAP1.

of mouse MRAP1 (Fig. 1A; motif A) and an as yet undetermined site on the MC2R ortholog.

The later conclusion is based on two sets of observations. The first is that the LDYI motif is absent from mammalian MRAP2 orthologs (Chan et al., 2009). Although this paralog of MRAP1 can mediate the trafficking of mammalian MC2R orthologs to the plasma membrane, MRAP2 cannot efficiently facilitate the activation of the MC2R orthologs (Hinkle and Sebag, 2009). The second observation is that when the LDYI motif in mouse MRAP1 was replaced with alanine residues, the activation of human MC2R was blocked (Sebag and Hinkle, 2009). An interesting finding of this study was that single alanine mutants of mouse MRAP1 at L^{18}, D^{19}, or I^{21} (Fig. 1A) were fully active, yet alanine substitution at Y^{20} resulted in a 50% drop in activation of MC2R relative to the activation of MC2R in the presence of the wild type mouse MRAP1. Clearly Y^{20} plays a critical role in activation of MC2R. However, since this single alanine analog did not completely block activation of the MC2R, the other amino acids in the motif are making some contribution to the activation process (Sebag and Hinkle, 2009).

Following the discovery of mammalian MRAP1 orthologs, *mrap1* genes were detected in the genomes of teleosts including, but not limited to, zebrafish (*Danio rerio*, Agulleiro et al., 2010) and rainbow trout (*Oncorhyncus mykiss*, accession number: FR837908). An alignment of the deduced amino acid sequences for mouse, rainbow trout, and zebrafish MRAP1 orthologs indicates that the transmembrane domains of rainbow trout and zebrafish MRAP1 orthologs have respectively 55% and 45% sequence identity with the corresponding domain of mouse MRAP1 (Fig. 1A; motif C). In addition, a putative dual-topology motif can also be seen in the rainbow trout and zebrafish MRAP1 orthologs (Fig. 1A; motif B). Finally, rainbow trout MRAP1 has the sequence YDYL that corresponds to the activation sequence for mouse MRAP1, and zebrafish MRAP1 has the sequence YDYV as a potential activation sequence (Fig. 1A; motif A). With respect to the activation motif, a feature common to mammalian and teleost MRAP1 orthologs was the DY motif.

Given these observations, the objective of this study was to determine the relative importance of the D and Y residues in the putative activation motifs of rainbow trout and zebrafish MRAP1. To test this hypothesis, single and multiple alanine substitutions were made in the cDNAs for rainbow trout and zebrafish MRAP1 to determine if one or more of the residues in these putative activation motifs is critical for the activation of rainbow trout MC2R.

2. Materials and methods

Rainbow trout MC2R (rtMC2R; accession number EU1198701) was synthesized with a V-5 epitope tag at the N-terminal of the receptor, and the rainbow trout MRAP1 (rtMRAP1; accession number FR837908), and zebrafish MRAP1 (zfMRAP1; Agulleiro et al., 2010) were synthesized with FLAG epitope tags at the N-terminal of the accessory proteins, respectively (GenScript; Piscataway, NJ). In addition alanine mutants of rtMRAP1 and zfMRAP1 were also synthesized by GenScript, and each mutant construct had a FLAG epitope tag at the N-terminal of the mutant MRAP1. The amino acids that were targeted for alanine substitution are numbered in Fig. 1B. Each cDNA construct was individually inserted in a pcDNA3.1+ vec-