Molecular cloning of rat transmembrane domain protein of 40 kDa regulated in adipocytes and its expression in H9c2 cells exposed to ischemic hypoxia and reoxygenation

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Abstract

We clone a 1230 bp complementary DNA encoding rat transmembrane domain protein of 40 kDa regulated in adipocytes (TPRA40), an orphan receptor, by reverse transcription-polymerase chain reaction using H9c2 cells derived from embryonic rat heart. The deduced amino acid sequence of rat TPRA40 consists of 369 amino acids and has a longer carboxyl terminus than that of the mouse protein. The level of TPRA40 mRNA decreases significantly throughout ischemic hypoxia and reoxygenation.

Keywords: Orphan receptor; TPRA40; Heart; Cloning; Ischemic hypoxia; mRNA expression

Various pathways in mammalian cells are induced in response to ischemia and reperfusion leading to cell death and organ dysfunction. In the heart, prolonged ischemia causes necrosis and contractile dysfunction, but the heart can recover from injury following brief ischemia. Many genes are expressed in the heart during recovery from ischemia. In particular, the expression of immediate early genes (e.g. c-fos, c-jun, and Egr-1) is rapidly up-regulated during post-ischemic reperfusion [1-4]. Among transcription factors, the induction of c-fos is regulated by mitogen-activated protein kinase (MAPK) [5]. We have demonstrated that ischemic hypoxia induces the nuclear translocation of MAPK [6], which is activated by nuclear atypical protein kinase C (PKC), PKCζ, activated by phosphoinositide 3-kinase (PI3-K) during reoxygenation [7,8]. However, the upstream factor of PI3-K involved in the response to ischemia and reoxygenation remains unknown, although it has been reported that PI3-K acts downstream of a seven transmembrane receptor [9,10].

Recently, an orphan receptor, transmembrane domain protein of 40 kDa regulated in adipocytes (TPRA40) in mouse, was cloned, and the expression of TPRA40 was found to be significantly increased in diabetic mice [11]. However, the TPRA40 cDNA in rat has not been cloned and the expression of TPRA40 during ischemic hypoxia and reperfusion also remains unknown. The present study uses an ischemia and reperfusion cell model to examine the expression of the TPRA40 mRNA. Here we report that the cloned rat TPRA40 cDNA shows a longer putative carboxyl terminus than that of the mouse protein, and that the level of TPRA40 mRNA decreases significantly throughout ischemic hypoxia and reoxygenation.

To isolate cDNA fragments of the mouse TPRA40-related protein expressed in H9c2 cells derived from embryonic rat heart, we carried out reverse transcription-polymerase chain reaction (RT-PCR) using primers a and i (Fig. 1) designed according to the sequence of mouse TPRA40 [11]. A DNA fragment of approximately 1.25 kbp was amplified. The band was excised from the gel, directly sequenced, and subcloned into pGEX-2T, a GST-fusion protein expression vector. As shown in Fig. 2, the open reading frame (ORF) consists of 1107 bp and encodes a protein of 369 amino acids with a predicted molecular mass of 40.5 kDa. The clone contains the cri-
teria for a Kozak consensus translation initiation site, GGAATGG [12]. The amino acid sequence demonstrates 98.2% homology to mouse TPRA40, and the cDNA shows 99.5% homology to that for mouse TPRA40. Therefore, we have designated this rat gene TPRA40. The rat TPRA40 cDNA has one cytosine at position 867 from the first nucleotide of the ORF, whereas the mouse cDNA has two cytosines at the corresponding position, causing the cDNA sequence of rat TPRA40 to encode a glutamic acid at the position where the mouse TPRA40 is terminated. Recently, a full-length cDNA encoding a human seven transmembrane domain orphan receptor (GenBank accession number AB037108) was isolated from human fetal brain by Saito T. et al. The deduced amino acid sequence shows 85.8% homology to rat TPRA40, and 83.3% homology to mouse TPRA40 (Fig. 3). The human cDNA at nucleotide position 876 counted from the first nucleotide of the ORF also has one cytosine, as in the case of the rat cDNA for TPRA40. As shown in Fig. 2, analysis of the amino acid sequence of rat TPRA40 indicates seven putative transmembrane domains and a longer carboxyl terminus (71 amino acid residues) than that of mouse. Four putative N-glycosylation sites (two at the amino terminus, the other two at the carboxyl terminus) are present, suggesting that rat TPRA40 may be a membrane receptor. The protein contains four serine residues that can act as possible PKC phosphorylation sites, and all of these residues are located in putative intracellular sites (two at the carboxyl terminus, one at the putative intracellular loop 2, one at the putative intracellular loop 3). These sites in rat TPRA40 may be associated with signaling pathways, since protein phosphorylation by PKC plays important roles in intracellular signal transduction [7,8,13,14]. In addition, rat TPRA40 has six putative myristoylation sites in the second, third, and sixth transmembrane domains, and the carboxyl terminus. Rat TPRA40 demonstrates 100% identity to mouse TPRA40 at amino acid positions 1–289 (Fig. 3), and the longer carboxyl terminus demonstrates 33.3% identity to cathepsin L.

Yang et al. demonstrated that the level of TPRA40 mRNA is significantly increased in the epididymal fat of animal models of diabetes [11]. To investigate the expression of TPRA40 mRNA during ischemic hypoxia and reoxygenation, we performed RT-PCR under the conditions described in the legend to Fig. 4, using total RNA derived from H9c2 cells exposed to ischemic hypoxia and reoxygenation for the indicated time (Fig. 4A,B). We performed RT-PCR of c-fos and c-jun using the same samples examined above to confirm that the cells were exposed to ischemic hypoxia and reoxygenation. In previous studies, the c-fos and c-jun genes were shown to be induced under conditions of ischemia and reperfusion in the heart [1,4], or in cardiac myocytes exposed to hypoxia [2]. The expression of the c-fos and c-jun mRNAs are significantly induced by ischemic hypoxia and reoxygenation (Fig. 4A). In addition, we observed that reactive oxygen species increased during ischemic hypoxia and were decreased by reoxygenation. Reactive oxygen species generation in cells was observed using diacetate form of 2,7-dichlorofluorescein (Molecular Probes) with a fluorescent microscope (Axioplan 2, Carl Zeiss Co., Ltd.) (data not shown). The ATP level decreased slightly during ischemic hypoxia and returned to the initial level by reoxygenation (data not shown). These findings are consistent with previous reports [15,16]. The level of TPRA40 mRNA was significantly decreased at 2 h of ischemic hypoxia and the decrease continued until at least 3 h after reoxygenation as shown in Fig. 4. Ischemic hypoxia for 5 h resulted in the decrease of TPRA40 mRNA by approximately 55% of control level (Fig. 4C), whereas the expression of TPRA40 mRNA at reoxygenation for 3 h after ischemic hypoxia for 2 h was approximately 25% of control level (Fig. 4A,B). These findings suggest that both ischemic hypoxia and reoxygenation may be involved in the inhibition of expression of TPRA40 mRNA. The expression of TPRA40 mRNA in H9c2 cells exposed to medium including low concentration of glucose decreased by approximately 85% of control level, suggesting that the decrease of glucose concentration in medium induces the inhibition of the expression of TPRA40 mRNA during ischemic hypoxia. The level of TPRA40 mRNA recovered to the control level at 12 h after reoxygenation (data not shown).
The level of G3PDH mRNA used as an internal control remained almost the same throughout ischemic hypoxia and reoxygenation under the conditions used in the study (Fig. 4). We confirmed that the increase in PCR product was linear up to 28 cycles for G3PDH, and up to 30 cycles for TPRA40 (data not shown).

What are the factors regulating the expression of the TPRA40 mRNA? It is possible that the extracellular glucose level may be involved in the regulation of TPRA40 expression for the following reasons. First, the expression of the TPRA40 mRNA began to decrease after the cells were incubated in glucose-free medium during ischemic hypoxia as described in the legend to Fig. 4. Second, during reoxygenation, the level of TPRA40 mRNA recovered...
when the cells were incubated in medium containing glucose. Third, the expression of TPRA40 mRNA was partially inhibited by lowering glucose concentration in medium, as shown in Fig. 4C. Finally, an increase in the level of TPRA40 mRNA has been reported in an animal model of diabetes [11]. Further investigations are required to clarify the regulation of TPRA40 expression.

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References


