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Molecules and Cells

C1qTNF-Related Protein-6 Increases the Expression of Interleukin-10 in Macrophages

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C1qTNF-Related proteins (CTRPs), a new highly conserved family of adiponectin paralogs, were recently identified as being involved in diverse processes including metabolism, host defense, apoptosis, cell differentiation, and organogenesis. However, the functional role of CTRP6 remains poorly identified. Here we provide evidence that CTRP6 induces the expression of interleukin-10 (IL-10) in macrophages. Conditioned medium from CTRP6-expressing HEK293 cells increased IL-10 expression in Raw264.7 cells. The globular domain of CTRP6 (gCTRP6) also dose-dependently increased both IL-10 mRNA and protein expression levels, with transcript levels increasing within 2 h. Furthermore, the globular domain of CTRP6 rapidly induced phosphorylation of ERK1/2 in Raw264.7 cells. Treatment with U0126, a selective inhibitor, abolished CTRP6-stimulated IL-10 induction. Taken together, there results demonstrate that CTRP6 induces expression of IL-10 via ERK1/2 activation. Considering that IL-10 is a potent anti-inflammatory cytokine that modulates inflammatory signaling pathways, CTRP6 may be a novel target for pharmacological drugs in inflammatory diseases.

INTRODUCTION

C1q-TNF-related protein-6 (CTRP6) is a member of a new highly conserved family of adiponectin paralogs, which are comprised of a signal peptide, a short variable region, a collagen-like region, and a C-terminal globular domain (Wong et al., 2004; 2008). Although CTRPs share a similar domain structure, they are involved in diverse physiological processes, including fatty acid and glucose metabolism (Park et al., 2009; Wong et al., 2004), inflammation host defense (Weigert et al., 2005), platelet aggregation (Lasser et al., 2006), chondrogenesis and cartilage development (Maeda et al., 2006), aldosterone pro-duction (Wong et al., 2009), cell proliferation (Akiyama et al., 2007), and extracellular matrix-related functions (Ayyagari et al., 2005; Hayward et al., 2003). However, the functional role of CTRP6 remains poorly investigated.

Adiponectin is an adipokine secreted from adipose tissues and plays a key role in regulating glucose and lipid metabolism (Kadowaki et al., 2006). It also regulates the functions of macrophages and monocytes via induction of the anti-inflammatory cytokine IL-10 (Kumada et al., 2004; Wolf et al., 2004). Accumulating data show that CTRPs are also secreted from adipose tissues and associated with the immune response (Schaffler et al., 2007). CTRP1 is expressed in preadipocytes and upregulated by TNF-α and IL-1β (Kim et al., 2006). CTRP3/CORS26/Cartducin is expressed in mature adipocytes and has anti-inflammatory properties (Weigert et al., 2005). Whereas adiponectin, CTRP1, and CTRP3 are predominantly expressed in adipose tissues, CTRP6 is widely expressed in many tissues (Wong et al., 2008). However, the serum level of CTRP6 is significantly increased in adiponectin-null mice (Wong et al., 2008). 12-week-old *ob/ob* mice, which have decreased expression of adiponectin relative to their lean controls (Wong et al., 2008), have increased CTRP6 expression in adipose tissue, suggesting that CTRP6 plays a role in compensating for the loss of adiponectin under certain conditions. More recently, we demonstrated that CTRP6 is involved in fatty acid metabolism via activation of AMP-protein kinase (AMPK), similar to adiponectin function (Lee et al., 2010). These findings led us to speculate that CTRP6 may be involved in anti-inflammatory cytokine production. In this study, we present evidence that CTRP6 stimulates the expression of IL-10 in macrophages.

MATERIALS AND METHODS

Reagents

DMEM medium was obtained from Invitrogen (USA). Polyclonal antibody directed against human CTRP6 was purchased from Abcam (UK). Polyclonal antibodies directed against phospho-p38 and phospho-JNK were obtained from Cell Signaling Technology (USA). Polyclonal antibody directed against phospho-Erk (E-4) was obtained from Santa Cruz Biotechnology (USA). Anti-actin antibody was obtained from Sigma (USA). Horseradish peroxidase (HRP)-conjugated anti-mouse IgG and anti-rabbit IgG were obtained from Santa Cruz Biotechnology. The IL-10 ELISA kit was purchased from R&D System (USA). Oligonucleotides primers were synthesized by Bionics (Korea).

Cell culture

Raw264.7 and HEK293 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) heat-

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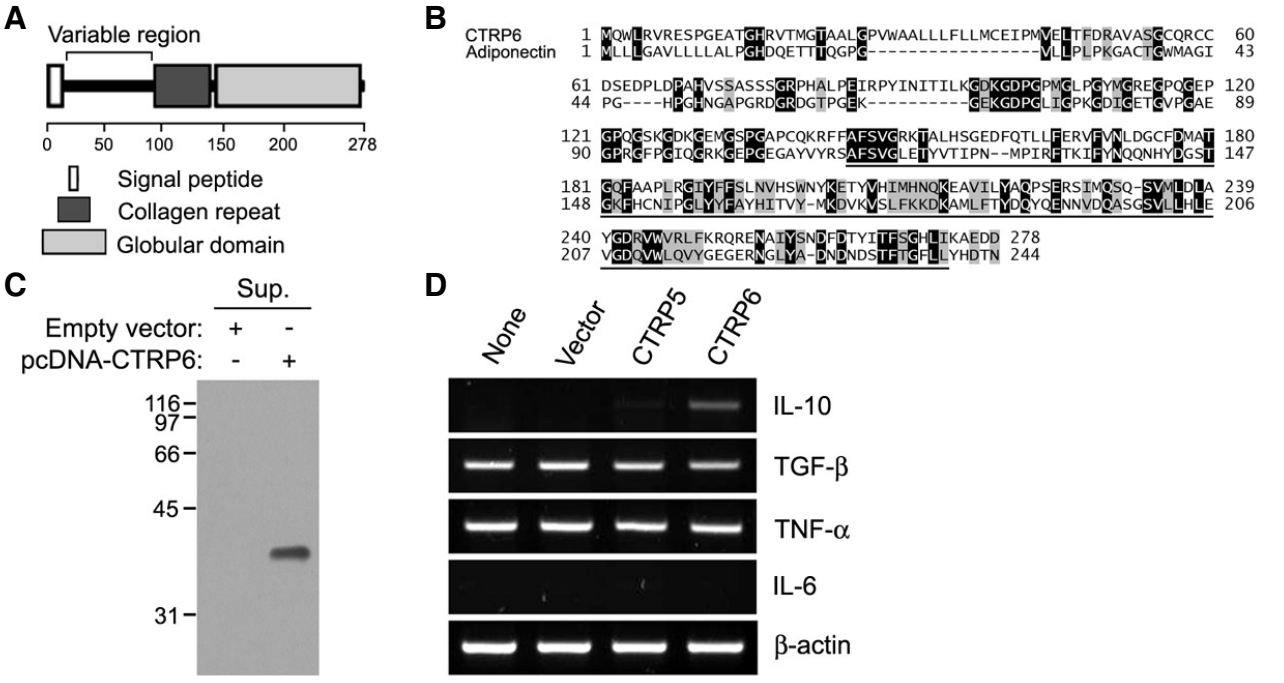

Fig. 1. Secreted CTRP6 induces expression of interleukin-10 in Raw264.7 macrophages. (A) Schematic diagram of human CTRP6 protein. Numbers indicate amino acid positions in CTRP6. (B) Amino acid sequences of CTRP6 and adiponectin were aligned using the ClustalW program. The region corresponding to C-terminal globular domain is indicated by underlines. Amino acids in black boxes indicate matched amino acids between CTRP6 and adiponectin. Numbers indicate amino acid positions in the CTRP6 protein. (C) HEK293 cells were transfected with an expression vector encoding human CTRP6 or empty vector. Conditioned media from transfected cells were collected 48 h later. Each sample was analyzed by 10% SDS-PAGE and detected by immunoblotting with an anti-CTRP6 antibody. (D) HEK293 cells were then transfected with a vector encoding human CTRP6, CTRP5, or empty vector. The 24 h after transfection, culture medium was exchanged with serum-free DMEM. The supernatant was collected after 48 h and then added to Raw264.7 cells. After 4 h, the cells were harvested, and the mRNA levels of IL-10, IL-6, TNF-α, and TGF-β were analyzed by RT-PCR. A representative result from two independent experiments is shown.

inactivated FBS (Invitrogen) and the appropriate antibiotics.

Transient transfection

The complete CTRP6 cDNA was amplified from human muscle cDNA and cloned into pcDNA(+) 3.1 (Invitrogen). The mammalian expression vector encoding human CTRP5 was previously described (Park et al., 2009). HEK293 cells were transiently transfected with a vector encoding human CTRP6 or CTRP5 in OptiMEM I medium using Lipofectamine (Invitrogen), in accordance with the manufacturer's instructions. The 24 h after transfection, culture medium was exchanged with serum-free DMEM. The supernatant was collected after 48 h and used for further experiments.

Generation of recombinant CTRP6 proteins

To produce GST-fusion protein corresponding to the globular domain of human CTRP-6, the fragment of CTRP-6 cDNA encoding amino acids 145-273 was generated by PCR and cloned into the *Bam*HI and *Xho*I sites of pGEX 4T-1. The recombinant protein was expressed in *E. coli* BL21, harvested, and purified using Glutathione Sepharose™ Fast Flow, in accordance with the manufacturer's instructions (Pharmacia, Sweden). To remove endotoxins, the purified proteins were loaded in Detoxi-Gel (USA).

RNA isolation and reverse transcription-PCR

Total RNA was extracted from Raw264.7 cells using Trizol reagent, in accordance with the manufacturer's instructions (Invitrogen). The reverse transcription reaction was performed in a final volume of 30 μl comprised of 2 μg of total RNA, 200 ng of oligo(dT)$_{15}$ primer, 1$\times$ reverse transcription buffer, 0.5 mM deoxynucleotide triphosphate mixture, RNasin recombinant ribonuclease inhibitor (Promega, USA), 200 units of M-MLV reverse transcriptase (Promega), and diethylpyrocarbon-ate-treated water. After incubation at 42°C for 50 min, the re-verse transcription reaction was terminated by heating at 70°C for 15 min. The newly synthesized cDNA was amplified by PCR in a reaction mixture comprised of 2 μl of cDNA template, 1.5 mM $MgCl_2$, 2.5 units of Taq polymerase, and 0.3 μM of primers. The primers used in this study are shown in Table 1. Amplifications were performed under the following conditions: initial denaturation at 94°C for 2 min; 45 cycles of amplification with denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min; and final extension at 72°C for 10 min. PCR products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining.

Quantitative real-time PCR

For quantitative real-time PCR, cDNA produced by reverse transcription was diluted 2.5-fold, and SYBR green master mix (Roche Applied Science) was used to amplify IL-10 or a β-actin control. Real time PCR amplification was carried out in a LightCycler 480 (Roche Applied Science) as follows: initial denaturation at 95°C for 5 min; 45 cycles of amplification with denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s; 1 cycle of melting curves at

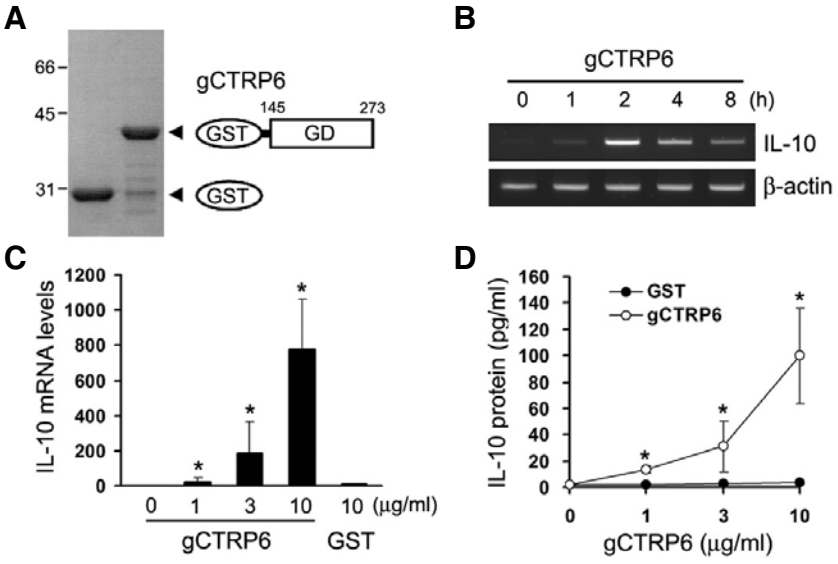

Fig. 2. The globular domain of CTRP6 also increases interleukin-10 expression at the mRNA and protein levels. (A) Schematic diagram of GST-fused globular CTRP6. Numbers indicate amino acid positions in CTRP6 protein. GD, globular domain. (B) Time course of effects of gCTRP6 on IL-10 expression. Raw264.7 cells were treated with GST-gCTRP6 (10 μg/ml) for the indicated times. The mRNA levels were analyzed by RT-PCR, as described in "Materials and Methods". A representative result from three indepen-dent experiments is shown. (C) Dose-res-ponse effects of gCTRP6 on IL-10 mRNA expression in Raw264.7 cells. The cells were treated for 4 h with the indicated concentrations of gCTRP6. The mRNA levels were measured by real-time quantitative RT-PCR, as described in "Materials and Methods". The results are expressed as the mean ± SD for three independent experiments. *t*-test: *, $P <$ < 0.05 compared with untreated control. (D) Dose-response effects of gCTRP6 on levels of IL-10 protein secreted into the media. The amount of IL-10 protein was determined by ELISA, as described in "Materials and Methods". The results are expressed as the mean ± SD for three independent experiments. *t*-test: *, $P < 0.05$ compared with GST control.

95°C for 5 s, 65°C for 1 min, and 97°C continuous; and a final cooling step at 40°C for 30 s. All samples were run in triplicate. The qRT-PCR results were analyzed as previously described (Lee et al., 2008; Park et al., 2005). Briefly, the comparative cycle threshold (C_T) method was used to analyze the data by generating relative values of the amount of target cDNA. Relative quantitation for any given gene, expressed as fold induction over control, was calculated after determination of the difference between C_T of the target gene A (IL-10) and that of the reference gene B (β-actin) in CTRP6-treated cells ($\Delta C_{T1} = \Delta C_{T1A} - \Delta C_{T1B}$) and untreated cells ($\Delta C_{T0} = \Delta C_{T0A} - \Delta C_{T0B}$) using the $2^{-\Delta\Delta CT(1-0)}$ formula.

Interleukin-10 assays

Raw264.7 cells were incubated with the indicated concentrations of gCTRP6 protein for 16 h. The supernatants were evaluated for cytokine production via ELISA 18 h later (R&D Systems, USA) as previously described (Kim et al., 2003).

Phosphoprotein analysis

Raw264.7 cells were serum-starved for 4 h and treated with gCTRP6 (10 μg/ml) recombinant protein. The cells were immediately washed three times with ice-cold phosphate-buffered saline (PBS) and lysed in cold lysis buffer [20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulphonyl fluoride, 5 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 1 mM pyrophosphatase, 1 μM pepstatin A, 1 μM leupeptin, and 1 μM aprotinin] for 30 min with vortex mixing. Equal amounts (30 μg) of total cell lysates were resolved by 10% SDS-PAGE, and separated proteins were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad Laboratories, USA). The membranes were incubated in blocking solution consisting of 5% skim milk in TBS-T [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% Tween 20] for 1 h at room temperature, and then immunoblotted with anti-phospho-Erk1/2, anti-phospho-p38, anti-phospho-JNK, or anti-actin antibodies. After overnight incubation, the membranes were extensively washed with TBS-T and incubated with HRP-conjugated anti-rabbit or anti-mouse IgG. Proteins were visualized using an enhanced chemiluminescent substrate kit (Pharmacia, Sweden).

Statistical analysis

The statistical significance was assessed using by the *t*-test. A *P* value of < 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

CTRP6 increases IL-10 expression in macrophages

C1qTNF related protein-6 (CTRP6) is a member of the CTRP family and consists of an N-terminal signal peptide, a collagen repeat and a C-terminal C1q-like globular domain (Fig. 1A). The globular domain of human CTRP6 shares a high degree of amino acid identity (33%) to adiponectin (Fig. 1B). A recent study showed that mouse C1QTNF isoforms are secretable proteins (Wong et al., 2004). To confirm that human CTRP6 is secreted from the cells where it is expressed, we cloned full-length human CTRP6 into the mammalian expression vector pcDNA3.1 and analyzed secretion of CTRP6 from transfected HEK293 cells. As shown in Fig. 1C, CTRP6 could be detected in culture medium by immunoblotting with an anti-CTRP6 antibody, demonstrating that CTRP6 is indeed secreted from mammalian cells. To examine the role of CTRP6 in the production of pro-inflammatory or anti-inflammatory cytokines, Raw264.7 cells were incubated with media derived from HEK293 cells transfected with a CTRP6 expression vector or a CTRP5 expression vector, or empty vector, and cytokine expression was analyzed using RT-PCR. As shown in Fig. 1D, the medium containing human CTRP6 significantly induces the expression of IL-10 mRNA. There is no detectable change in the expression levels of TNF-alpha, TGF-beta, and IL-6.

The globular C1q domain of CTRP6 is sufficient to increases IL-10 expression in macrophages

In a recent study, the migration of serum CTRP6 on immunoblots corresponds to the size of its globular head rather than that of the full-length protein (Wong et al., 2008). Therefore, to

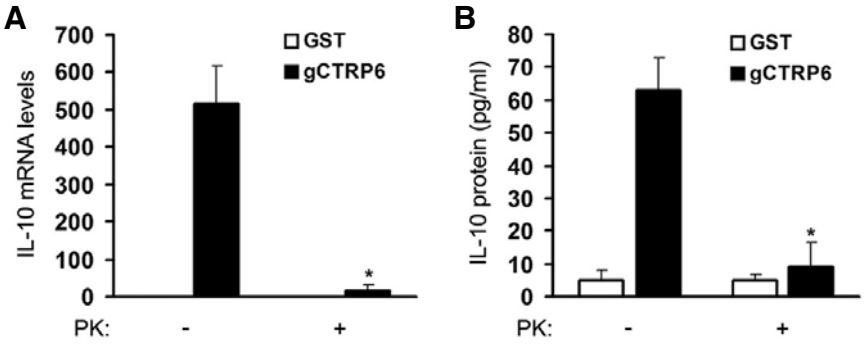

Fig. 3. Treatment of gCTRP6 with Proteinase K abrogates its effect in IL-10 expression. (A) Effect of protease-treated gCTRP6 on IL-10 mRNA expression. Raw264.7 cells were treated for 4 h with gCTRP6 (10 μg/ml) or Protease-treated gCTRP6 (10 μg/ml). The mRNA levels were measured by real-time quantitative RT-PCR, as described in "Materials and Methods". The results are expressed as the mean ± SD for three independent experiments. PK, heat digestion of the recombi- nant gCTRP6 with Proteinase K. $P < 0.05$ compared with protease-untreated gCTRP6. (B) Effect of protease-treated gCTRP6 on IL-10 protein expression. The Raw264.7 cells were treated for 16 h with gCTRP6 (10 μg/ml) or protease-treated gCTRP6 (10 μg/ml). The levels of IL-10 protein were determined by ELISA, as described in "Materials and Methods". PK, heat digestion of the recombinant gCTRP6 with Proteinase K. The results are expressed as the mean ± SD for three independent experiments. *t*-test: *, $P < 0.05$ compared with protease-untreated gCTRP6.

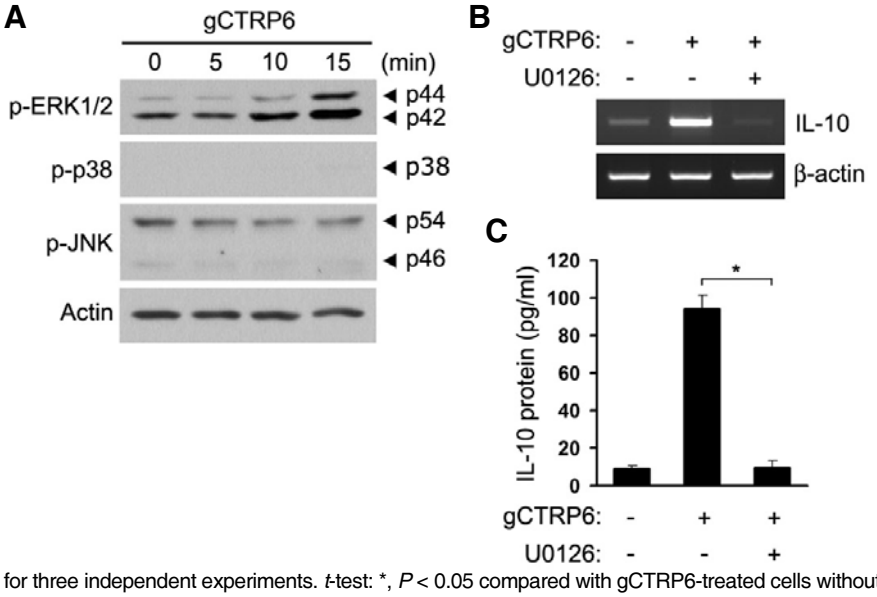

Fig. 4. ERK1/2 is involved in CTRP6-induced IL-10 expression. (A) Effect of gCTRP6 on phosphorylation of ERK1/2, p38, and JNK. Raw264.7 cells were treated with gCTRP6 (10 μg/ml) for the indicated times. Total cell lysates were subjected to 10% SDS-PAGE and immunoblotted with the indicated antibodies. (B) Effect of U0126 on CTRP6-induced IL-10 expression. Raw264.7 cells were pretreated with U0126 (10 μM) for 30 min and then incubated with gCTRP6 (10 μg/ml) for 4 h. Total RNA was prepared, and the expression level of IL-10 mRNA was analyzed with RT-PCR. A representative result from three independent experiments is shown. (C) Effect of U0126 on CTRP6-induced IL-10 expression. The levels of IL-10 protein were determined by ELISA, as described in "Materials and Methods". The results are expressed as the mean ± SD for three independent experiments. *t*-test: *, $P < 0.05$ compared with gCTRP6-treated cells without U0126 pretreatment.

further investigate the effect of CTRP6 on the production of IL-10, we generated the globular domain of human CTRP6 (gCTRP6) as a GST-fusion protein (Fig. 2A). Treatment of Raw264.7 cells with gCTRP6 induced expression of IL-10 mRNA within 1 h, with an apparent peak at 2 h (Fig. 2B). Further, recombinant gCTRP6 protein increased IL-10 expression at both the mRNA and protein levels in a dose-dependent manner (Figs. 2C and 2D). To verify whether the effect of recombinant gCTRP6 protein is due to bacterial endotoxin contamination, we examined the effect of protease-treated gCTRP6 protein on IL-10 expression. Protease-treated gCTRP6 was unable to induce IL-10 mRNA and protein expression, indicating that its effects are not due to endotoxin contamination (Figs. 3A and 3B).

ERK1/2 activation is required for CTRP6-mediated IL-10 induction

MAPKs are serine and threonine protein kinases, including the p42 and p44 ERKs, the p54 and p46 JNK1, and p38 MAPK. It has previously been found that LPS induces IL-10 synthesis in macrophages via MAPK activation (Liu et al., 2006). To investigate whether MAPKs are involved in CTRP6-induced IL-10 expression, we measured MAPK activation by western blotting. After treatment with gCTRP6, tyrosine phosphorylation of p44/p42 ERK significantly increased at 10 and 15 min (Fig. 4A), suggesting that p44/p42 ERK activation may play a significant role in CTRP6-mediated IL-10 expression. Phosphorylation of p38 MAPK also slightly increased at 15 min, but not remarkably. No activation of JNK was observed. To further investigate the role of p44/p42 ERK activation in gCTRP6-induced IL-10 expression, we pretreated Raw264.7 cells with U0126, a selective inhibitor of ERK1/2, and then examined its effect on gCTRP6-induced IL-10 expression. When Raw264.7 cells were pretreated with U0126, both IL-10 mRNA and protein expression were completely inhibited (Figs. 4B and 4C). These results show that gCTRP6 mediates the induction of IL-10 expression via activation of ERK1/2.

Adipose tissues secrete high amounts of adipokines into the blood, including leptin and adiponectin, that have regulatory functions for monocyte/macrophages. Adipose tissues also secrete molecules involved in the immune response, such as the C1qTNF-related proteins (CTRPs) (Schaffler et al., 2007). Among the CTRP isoforms, CTRP1 and CTRP3 are predominantly expressed in adipose tissues and involved in regulating

Table 1. DNA sequences of primers used for RT-PCR and real-time quantitative PCR.

Identity	Nucleotide sequences	Product size
IL-10	Forward: 5′-AGAAGCATGGCCCAGAAATC-3′	341
	Reverse: 5′-CCAAGGAGTTGTTTCCGTTAGC-3′	
IL-6	Forward: 5′-TGACAACCACGGCCTTC-3′	197
	Reverse: 5′-TTCTGCAAGTGCATCATCG-3′	
TNF-α	Forward: 5′-CCGATGGGTTGTACCTTGTC-3′	352
	Reverse: 5′-GGGCTGGGTAGAGAATGGAT-3′	
TGF-β	Forward: 5′-CTTTAGGAAGGACCTGGGTT-3′	258
	Reverse: 5′-CAGGAGCGCACAATCATGTT-3′	
β-actin	Forward: 5′-TCACCCACACTGTGCCCATCTACGA-3′	348
	Reverse: 5′-GGATGCCACAGGATTCCATACCCA-3′	

immune responses. In the present study, we provide evidence that CTRP6 can also increase the expression of Interleukin-10 in macrophages. First, media containing secreted CTRP6 induces expression of IL-10 mRNA in Raw264.7 macrophages. Second, recombinant gCTRP6 rapidly induces IL-10 expression and dose-dependently increases expression of IL-10 at the mRNA and protein levels. Third, gCTRP6 causes phosphorylation of p44/p42 ERK, and pretreatment of U0126, a selective inhibitor of ERK1/2, abrogates CTRP6-mediated IL-10 expression. Taken together, these results indicate that CTRP6 induces IL-10 expression in macrophages via the activation of ERK1/2.

MAPK signaling pathways are thought to play an important role in the regulation of IL-10 expression. Phosphorylation of ERK1/2 is involved in adiponectin-mediated IL-10 expression (Park et al., 2008). Consistent with this result, we found that CTRP6-stimulated IL-10 expression requires ERK1/2 activation, but not p38 MAPK or JNK activation. Considering that all three MAPKs contribute to LPS-mediated IL-10 induction in macrophages (Liu et al., 2006), CTRP6-induced IL-10 expression appears to be activated by a different signaling pathway than LPS-induced IL-10 expression. However, the mechanism by which IL-10 promoter is activated still needs further clarification. Recently, we showed that CTRP6 induces AMP-activated protein kinase (AMPK) activation in C2C12 cells (Lee et al., 2010). Another recent study demonstrated that AMPK acts as a potent counterregulator of inflammatory signaling pathways in macrophages, thereby decreasing proinflammatory cytokines and concomitantly increasing IL-10 (Sag et al., 2008). Thus, CTRP6 is possibly also involved in regulating inflammatory signaling pathways via AMPK activation. Furthermore, adiponectin exerts anti-inflammatory effects by reducing the release of IL-6 and TNF-α and increasing IL-10 in LPS-treated THP-1 cells (Wulster-Radcliffe et al., 2004). CTRP3/CORS-26 also has an anti-inflammatory effect by reducing IL-6 and TNF-α secretion (Schaffler et al., 2007). However, our results showed that the conditioned media containing CTRP6 does not regulate the expression levels of TNF-alpha and IL-6 in Raw264.7 cells. This finding may be explained by the different experimental conditions. In this study, we have performed all experiments under non-inflammatory conditions but not LPS-treated inflammatory conditions because treatment of LPS can induce IL-10 expression in macrophages. Thus, whether CTRP6 regulates pro-inflammatory signaling pathways is currently unknown and will require further study.

In conclusion, we have reported that CTRP6 plays a role in inducing IL-10 expression in Raw264.7 macrophages. Furthermore, we have demonstrated that ERK1/2 activation is involved in CTRP6-stimulated IL-10 expression. Because IL-10 is a potent anti-inflammatory cytokine that modulates the inflammatory signaling pathway, CTRP6 may be a novel target for pharmacological drugs in inflammatory diseases.

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