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# Insulin-like growth factor-binding protein-5 (IGFBP-5) inhibits TNF-α-induced NF-κB activity by binding to TNFR1

Jae Ryoung Hwang [a,1], Jae Ho Huh [a,1,2], Yoonna Lee [a], Sang Il Lee [a], Seung Bae Rho [b], Je-Ho Lee [a,c,*]

[a] Molecular Therapy Research Center, Sungkyunkwan University, Seoul 135-710, Republic of Korea
[b] Research Institute, National Cancer Center, Goyang-si, Gyeonggi-do 411-769, Republic of Korea
[c] Department of Obstetrics and Gynecology, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Republic of Korea

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ABSTRACT

IGFBP-5 is known to be involved in various cell phenomena such as proliferation, differentiation, and apoptosis. However, the exact mechanisms by which IGFBP-5 exerts its functions are unclear. In this study, we demonstrate for the first time that IGFBP-5 is a TNFR1-interacting protein. We found that ectopic expression of IGFBP-5 induced *TNFR1* gene expression, and that IGFBP-5 interacted with TNFR1 in both an *in vivo* and an *in vitro* system. Secreted IGFBP-5 interacted with GST-TNFR1 and this interaction was blocked by TNF-α, demonstrating that IGFBP-5 might be a TNFR1 ligand. Furthermore, conditioned media containing secreted IGFBP-5 inhibited PMA-induced NF-κB activity and IL-6 expression in U-937 cells. Coimmunoprecipitation assays of TNFR1 and IGFBP-5 wild-type and truncation mutants revealed that IGFBP-5 interacts with TNFR1 through its N- and L-domains. However, only the interaction between the L-domain of IGFBP-5 and TNFR1 was blocked by TNF-α in a dose-dependent manner, suggesting that the L-domain of IGFBP-5 can function as a TNFR1 ligand. Competition between the L-domain of IGFBP-5 and TNF-α resulted in inhibition of TNF-α-induced NF-κB activity. Taken together, our results suggest that the L-domain of IGFBP-5 is a novel TNFR1 ligand that functions as a competitive TNF-α inhibitor.



## 1. Introduction

Insulin-like growth factor binding protein-5 (IGFBP-5) is a secreted, multifunctional protein that functions in both an IGF-dependent and an IGF-independent manner in bone formation, differentiation, proliferation, and cell death [1,2]. IGFBP-5 is found in both the nucleus and cytoplasm, although the functional relevance of these different locations is unclear. However, a recent study reported that IGFBP-5 might have different functions depending on its subcellular localization [3]. IGFBP-5 has three domains: an N-terminal domain, an L-domain, and a C-terminal domain. The N-terminal domain of IGFBP-5 possesses an IGF-I binding site and a signal peptide, while the C-terminus contains a nuclear localization signal and a heparin-binding site [4,5]. Protein degradation and posttranslational modifications such as phosphorylation and glycosylation occur in the L-domain [6]. Truncated forms of IGFBP-5 have been detected in serum [7] and conditioned media [8]. In concert with these findings, it has been reported that IGFBP-5 is cleaved by several proteases including MMPs and ADAM family proteases, as well as unknown proteases [8–10]. However, the functional roles of truncated forms of IGFBP-5 have not been evaluated.

Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine mostly produced by activated macrophages and T lymphocytes [11]. Binding of TNF-α to tumor necrosis factor receptor 1 (TNFR1) triggers a signaling cascade that results in cell death or cell survival, depending on the interacting proteins. TNFR1 can activate cell proliferation via activation of the NF-κB signal pathway or cell death via activation of the Fas-associated death domain (FADD) protein and caspases [12]. Increased expression of TNF-α is a hallmark of several diseases including inflammatory diseases and certain types of cancer [11,13].

In this study, we investigated the functional mechanisms underlying IGFBP-5-induced *TNFR1* gene expression. We found that IGFBP-5 competed with TNF-α for binding to TNFR1 via its L-domain and inhibited the TNF-α-TNFR1 downstream signaling pathway. IGFBP-5 is therefore a potential therapeutic molecule that can be used to treat diseases characterized by dysregulation of TNF-α such as rheumatoid arthritis and cancer.

* Corresponding author at: Molecular Therapy Research Center, Sungkyunkwan University, Annex 3F, Samsung Medical Center, Ilwon-dong, Kangnamgu, Seoul 135-710, Republic of Korea. Fax: +82 2 3410 0044.
*E-mail address:* jeholee@gmail.com (J.-H. Lee).
[1] These authors contributed equally to this work.
[2] Present address: CELLTRION Inc., Songdo-dong, Yeonsu-gu, Incheon 406–840, Republic of Korea.

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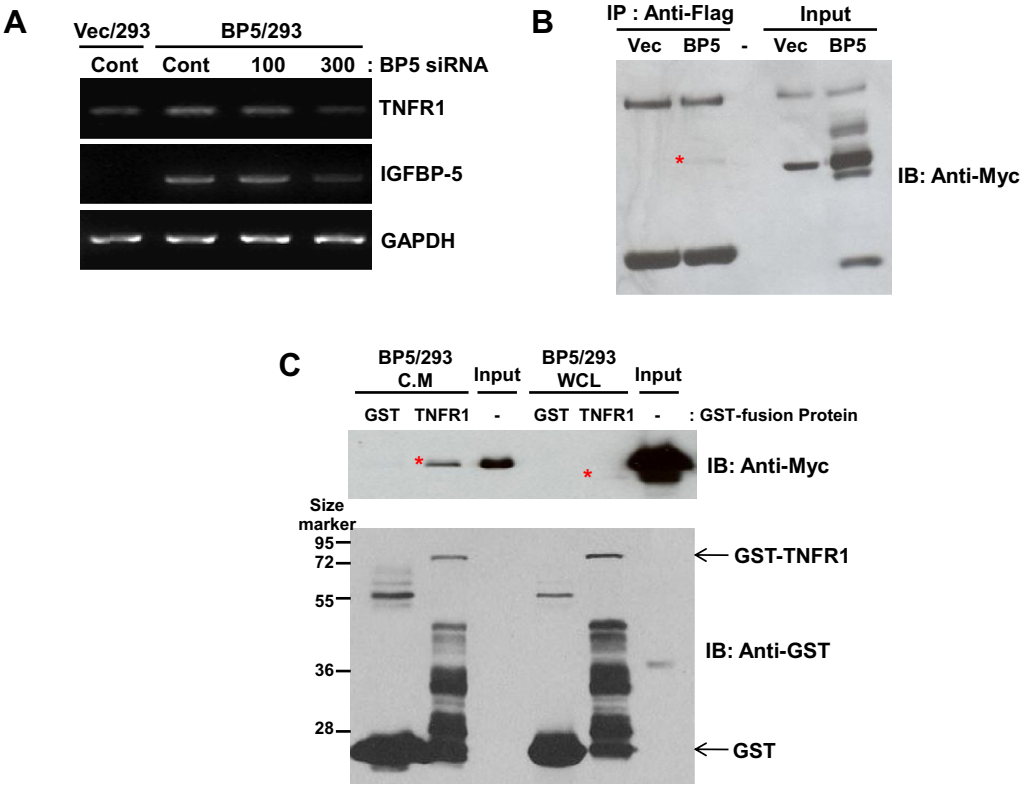


**Fig. 1.** IGFBP-5 up-regulates TNFR1 mRNA expression and interacts with TNFR1. (A) IGFBP-5-specific siRNA or control siRNA was transfected into BP5/293 and Vec/293 stable cells at the concentrations indicated. Total RNA was isolated and reverse transcription-PCR was performed to detect *TNFR1*, *IGFBP-5*, and *GAPDH* expression. (B) IGFBP-5 and TNFR1 in BP5/293 and Vec/293 cells were coimmunoprecipitated using anti-Flag antibody and Western blot analysis was performed using anti-Myc. (C) GST-fused TNFR1 and GST protein alone (negative control) were purified from *E. coli*. Conditioned media and whole cell lysates from BP5/293 stable cells were incubated with either GST-TNFR1 or GST immobilized on GST-beads. Bound IGFBP-5 was detected by Western blotting with anti-Myc. The asterisk indicates TNFR1-bound IGFBP-5. CM and WCL refer to conditioned media and whole cell lysate, respectively. IB refers to immunoblotting.

## 2. Materials and methods

### 2.1. Plasmids and construction of truncation mutants

Human IGFBP-5 and TNFR1 were obtained by reverse transcription-PCR from HEK293 cells and cloned into a TA vector (Invitrogen, Carlsbad, CA, USA). Flag-TNFR1 was constructed by PCR using TNFR1 in a TA vector. The resulting PCR product was cloned into a pcDNA3 vector containing Flag (kindly provided by Prof. H.S. Choi, Chonnam University, South Korea). To construct glutathione S-transferase (GST)-fused TNFR1, TNFR1 excluding the signal peptide was subcloned into pGEX-2T vector (Amersham). Myc- and GFP-tagged IGFBP-5 were incorporated into pcDNA 3.1/myc-His (Invitrogen) and pEGFP-N1 (Clontech, Mountain View, CA, USA) vectors, respectively, by PCR using IGFBP-5 in the TA vector as a template. IGFBP-5-truncation mutants containing $N^{1-80}$, $L^{81-168}$, or $C^{169-252}$ domains were constructed by PCR using IGFBP-5-Myc as a template. The resulting PCR products were cloned into pSec-Taq2/Hygro A (Invitrogen). All of the cloned constructs were verified by sequencing.

### 2.2. GST-TNFR1 expression and binding assays

Plasmids expressing either GST-TNFR1 or GST vector alone were expressed in pLys S cells (Promega). Cells were grown until the optical density was between 0.6 and 0.8 at 600 nm, and then the cells were induced with 0.5 mM IPTG for 3 h at 28 °C. Cells were centrifuged and the cell pellet was resuspended in lysis buffer (25 mM Tris, pH 7.5, 150 mM NaCl, and 1 mM EDTA) containing 1 mM PMSF, sonicated on ice, and then the insoluble proteins were removed by centrifugation. Soluble proteins were incubated with GST beads for 2 h at 4 °C and the beads were then washed with lysis buffer.

For binding assays, whole cell lysates or overnight conditioned media obtained from BP5/293 stable cells were incubated with GST-TNFR1-bound GST beads for 3 h at 4 °C. The beads were then washed with lysis buffer containing 0.5% Triton X-100, boiled in Laemmli sample buffer, and the bound proteins were detected by Western blot analysis.

### 2.3. Competition assays

To assess the competition between IGFBP-5 and TNF-α for binding to TNFR1, HEK293 cells were transiently transfected. Twenty-four hours after transfection, cells were washed with 1× PBS to remove FBS, and the cells were then treated with various concentrations of TNF-α (eBioscience, San Diego, CA, USA) for 4 h. Cells were lysed in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EGTA, 0.25% sodium deoxycholate, 0.2% Nonidet P-40) supplemented with protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA) and 1 mM PMSF, and soluble proteins were immunoprecipitated with anti-Flag-conjugated agarose beads (Sigma, St. Louis, MO, USA). Western blotting was performed using mouse monoclonal anti-Myc antibody (9E10).

For the *in vitro* competition assay, conditioned media from BP5/293 cells was incubated with GST-TNFR1 immobilized on GST beads in the presence of increasing amounts of TNF-α for 3 h at 4 °C. The beads were then washed with lysis buffer containing 0.5% Triton X-100, boiled, and the bound proteins were analyzed by Western blotting.