

## DIFERENCIAS EN LA ACTIVACIÓN DEL FACTOR DE CRECIMIENTO TRANSFORMANTE BETA-1 (TGF- $\beta$ 1) POR EL ÁCIDO Y EL CALOR

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### Resumen

El factor de crecimiento transformante beta-1 (TGF- $\beta$ 1) es una citoquina con diversas funciones en procesos inflamatorios y el sistema inmune. Un método comercial comúnmente usado para la determinación de TGF- $\beta$ 1 es el ensayo de inmunoabsorción enzimática (ELISA), que detecta directamente la forma activa, o la forma inactiva (latente), después de activarla por acidificación o por tratamiento con urea. Este método constituye la mejor opción debido a su simplicidad, especificidad y sensibilidad, sin embargo, existen algunas discrepancias en la literatura científica relacionadas con los factores que activan el TGF- $\beta$ 1 latente *in vitro* e *in vivo*. Por este motivo decidimos comparar los efectos del calor y de la acidificación en la activación del complejo inactivo. Los resultados muestran que aunque ambos tratamientos activan el TGF- $\beta$ 1 latente, la activación térmica es más eficiente que la acidificación. Estos resultados sugieren que los datos publicados reportando valores absolutos de TGF- $\beta$ 1, basados sólo en ELISA, se deben interpretar cautelosamente. Asimismo, para la detección de TGF- $\beta$ 1 por este método es recomendable usar como control positivo tanto la activación térmica, como la acidificación.

**Palabras clave:** proteínas de unión TGF- $\beta$ 1 latente, ELISA

## DIFFERENTIAL ACTIVATION OF RECOMBINANT HUMAN LATENT TRANSFORMING GROWTH FACTOR- $\beta$ 1 (TGF- $\beta$ 1) BY ACID AND HEAT

### Abstract

Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) is a cytokine with many effects on inflammation and immunity. A common and commercially-available method for the detection of TGF- $\beta$ 1 consists of an enzyme-linked immunosorbent assay (ELISA), which detects active TGF- $\beta$ 1 or total TGF- $\beta$ 1 after activation by transient acidification or treatment with urea. Because of its simplicity, specificity and sensitivity, this method is the best option when working on a cell-free system. Given that some discrepancies can be found in the literature regarding the factors that activate the latent TGF- $\beta$ 1 complex both *in vitro* and *in vivo*, this study was performed to compare the effectiveness of acidification vs. heat treatment in activating latent TGF- $\beta$ 1. Our results demonstrate that while both heat and acid treatment activate latent TGF- $\beta$ 1, the former is a more efficient activator of the complex. Our results suggest that published data reporting absolute values of total and active TGF- $\beta$ 1 based solely on this method should be interpreted cautiously and recommend the use of both heat and acidification as positive controls when assaying activation of TGF- $\beta$ 1 using the ELISA detection system.

**Key words:** latent TGF- $\beta$ 1 binding proteins, ELISA

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The transforming growth factor- $\beta$  (TGF- $\beta$ ) family of three related mammalian peptides exerts a multitude of effects on most cell types (1-3). Of these, the TGF- $\beta$ 1 isoform is the one most closely associated with immune modulation (4). The numerous biological functions of all TGF- $\beta$ 's require a set of post-translational modifications termed "activation." The bioactive forms of the TGF- $\beta$ 's are 25 kDa homodimers produced from 50 kDa monomers that dimerize to form the ca. 100 kDa TGF- $\beta$  precursor. This dimeric precursor is cleaved intracellularly by furin proteases to yield the 25 kDa active TGF- $\beta$  dimer, which remains associated with the remaining portion of its own pro-form, the latency-associated peptide (LAP, ca. 75 kDa). This complex is termed "latent TGF- $\beta$ ," and is secreted in this form. Other proteins, such as latent TGF- $\beta$  binding proteins (LTBP, which targets TGF- $\beta$ 's to the extracellular matrix) or  $\beta$ 2 macroglobulin (which is associated with circulating TGF- $\beta$ 1) can bind to this complex, creating the so-called large latent complex (5). Latent TGF- $\beta$  is activated by a process that involves dissociation and degradation of LAP by proteins (e.g. plasmin and transglutaminase), heat, chaotropic agents, acid, and oxygen and nitrogen free radicals (1,5-8). This post-translational control of TGF- $\beta$ 1 through activation is arguably the most potent regulatory mechanism for this cytokine (5). Once activated, TGF- $\beta$ 1 binds to its signaling receptor complex (type I, type II, and type III in concert) (9).

A common and commercially-available method for the detection of TGF- $\beta$ 1 consists of an enzyme-linked immunosorbent assay (ELISA), which detects active TGF- $\beta$ 1 (or total TGF- $\beta$ 1 after activation by transient acidification or treatment with urea (10)). In our own previous studies on the activation of latent TGF- $\beta$ 1 by nitrogen free radicals, we had used heat as the positive control for TGF- $\beta$ 1 activation (8,11). This assay is based on the binding of active TGF- $\beta$ 1 by the immobilized specific monoclonal antibody that been pre-coated onto a microplate. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TGF- $\beta$ 1 is added to the wells to sandwich the TGF- $\beta$ 1 immobilized during the first incubation. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of TGF- $\beta$ 1 bound in the initial step. Because of its simplicity, specificity and sensitivity, this method is the best option when working on a cell-free system.

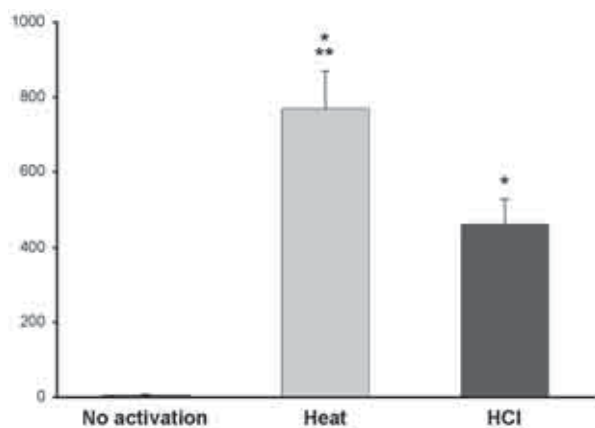
As mentioned above, latent TGF- $\beta$ 1 can be activated by heat, acidification, alkalinization or action of

chaotropic agents *in vitro* (5), but some discrepancies can be found in the literature regarding this process. Using fibroblastic (NRK-49F and AKR-MCA) cell-conditioned medium as a model and radioreceptor and soft agar assays for monitoring activation, it was suggested that conditioned medium may contain at least two different pools of latent TGF $\beta$ 1: one pool resistant to mild acid and/or plasmin that requires strong acid or alkali treatment for activation, and a second pool activated by mild pH change and/or plasmin (12). Similarly, incubations of the latent form of TGF- $\beta$ 1 at extreme pH values, in 0.02% SDS or in 8 M urea, lead to activation of TGF- $\beta$ 1, whereas the complex was resistant to treatment with 5 M NaCl or heat (3 min at 95°C) (13). In contrast, others have reported that thermal activation of native and recombinant latent TGF- $\beta$ 1 occurs over the temperature ranges of 75-100 °C and 65-100 °C, respectively, with complete activation after 5 min at 80°C. Temperatures above 90 °C result in thermal denaturation of TGF- $\beta$ 1 itself (14).

The present study was performed to compare the effectiveness of acidification vs. heat treatment in activating latent TGF- $\beta$ 1 in a cell-free system. The ELISA assay was carried out using recombinant human latent TGF- $\beta$ 1 and the Quantikine® mouse/rat/porcine TGF- $\beta$ 1 Immunoassay Kit, both from R&D Systems, Inc. (Minneapolis, MN). The samples (5000 pg/ml latent TGF- $\beta$ 1 in PBS) were activated by treatment with 1N HCl and then neutralized by 1.2 N NaOH/0.5 mol/L HEPES according to the instructions of the manufacturer, or by incubation at 80°C for 5 min in a water bath. The levels of active TGF- $\beta$ 1 in the samples were calculated by interpolation from the standard curve as per the kit instructions. We found that while both heat and HCl lead to activation of latent TGF- $\beta$ 1 (Figure 1), heat treatment was significantly more effective than acidification (15% vs. nearly 10%, respectively). Interestingly, neither treatment induced full activation. We cannot explain this observation, but it should be noted that R&D Systems is the only commercial supplier of recombinant latent TGF- $\beta$ 1 available, and this company's instructions for the use of Quantikine® Human TGF- $\beta$ 1 Immunoassay (Cat. No. DB100) report 15% cross-reactivity with recombinant human latent TGF- $\beta$ 1.

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**FIGURE 1.** Levels of active TGF- $\beta$ 1 recovered after heat or acid-activation of human recombinant latent TGF- $\beta$ 1. Two samples of recombinant human latent TGF- $\beta$ 1 (5000 pg/ml in PBS, R&D Systems, Cat. No. 299-LT) were heat activated for 5 min at 80°C or acid-activated with 1N HCl. Active TGF- $\beta$ 1 was detected using a Quantikine® kit (R&D Systems, Cat. No. MB100) as per the manufacturer's instruction. Untreated samples in PBS served as negative control (No activation). Results are the mean  $\pm$  SEM of four independent experiments (\* $P$ <0.001 vs. Control, \*\* $P$ <0.05 vs. HCl by  $t$  test). Taken together, our results demonstrate that while both heat and acid treatment differentially activate latent TGF- $\beta$ 1, the former is a more efficient activator of the complex. Since acidification is commonly used as positive control when assaying cell-free samples, our results suggest that the published data reporting absolute values of total and active TGF- $\beta$ 1 based solely on this method should be interpreted cautiously. It is recommended therefore, to use both heat and acidification when estimating the levels of active TGF $\beta$ 1 using the ELISA detection system.

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