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Expression and Receptor Binding Activity of Fusion Protein from Transforming Growth Factor-β1 and GFP

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Abstract A TGF-β1/GFP monomeric fusion protein was cloned from pPK9A and pGFP-C1 plasmid by PCR amplification. The fusion protein was expressed in a Bac-To-Bac™ baculovirus expression system. A 45 kDa fusion protein was purified using an Ni-NTA column with 300 mM imidazol from a cell lysate infected with recombinant viruses for 72 h post-infection. The fusion protein cross-reacted with the commercial TGF-β1 polyclonal Ab as well as Ab raised against a precursor, monomeric TGF-β1, and GFP. The binding activity of the fusion protein with a TGF-β1 receptor was examined. Fluorescence was observed in Mv1Lu cells, yet not in insect cells treated with the fusion protein. No fluorescence was detected in Mv1Lu cells incubated with the fusion protein treated with Ab prior to the binding reaction, or with GFP alone, thereby indicating that the binding of the fusion protein was specific to TGF-β1 with a receptor.

Key words: TGF-β1/GFP fusion protein, baculovirus expression, receptor binding

Transforming growth factor beta (TGF-β) is a multifunctional polypeptide whose diverse functions include the modulation of cellular, physiological, and immunological processes. The vast majority of studies performed to date also indicates that TGF-β is involved in cell growth, differentiation, and the regulation of an extracelluar matrix along with various metabolic effects depending on the cell type and differentiation stages [14, 18, 19].

TGF-β is a member of a large superfamily of functionally and structurally related regulatory proteins, which includes three mammalian isoforms of TGF-β, activin/inhibins, and bone morphogenic proteins (BMP). The proteins of this family mediate many key events in the normal cell growth and development of organisms as diverse as *Drosophila*, *Xenopus*, and mammals. Three mammalian isoforms of TGF-β exist as homodimers linked with one interchain and four intrachain disulfide bonds [19] and are also found as heterodimers in certain cell types. So far, three different human isoforms of TGF-β have been reported (TGF-β1, TGF-β2, and TGF-β3), and TGF-β4 and TGF-β5 have also been identified in chickens and frogs, respectively [14, 19]. Each TGF-β is produced as a large precursor protein, approximately 400 amino acids in length, with an N-terminal signal sequence and mature C-terminal domain of 112 amino acids. The TGF-β precursor proteins undergo a number of processing steps prior to secretion by a cell. The most important processing step is intracellular proteolytic cleavage of the N-terminal sequences. The proteolysis yields two products that assemble into a dimer. The 65–75 kDa dimer protein from the N-terminal region is called a latent associated protein (LAP), while the 25 kDa dimer protein from the C-terminal region is the mature TGF-β (reviewed in [10]). The cleavage is carried out by proteases (plasmin, cathepsin D, glycosidase, and furin), resulting in the activation of TGF-β. The LAP can also be removed by an extreme pH or heating *in vitro* [7, 13, 15]. A comparison of amino acid sequences among mammalian TGF-β1 revealed a remarkably high degree of sequence conservation. Amino acid sequences of mature TGF-β1 protein in human, bovine, and porcine are almost identical, with only one or two amino acid differences in the pro-region [6, 18].

Since TGF-β mediates such an array of cellular and immunological functions, the signal pathways controlled by the TGF-β superfamily is only slowly being elucidated [14, 19]. TGF-β proteins initiate a signal network by assembling receptor complexes that activate Smad transcription factors [14, 21]. The ligand brings together members from two families of receptor serine/threonine kinase, known as type I and type II receptors. A type II receptor activates a type I receptor, which then initiates the signal pathway by the phosphorylation of a Smad protein. Smad proteins are the

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only known TGF-β receptor substrates capable of signal transduction [14, 21]. Smad proteins transduce signals from TGF-β receptors and regulate the transcription of target genes either directly or in combination with other sequence specific transcription factors [21]. It has also been reported that TGF-β1 induces IgA isotype switching both *in vitro* and *in vivo* [18, 19]. Recently, the involvement of Smad proteins in TGF-β1 induced isotype switching has been reported [16]. Hundreds of papers have also been published on the diseases, heritable disorders, and cancers caused by TGF-β itself or TGF-β receptors [14].

Therefore, clinical interest in TGF-β1 as a potential therapeutic agent and as a research tool has grown tremendously. Unfortunately, the supplies of TGF-β1 from natural sources are relatively limited. Only a small amount of TGF-β1 can be extracted from platelets and demineralized bone [1]. Recombinant TGF-β1 has been successfully expressed in Chinese hamster ovary (CHO) cells and partially in insect cells, which is commercially available [2, 8]. Yet these recombinant TGF-β1 proteins are still very expensive, which hampers research on TGF-β1.

Accordingly, to find a method for mass production, attempts have been made to express biologically active TGF-β1 in cells other than mammalian type. A TGF-β1 fusion protein with a collagen binding protein was expressed in *E. coli* in the form of inclusion bodies, but the solubilization with 8 M urea and renaturation made it only partially active [20]. A baculovirus expression system was used to express either a monomer or precursor form of TGF-β1, but these proteins were not biologically active and only cross-reactive with TGF-β1 polyclonal antibodies [12]. Nevertheless, such proteins would be good candidates for making monoclonal antibodies against TGF-β1. The TGF-β1 with a visual marker such as GFP would also be good research tools for the tracing of the protein binding or identifying the functional domain of TGF-β1 [4].

The current paper reports on the expression of a TGF-β1 fusion protein with GFP in a baculovirus system, and the capability of this fusion protein to bind the TGF-β1 receptor.

Materials and Methods

Cloning of TGF-GFP Fusion Protein

The recombinant porcine TGF-β1 gene was from pPK9A [10], which is TGF-β1 cDNA with two point mutations resulting in amino acid changes from Cys to Ser at residues 233 and 225 in the prepeptide region [3]. A monomer form of the TGF-β1 gene was amplified through a PCR using primers containing *Hin*dIII and *Pst*I linkers as described previously [12]. The PCR product was digested by *Hin*dIII and *Pst*I and inserted in pGFP-C1 (CLONETECH Inc., Palo Alto, U.S.A.) in frame. The cloned product was identified using restriction enzyme digestion and the presence of fluorescence in *E. coli*. The expression of the fusion protein was carried out in a baculovirus expression system.

Generation of Recombinant Baculovirus for Expression of TGF-GFP Fusion Protein

A Bac-to-Bac™ (Life Technologies, Inc., U.S.A.) expression system was used for the expression of the TGF-β1/GFP fusion protein. The fusion protein gene was amplified from pGFP-TGF through a PCR using primers containing *Eco*RI and *Pst*I linkers. The subcloning and generation of the recombinant virus was carried out according to the manufacturer's manual and as described previously [12]. Briefly, the recombinant bacmid DNA was purified and transfected in *Spodoptera frugiferda* (Sf-9) cells using Lipofectin (Life Technologies, Inc., U.S.A.). The cells were infected at 27°C for 5 h, and incubation was then continued for 72 h. The recombinant plaques were identified visually by the absence of occlusion bodies and the presence of fluorescence under longwave UV-light. The cells were harvested and the recombinant virus was purified with three rounds of plaque isolation. The virus titer was determined with a plaque assay and amplified up to 10^8–10^9 pfu/ml using serial infection in a liquid culture of insect cells as necessary.

Identification of Recombinant Protein

Monolayers of Sf-9 cells were infected with the recombinant virus at m.o.i of 10 in 75 cm^2 tissue culture flasks containing 9×10^6 cells/flask or liquid culture flasks containing $1-2\times10^6$ cells/ml. For a time course analysis, the cells were harvested at various intervals up to 120 h, and lysed with lysis buffer I (2% SDS, 62.5 mM Tris-HCl, pH 6.8), and the total proteins were analyzed by 12% SDS-PAGE, as described previously [12].

Purification of Fusion Protein

The cells infected with the recombinant virus were lysed with lysis buffer II (8 M Gu-HCl, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl, pH 8.0, 1% Tween 20, 30 mM imidazole) and any cell debris was removed by centrifugation at 15,000 rpm for 15 min. The recombinant protein was purified using a batch purification method with an Ni-NTA agarose column. Briefly, the cell lysates were equilibriated with an Ni-NTA agarose for 1 h at room temperature with occasional shaking. The column was packed in a 1 ml syringe, and washed twice with 5 ml of a washing buffer (8 M urea, 0.1 M NaH_2PO_4, Tris-HCl, pH 8.0, 30 mM imidazole) and 5 ml of the same buffer at pH 6.3. The recombinant fusion protein was then eluted with a washing buffer (pH 6.3) containing different concentrations of imidazole up to 500 mM.

Generation of Antiserum Against Fusion Protein

To produce a TGF-β1/GFP fusion protein antibody, about 100 μg and 50 μg of protein were injected four times into

two rabbits and two mice, respectively. The serum was collected and tested for Ab production using ELISA and a Western blot analysis.

Western Blot

The cell lysates were separated, as described above, and the proteins were then transferred to a nitrocellulose membrane. The blots were blocked by immersion in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 2.5% BSA for 1 h and immunological detection was carried out with a TGF polyclonal antibody and GFP antibody as described previously [12], using alkaline phosphatase and ECL™.

Receptor Binding Experiment of Fusion Protein

To test the receptor binding activity of the fusion protein, Mv1Lu cells, the standard cell line for testing the biological activity of TGF-β1, were used along with Sf9 cells, which have no TGF-β1 receptors, as the negative control. Thus, the Mv1Lu cells were attached in a 6-well plate containing 0.5% FBS-DMEM for 20 h at 37°C, while the Sf9 cells were attached with SFM at 27°C. About 30 μg of the fusion protein was added to both plates, which were then incubated for 1 h with slow shaking. The culture supernatant was removed and the plates were washed twice with PBS. Both cells were harvested and fixed with 3% paraformaldehyde for 30 min on a glass slide. The presence of fluorescence was examined using a fluorescent microscope (Polyvar, Cambridge Inst. Co., U.S.A.).

RESULTS

Construction and Recovery of Recombinant Virus Expressing TGF-β1/GFP Fusion Protein

To construct a gene encoding the porcine TGF-β1/GFP fusion protein, a monomer form of the TGF-β1 gene from pPK9A was amplified with a primer containing *Hin*dIII and *Pst*I sites. The PCR product was digested and inserted into pGFP-C1. The TGF-β1/GFP fragment was then amplified again with a primer containing *Eco*R1 and *Pst*I sites. The PCR product was digested and inserted into a transfer vector of the baculovirus expression system, pFBa. The cloned gene was then identified using restriction enzyme digestion and a PCR. The transfer vector was transformed to DH10Bac to generate a recombinant bacmid. The bacmid DNA was isolated and transfected to a Sf-9 cell using Lipofectin. The recombinant baculovirus plaque was isolated and the TGF-β1 gene and GFP expression were confirmed by a PCR and fluorescent microscopy, respectively (Fig. 1). The cells infected with the recombinant viruses exhibited a diffuse green fluorescence throughout the cell, as expected for soluble GFP, which is a good candidate as a visual marker for the TGF-β1 monomer.

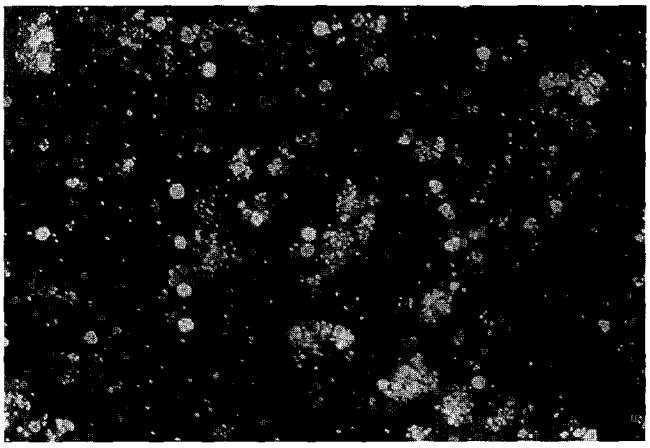

Fig. 1. Fluorescence micrographs of fusion protein in Sf9 cells.
Sf9 cells infected with the recombinant virus were photographed using a UV excitation filter (475–495 nm), dichroic mirror (510 nm), and barrier filter (520–560 nm), magnification ×100.

Expression of TGF-β1/GFP Fusion Protein

The recombinant virus was infected in Sf-9 cells grown in a flask culture at an m.o.i. of 10. The cells were harvested 24, 48, 72, 96, and 120 h post-infection. The total protein in the cell lysates was analyzed on 12% PAGE. A 42 kDa fusion protein band, corresponding to a TGF-β1 monomer and GFP, was detected 48 and 72 h post-infection, while the amount of protein decreased (Fig. 2). Since the fusion protein contained the GFP protein as a visual marker, the intensity of the fluorescence was the highest 72 h post-infection, indicating that 72 h was the optimal infection time (data not shown).

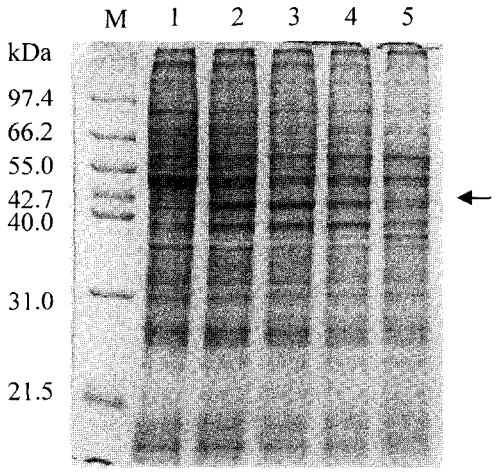

Fig. 2. Time course expression of fusion protein in the Baculovirus expression system.
Sf9 cells were infected with the recombinant virus at m.o.i. 10. The total proteins were analyzed by 12% SDS-PAGE at various periods post-infection. **1**, 24 h post-infection; **2**, 48 h post-infection; **3**, 72 h post-infection; **4**, 96 h post-infection; **5**, 120 h post-infection.

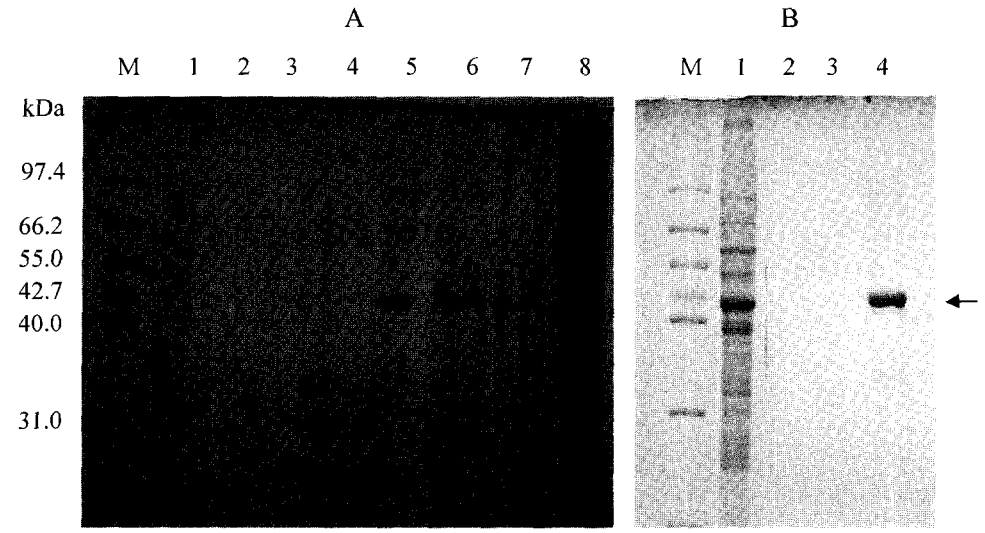

Fig. 3. Purification of fusion protein by an Ni-NTA affinity column chromatography.
A. The fusion protein was eluted using an Ni-NTA column with a linear concentration of imidazole ranging from 100 mM to 500 mM. 1, unbound; 2, washing fraction; 3, 100 mM; 4, 200 mM; 5, 300 mM; 6, 400 mM; 7, 500 mM; 8, cells infected with recombinant virus. B. The fusion protein was eluted with 300 mM of imidazole. 1, cells infected with recombinant virus; 2, washing fraction a; 3, washing fraction b; 4, elution fraction with 300 mM imidazole.

Purification of Fusion Protein

Since the expression system contained 6x His tag at the N-terminus, the fusion protein was purified using an Ni-NTA column. Thus, a cell lysate of 72 h post-infection was incubated with Ni-NTA agarose and the fusion protein was eluted with different concentrations of imidazole, followed by an analysis of each fraction using 12% PAGE. Most of the fusion protein was eluted with 300 mM of imidazole, and no protein was detected in the washing fraction with either lower or higher concentration of imidazole (Fig. 4). Only a 42 kDa fusion protein was clearly visible in Coomassie stained gel (panel A), and the fusion protein was found to react with TGF-β1 polyclonal antibody (panel B).

Antibody Production and Western Blot Analysis

To identify immunological cross-reactivity of the fusion protein, Western blot was carried out using Ab raised against the TGF-β1 monomer, precursor [12], and GFP/TGF-β1 fusion protein with rabbit and mouse antisera. Due to strong immunogenicity of GFP, the fusion protein

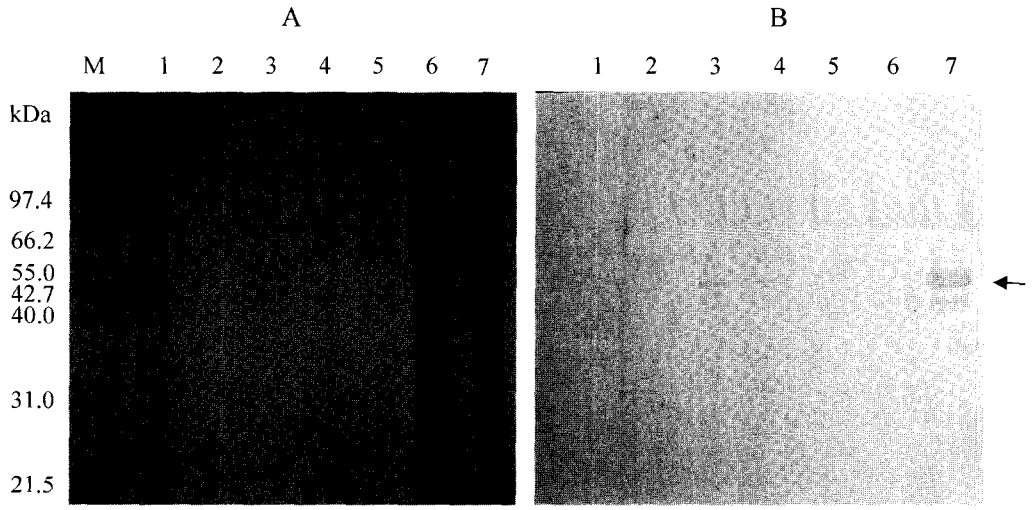

Fig. 4. Immunoblot analysis of recombinant fusion protein.
The purified protein and total proteins of the cells infected with the recombinant virus were analyzed 72 h post-infection using 12% SDS-PAGE and stained with (A) Coomassie blue. (B) The proteins were transferred to a nitrocellulose membrane and reacted with a TGF-β1 polyclonal antibody. 1, unbound; 2, wash; 3, 300 mM imidazole; 4, 400 mM imidazole; 5, 500 mM imidazole; 6, cells infected with the wild-type virus; 7, cells infected with the recombinant virus.

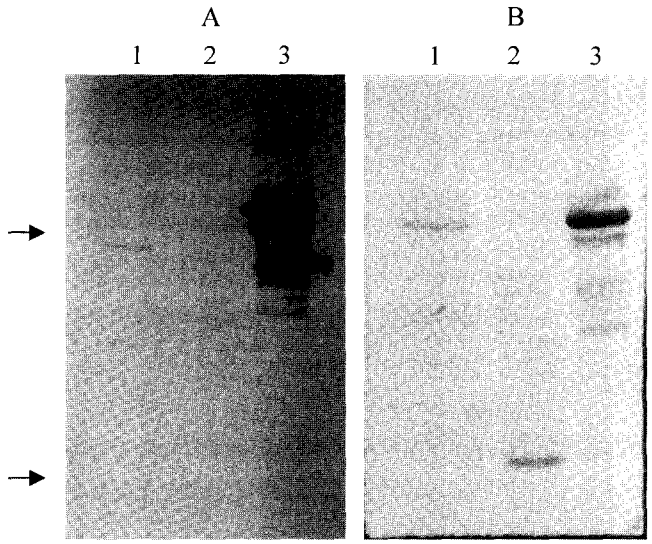

Fig. 5. Immunoblot of precursor, monomer, and fusion protein with immunized serum.
Rabbit serum (A) and mouse serum (B) immunized with fusion protein. 1, TGF-β1 precursor; 2, TGF-β1 monomer; 3, GFP-TGF-β1 monomer fusion protein.

exhibited much better reaction than the TGF-β1 protein alone (Fig. 5). When comparing the antiserum from the two different animal sources, the mouse antiserum exhibited stronger reactivity than the rabbit antiserum.

Receptor Binding Assay of Fusion Protein

Since the fusion protein contained GFP at the C-terminus and the TGF-β1 monomer protein at the N-terminus, the fusion protein could be used as a visual marker for the TGF-β1 receptor. As such, the binding of the fusion protein to TGF-receptor was examined. The fusion protein was purified using an Ni-NTA column and incubated with Mv1Lu and Sf-9 cells for 1 h, followed by excessive washing. Both cell types were examined using a fluorescent microscope. The fluorescence caused by GFP was clearly visible in the Mv1Lu cells (Fig. 6), yet no signal was detected in the insect cells (data not shown), indicating that the fusion protein did bind with a TGF-β1 receptor. When the polyclonal Ab raised against the TGF-β1 monomer was treated with the fusion protein prior to a binding assay or simply after treatment with GFP alone, no fluorescence was detected (data not shown), indicating that the binding of the TGF-β1/GFP fusion protein was receptor specific.

Fig. 6. Fluorescence micrographs of Mv1Lu cells incubated with fusion protein (magnification ×100).
The cells were photographed using a UV excitation filter (475–496 nm), dichromic mirror (510 nm), and barrier filter (520–560 nm).

DISCUSSION

TGF-β1 is an immune modulating protein that can control growth and differentiation. It is also a bifunctional growth regulator, inhibiting the cell growth of most cell types while stimulating the proliferation of others. The isoforms in mammalian species, including TGF-β1, TGF-β2, and TGF-β3, exhibit very high sequence homologies and have similar biological activities. Most researches on the TGF-β1 have been focused on the activation mechanism of precursor proteins, the biological activities, and the human diseases caused by the TGF-β1 and its receptors. The active form of TGF-β1 is the homodimeric form of a 25 kDa monomer linked with one interchain and four intrachain disulfide bonds. The two most important functions of TGF-β1 are binding to type I receptors and the recruitment of type II receptor, which initiate signal transduction via a complicated network of Smad proteins. The functional domains of TGF-β1 are not yet clearly determined. However, it has been reported that the tetrapeptide of WSLD (aa 52 to 55) would appear to be the receptor binding motif of TGF-β1 when using the peptide antibody. Treatment with this antibody completely blocked both ^{125}I-TGF-β1 binding to its receptor and TGF-β1 induced growth inhibition in Mv1Lu cells [9]. A site-directed mutagenesis analysis also revealed that the replacement of Trp^{52} and Asp^{55} by alanine residues diminished the growth inhibitory activity of TGF-β1 by 90%.

The current authors previously attempted to express biologically active TGF-β1 in a system other than a mammalian expression system. When TGF-β1 was expressed in *E. coli*, this only resulted in a biologically inactive and insoluble form [5]. When a baculovirus system was used, the protein exhibited no biological activity, while the recombinant protein was immunologically cross-reactive with TGF-β1 polyclonal antibodies. In the current study, the GFP/TGF-β1 chimeric protein was expressed in a baculovirus system and the binding activity to a receptor was examined. The active form of TGF-β1 had a homodimeric structure linked with five disulfide bonds. However, the

fusion protein contained a monomeric subunit, indicating that the monomeric form of TGF-β1 would bind to a receptor. Therefore, the fusion protein has potential as a visual marker for determining a TGF-β binding site to a receptor. Furthermore, the protein can also be used as a competitive molecule for the biological activity of the TGF-β1. Even though the fusion protein appeared to have no biological activity, it was cross-reactive with a TGF-β1 polyclonal antibody, and may be a good candidate for the production of a TGF-β1 monoclonal antibody. Using a truncated form of the fusion protein expressed in insect cells, a study to determine the binding domain of TGF-β1 is currently in progress.

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