Use of FlashPlate Technology for 

*In Vitro* Measurement of $^{125}$I-Labeled 

TGF-β1 Binding on Chimeric 

Extracellular Domain of Type II 

Transforming Growth Factor Receptor 

*Sylviane Komesli, Martine Page, and Patrick Dutartre* 

*Département d’Immunologie, Laboratoires Fournier S.A., France*
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Abstract

Transforming growth factor-β (TGF-β) receptors are transmembrane proteins that play a crucial role in the regulation of cell growth, differentiation, and apoptosis. The TGF-β family includes at least three members: TGF-β1, TGF-β2, and TGF-β3, which are involved in various physiological processes. The TGF-β receptors are complex signaling proteins that consist of type I and type II transmembrane receptors. The type II receptor (TβRII) is responsible for binding TGF-β and activating the type I receptor (TβRI), which then initiates the signaling cascade through Smad proteins.

In the present study, we investigated the feasibility of using the FlashPlate platform to assay a chimeric soluble type II receptor fused with the Fc regions of human immunoglobulin (TβRII-Fc), in order to screen for potent agonists and antagonists of TGF-β.

Introduction

Transforming growth factors β1, β2 and β3 (TGF-βs) are multifunctional cytokines involved in the regulation of cell proliferation, differentiation and extracellular matrix production. In mammalian cells, responses to TGF-β are mediated by types I and II cell surface receptors (TβRI and TβRII respectively) which are expressed in most cell types and tissues. TGF-β binds directly to TβRII, allowing this receptor to associate with and phosphorylate TβRI, which then propagates the signal through activation of Smad2 and Smad4 heteromeric complexes1.

At this time, there are no known, clinically useful TGF-β agonists or antagonists. A better understanding of the mechanism of activation of the TGF-β receptor complexes may be useful, therefore, as a step towards the development of TGF-β antagonist or agonist drugs. We have investigated the possibility of using the soluble extracellular domain of TβRII in a nonseparation microplate receptor binding assay.

To facilitate this approach and increase the probability of success, we constructed a chimeric soluble receptor, by fusing the extracellular domain of TβRII to the Fc regions of human immunoglobulin (TβRII-Fc). Through the Fc region, the chimeric receptor, TβRII-Fc, expressed in a transiently transfected Cos-7 cell line, was easily purified by one-step protein A affinity chromatography, then coated with high efficiency into the wells of a Protein A FlashPlate microplate (SMP102). This protein was biochemically characterized; then its ability to bind ¹²⁵I-labeled TGF-β1 was studied2.

Methods

Construction of vector: The cDNA encoding the extracellular domain of the human TGF-β type II receptor (TβRII) was amplified by PCR from the plasmid, which contained a full-length cDNA of the receptor. The resulting amplified PCR product was digested and ligated into the cloning sites of the vector pIg-Tail (R&D System).

Transient transfection of Cos-7 cells: Cos-7 cells (American Type Culture Collection CRL 1651) were transiently transfected with the expression plasmid pIg-Tail containing the cDNA encoding for the truncated TGF-β type II receptor. The recombinant protein was expressed in the transfected cells and secreted into the medium.

Protein purification and analysis: The human recombinant protein TβRII-Fc was purified by one-step protein A affinity chromatography. The eluted protein was dialyzed overnight against 0.1 x PBS and lyophilized.

Protein A FlashPlate binding assay: Protein A FlashPlate microplates are precoated with protein A. This was allowed to immobilize the chimeric protein (100 µl of affinity purified protein at 2.5 µg/ml in pH 7.2 NaCl/Pi) by the Fc portion for two hours at room temperature. After 2 washes with binding buffer (128 mM NaCl; 5 mM KCl; 5 mM MgSO₄; 1.3 mM CaCl₂; 50 mM Hepes, pH 7.6), the wells were treated for two hours at room temperature with blocking solution consisting of binding buffer containing 5% BSA. After incubation, the wells were washed three times with binding buffer containing 1% BSA.
For the binding studies, \(^{125}\text{I}\)-labeled TGF-β1 (Specific activity 3,000-4,500 Ci/m mole, NEX267) was diluted in binding buffer just prior to its addition into the wells. The final concentrations of \(^{125}\text{I}\)-labeled TGF-β1 ranged from 50 pM to 2.5 nM. The plates were incubated at room temperature for two hours, then sealed and counted on a Packard Top Count® Microplate Scintillation Counter. A one minute counting period was used. All determinations were carried out at least in duplicate. Nonspecific binding was determined with an excess of unlabeled TGF-β1 (100-fold). The counting efficiency was determined through the use of \(^{125}\text{I}\)-labeled IgG directly coated into Protein A FlashPlates. The cpm obtained at equilibrium, divided by the dpm added to the well, gave a measure of the counting efficiency, which was evaluated at 5%. For the competition binding assay, the final concentration of radiolabeled ligand was 500 pM (specific activity 300-450 Ci/m mole), with final concentrations ranging from 0.05 to 200 nM for TGF-β1, TGF-β2 and TGF-β3.

**Binding of \(^{125}\text{I}\)-labeled TGF-β1 to cell:** Ligand binding of cell monolayers with \(^{125}\text{I}\)-labeled TGF-β1 (20 pM to 1 nM) were carried out as previously described, then scatchard plotted with values obtained after gamma scintillation counting. Scatchard experiments were performed on Cos-1 cells (American Type Culture Collection CRL1650) transiently transfected either with the TβRII (pCMV5 TβRII), or with both TβRI and TβRII containing plasmids (pCMV5 TβRI + pCMV5 TβRII), and compared to Cos-1 cells transfected with the empty pCMV5 (Invitrogen) in order to measure the nonspecific binding.

### Results

**Comparative studies on binding of \(^{125}\text{I}\)-labeled TGF-β1 to human TβRIIs-Fc and wild type TβRII:** Ligand binding activity of the recombinant hTβRIIs-Fc receptor was tested in a Protein A FlashPlate binding assay. Protein A precoated plastic surfaces within the wells were coated with the Fc portion of the affinity purified recombinant chimeric receptor. The background level was measured in non-coated wells of Protein A FlashPlate, and shown to represent 50% of the nonspecific binding obtained upon TβRIIs-Fc coating. The soluble chimeric receptor secreted by Cos-7 cells was able to effectively bind \(^{125}\text{I}\)-labeled TGF-β1 (Figure 1). The Protein A FlashPlate assay showed a typical saturation curve of TGF-β1 binding to hTβRIIs-Fc (Figure 2). The kinetics of \(^{125}\text{I}\)-labeled TGF-β1 binding, performed at room temperature, were relatively rapid, since equilibrium binding was usually achieved after just two to three hours of incubation.

**Figure 1: Binding Curves**

**Total and nonspecific binding of \(^{125}\text{I}\)-labeled TGF-β1 to recombinant soluble chimeric hTβRIIs-Fc.**

The recombinant hTβRIIs-Fc from transfected Cos-7 cell line was tested using a Protein A FlashPlate binding assay in the presence of increasing concentrations of \(^{125}\text{I}\) TGF-β1.
Equilibrium binding of 125I-labeled TGF-ß1 to recombinant soluble chimeric hTßRIIs-Fc and to cell-surface transfected receptor complex.

Fig. 2a: The specific binding curve.
Fig. 2b: Scatchard analysis of the binding data in (2a).
Figs. 3a and 4a: Specific binding curve obtained from Cos-1 transfected with either the cDNA of TßRII alone or in association with the cDNA of TßRI respectively.
Figs. 3b and 4b: Scatchard analysis of the binding data obtained in (3a) and (4a).

Data in Figures 2a, 3a, and 4a are from representative experiments for which the calculated $K_d$ was $1370 \pm 363$ pM, $1123 \pm 413$ pM and $470 \pm 32$ pM respectively. The $K_d$ values were determined with GraphPad Prism 2.0 program.
Meaningful values for binding affinity constants could be derived from the obtained binding data. Scatchard analysis (Figure 2b) revealed a single class of binding sites giving a $K_d$ value of $1370 \pm 363$ pM. The data obtained from the scatchard analysis indicated that approximately 180-200 fmoles of recombinant hTßRIIs-Fc were immobilized in each coated well.

In order to understand the relevance of our results, we compared the $K_d$ value of the hTßRIIs-Fc for TGF-ß1 with the $K_d$ value obtained with recombinant hTßRII expressed in the Cos-1 cell line. Previous data showed that hTßRI was able to enhance the affinity of complexes formed with hTßRII in comparison to TßRII alone. Here, we have investigated the affinity of Cos-1 cells expressing TßRII alone, or expressing both TßRI and TßRII. As shown in Figures 3 and 4, although the relative affinity of Cos-1 cells for $^{125}$I-labeled TGF-ß1 was low ($K_d$ 1123 $\pm$ 413 pM) when TßRII was expressed alone (Figures 3a and 3b), co-expression of both TßRI and TßRII (Figures 4a and 4b) converted the system to a higher affinity model (470 $\pm$ 32 pM).

**Differential binding affinity of TGF-ß isoforms for soluble chimeric receptor TßRIIs-Fc:**

It has previously been shown that TGF-ß1 and TGF-ß3 bind with much higher affinities than TGF-ß2 to TßRII. To identify the selectivity of hTßRIIs-Fc, TGF-ß isoforms were compared in competition experiments with $^{125}$I-labeled TGF-ß1 binding in the Protein A FlashPlate assay, where $^{125}$I-labeled TGF-ß1 (final concentration 500 pM) was mixed with serial dilutions of recombinant TGF-ß isoforms. Figure 5 shows the displacement curve of three different TGF-ß isoforms: TGF-ß1, TGF-ß2, and TGF-ß3. The relative inhibitory concentration ($IC_{50}$) was determined as the TGF-ß concentration which inhibited 50% of $^{125}$I-labeled TGF-ß1 binding. The $IC_{50}$ values obtained in this test were $3.3 \pm 0.06$ nM and $3.9 \pm 0.09$ nM for TGF-ß1 and TGF-ß3, respectively, whereas TGF-ß2 was unable to compete, even at a concentration of 200 nM, with the binding of 500 pM of $^{125}$I-labeled TGF-ß1 to recombinant TßRIIs-Fc. These results indicated that the selectivity for binding TGF-ß isoforms was maintained by this chimeric recombinant receptor.

**Discussion**

In this study, we have analyzed the binding properties of recombinant hTßRIIs-Fc for TGF-ß1 using FlashPlate technology. For our experiments, the hTßRIIs-Fc component was coated onto the wells of Protein A FlashPlates and incubated with $^{125}$I-labeled TGF-ß1. The key feature of this Protein A FlashPlate binding assay, as described here, is its simplicity – comprising only the addition of the $^{125}$I-labeled TGF-ß1, incubation, and measurement. Unlike separation assays, this assay does not disturb the equilibrium, while the CPM obtained are directly correlated to the bound $^{125}$I-labeled TGF-ß1. In our assay, we have determined a counting efficiency of about 5%; this result may be explained by the increased distance between $^{125}$I-labeled TGF-ß1 and solid scintillant, due to the coating of protein A and hTßRIIs-Fc on the wells of the FlashPlate. Nevertheless, despite the low counting efficiency obtained, the assay is accurate and its correlation with a conventional approach suggests that it is suitable for hTßRIIs-Fc binding studies.
Moreover, we compared the affinity of hTßRIIs-Fc (1370 \pm 363 pM) to the affinity of Cos-1 cells expressing either TßRII alone (1123 \pm 413 pM), or both TßRI and TßRII (470 \pm 32 pM). In these experiments, the affinity of the chimeric soluble receptor for TGF-ß1 was threefold lower than the affinity of the heterodimer receptor complex (TßRI and TßRII) on the cell surface, but was very similar to that of TßRII when expressed alone in the Cos-1 cell line.

One significant result is that the chimeric protein shows the same selectivity for different TGF-ß isoforms as the native protein. TGF-ß2 is not able to compete with the binding of 125I-labeled TGF-ß1, although TGF-ß1 and TGF-ß3 both show an IC_{50} value in the 3-4 nM range. Previous data have shown that TßRII binds TGF-ß2 with low affinity, and co-expression of TßRIII is required.

In conclusion, in vitro binding assays demonstrate that hTßRIIs-Fc is able to bind TGF-ß1 with a similar affinity to that of wild type hTßRII when overexpressed alone in Cos-1 cells, and can be used accordingly in Protein A FlashPlate microplates to search for TGF-ß agonist and antagonist compounds.

References


Acknowledgments

Most of these results have been published (Komesli et al. 1998 Chimeric extracellular domain of type II transforming growth factor (TGF)-ß receptor fused to the Fc region of human immunoglobulin as a TGF-ß antagonist. Eur. J. Bioch. 234, 505-513). We thank European Journal of Biochemistry for giving us the permission to reproduce published material.