Activated Thyroglobulin Possesses a Transforming Growth Factor-β Activity*

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Thyroglobulin (Tg), the thyroid hormone precursor, is a major protein component in the thyroid gland and may have other important functions. Here, we show that bovine Tg inhibited 125I-labeled transforming growth factor-β1 (125I-TGF-β1) binding to cell-surface TGF-β receptors in mink lung epithelial cells with an IC50 of ~300 nM. After disuccinimidyl suberate (DSS) modification, reduction/alkylation, treatment with 8 M urea, 0.1% SDS, or acidic pH (pH 4–5), Tg exhibited a ~5–10-fold increase of 125I-TGF-β1 binding inhibitory activity with IC50 of ~30–60 nM. This inhibitory activity was an intrinsic property of the Tg and could not be segregated from Tg protein by 5% SDS-polyacrylamide gel electrophoresis or by immunoprecipitation using antisera to Tg. Untreated Tg did not affect DNA synthesis but blocked the TGF-β-induced inhibition of DNA synthesis in mink lung epithelial cells. After DSS activation, Tg possessed TGF-β agonist activity and inhibited DNA synthesis of mink lung epithelial cells and rat thyroid cells. The activated Tg also exerted a small but significant TGF-β antagonist activity in transcriptional activation of plasminogen activator inhibitor-1. These results suggest that Tg possesses an authentic TGF-β activity which can be induced by chemical modifications and treatments with denaturing agents and acidic pH.

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The abbreviations used are: Tg, thyroglobulin; TGF-β, transforming growth factor-β; DSS, disuccinimidyl suberate; PAI-1, plasminogen activator inhibitor-1; α2M, α2-macroglobulin; PBS, phosphate-buffered saline; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; IGFBP, insulin-like growth factor-binding protein.

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TGF-β gels were then incubated with 0.2 ml of 0.2M NH₄HCO₃, pH 7.8, at 4 °C. The extracts were analyzed for 125I-TGF-β activity using mink lung epithelial cells. Mink lung epithelial cells have been used as a model system to examine TGF-β receptor binding activities (15, 16). As shown in Fig. 1A, untreated and DSS-activated Tg completely inhibited 125I-TGF-β binding to cell-surface TGF-β receptors with IC₅₀ of ~300 and ~30 nM, respectively. The analysis by 125I-TGF-β₁ affinity labeling of cell-surface TGF-β receptors revealed that DSS-activated Tg at 0.1 μM almost completely inhibited 125I-TGF-β binding to cell-surface receptors, including types I, II, and V TGF-β receptors (Fig. 1B, lane 1 versus lane 2).

To test the possibility that untreated and DSS-activated Tg might inhibit 125I-TGF-β₁ binding to cell-surface TGF-β receptors by directly binding 125I-TGF-β₁, we investigated the interaction of both proteins with 125I-TGF-β₁ using a 5% native polyacrylamide gel electrophoresis system (10). This gel system has been used to analyze the complex formation of 125I-TGF-β and high molecular weight binding proteins, e.g. α₂M (9). In this system, 125I-TGF-β₁-binding protein complex co-migrates with the binding protein toward the anode as free 125I-TGF-β₁ migrates to the cathode (9). As shown in Fig. 2, no 125I-TGF-β₁ radioactivity associated with untreated and DSS-activated Tg. (Fig. 2, A, lanes 1 and 2, and B, lanes 5 and 6). DSS-activated Tg migrated faster than untreated Tg toward the anode due to decrease of its positive charges after modification of α₂ and ε-amino groups by DSS (Fig. 2B, lane 5 versus lane 6). As a positive control, 125I-TGF-β₁ associated and co-migrated with α₂M (Fig. 2, A, lane 3, and B, lane 8).

The inability of untreated or DSS-activated Tg to form a complex with 125I-TGF-β₁ was further confirmed by the observation that untreated and DSS-activated Tg did not affect the chromatographic migration of 125I-TGF-β₁ on Sepharose 4B (data not shown). Tg has been shown to bind to an N-acetylglu-
cosamine (GlcNAc) receptor (17), mannose 6-phosphate/insulin-like growth factor-II receptor (18), and megalin (19). However, inhibitors of this receptor binding including mannnose 6-phosphate (5 mM), EDTA (5 mM), and lactoferrin (10 μM) failed to have a significant effect on the $^{125}\text{I}$-TGF-β binding inhibitory activities of untreated and activated Tg (data not shown). These results suggest that untreated and activated Tg inhibit $^{125}\text{I}$-TGF-β binding by direct interaction with cell-surface TGF-β receptors but not by sequestering $^{125}\text{I}$-TGF-β from binding to the TGF-β receptors or by interaction with other known Tg-binding receptors.

**Authenticity of $^{125}\text{I}$-TGF-β, Binding Inhibitory Activity of Tg—**TGF-β has been identified, primarily in a latent form, in platelets, tissues, and cultured cells (20–22). We considered the possibility that the preparations of untreated and DSS-activated Tg were contaminated with active TGF-β. To exclude this possibility, both preparations of untreated and DSS-activated Tg were subjected to 5% SDS-polyacrylamide gel electrophoresis under non-reducing conditions, eluted from the SDS-polyacrylamide gel, and analyzed for $^{125}\text{I}$-TGF-β binding inhibitory activity. As shown in Fig. 3, the $^{125}\text{I}$-TGF-β binding inhibitory activity was only detected where untreated and DSS-activated Tg migrated (close to the top of the separating gel, fraction 1). In this gel system, TGF-β migrated at the dye front because of its small molecular size (25 kDa). To further prove that the $^{125}\text{I}$-TGF-β binding inhibitory activity is an inherent property of DSS-activated Tg, we determined the effect of immunoprecipitation with antiserum to Tg on the activity of DSS-activated Tg. As shown in Table I, immunoprecipitation using antiserum to Tg but not non-immune serum completely depleted the $^{125}\text{I}$-TGF-β binding inhibitory activity from the activated Tg solution. These results suggest that the $^{125}\text{I}$-TGF-β binding activities of untreated and activated Tg are authentic.

**Activation of Tg by Chemical Modifications and Treatments with Denaturing Agents and Acidic pH—** As described previously, after modification of its α-amino and ε-amino groups with DSS, the $^{125}\text{I}$-TGF-β binding inhibitory activity of Tg was activated ~10-fold (IC$_{50}$ ~30 nM versus IC$_{50}$ ~300 nM prior to modification). This activation may result from a conformational change of Tg following modification by DSS. To test this possibility, we determined the effects of reduction and alkylation treatments with 8 M urea and 0.1% SDS on the $^{125}\text{I}$-TGF-β binding inhibitory activity of Tg. As shown in Table II, Tg treated by 8 M urea and 0.1% SDS exhibited $^{125}\text{I}$-TGF-β binding inhibitory activities with IC$_{50}$ similar to that of DSS-activated Tg (Table II). Interestingly, reduction and alkylation also activated the $^{125}\text{I}$-TGF-β binding inhibitory activity of Tg (Table II). These results suggest that Tg can be activated by chemical modifications and treatments with denaturing agents. The activation of Tg by reduction and alkylation excludes the possibility that the $^{125}\text{I}$-TGF-β binding inhibitory activity of Tg might be contributed by contamination with TGF-β and/or latent TGF-β in the Tg preparations. Modification by reduction and alkylation is known to abolish the activity of TGF-β (20–22).

To investigate the physiological relevance of the activation of Tg by denaturing agents, we examined the effect of acidic pH treatment on the $^{125}\text{I}$-TGF-β binding inhibitory activity of Tg. Acidic pH, like denaturing agents, may induce a conformational change of Tg. Furthermore, the acidic pH effect may be physiologically relevant since Tg encounters acidic intracellular compartments during its secretion, uptake, and recycling by
thyroid cells (23). As shown in Fig. 4, Tg was activated in a pH-dependent manner; treatment at decreasing pH enhanced Tg activity. The IC50 of Tg treated with 0.1 M Tris acetate, pH 7.4, 0.1 M acetate, pH 6.0, 0.1 M acetate, pH 5.0, and 0.1 M acetate, pH 4.2 for 4 °C for 16 h. The inhibitory activity of treated Tg was assayed by incubating mink lung epithelial cells with 0.1 nM 125I-TGF-β1 and 0.025, 0.05, and 0.1 μM DSS-activated Tg. The RNA analysis of PAI-1 mRNA were estimated based on the ratio of PAI-1 mRNA and G3PDH mRNA using PhosphorImager.

Fig. 4. Acidic pH-dependent activation of Tg. Tg was treated with 0.1 M Tris acetate, pH 7.4, 0.1 M acetate, pH 6.0, 0.1 M acetate, pH 5.0, and 0.1 M acetate, pH 4.2 for 4 °C for 16 h. The binding inhibitory activity of treated Tg was assayed by incubating mink lung epithelial cells with 0.1 nm 125I-TGF-β1 and various concentrations of treated Tg as indicated. The specific binding of 125I-TGF-β1 obtained in the absence of treated Tg was taken as 0% inhibition (5,634 ± 120 cpm/well). The error bars indicate means ± S.D. of triplicate cultures.

Fig. 5. DSS-activated Tg blocking of DNA synthesis (A), untreated Tg blocking of TGF-β-induced DNA synthesis (B), and DSS-activated Tg-induced PAI-1 expression (C) in mink lung epithelial cells. A, cells were incubated with various concentration of DSS-activated Tg at 37 °C for 16 h. The [methyl-3H]thymidine incorporated into cellular DNA was then determined. The [methyl-3H]thymidine incorporation in the presence and absence of 10 pm TGF-β1 were taken as 100% and 0% inhibition (1,459 ± 349 and 24,324 ± 1,597 cpm/well). B, cells were incubated with 0, 0.025, 0.125, 0.25, 1.25, and 2.5 pm TGF-β1 in the presence and absence of 2.5 pm TGF-β1 was taken as 100% and 0% inhibition (2,429 ± 400 and 25,329 ± 2,459 cpm/well, respectively). C, cells were incubated with TGF-β1 (0 and 0.5 pm) or 0.025, 0.05 and 0.1 μM DSS-activated Tg. The RNA analysis of PAI-1 and G3PDH were performed using specific probes. The relative levels of PAI-1 mRNA were estimated based on the ratio of PAI-1 mRNA and G3PDH mRNA using PhosphorImager.

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**TABLE I**

| Treatment | 125I-TGF-β1 binding | Inhibition |
|-----------|---------------------|-----------|
| Control   | 250 ± 40 (n=4)      |           |
| + DSS-activated Tg treated with non-immune IgG affinity gel | 40 ± 10 (n=4) |           |
| + DSS-activated Tg treated with immune IgG (anti-Tg IgG) affinity gel | 35 ± 15 (n=4) |           |
| + Reduction/alkylation | 72 ± 11 (n=4) |           |
| + DSS modification | 25 ± 12 (n=4) |           |

**TABLE II**

| Treatment | 125I-TGF-β1 binding inhibitory activity (IC50) |
|-----------|---------------------------------------------|
| Control   | 250 ± 40 (n=4) |
| + 0.1% SDS | 40 ± 10 (n=4) |
| + 8 M urea | 35 ± 15 (n=4) |
| + Reduction/alkylation | 72 ± 11 (n=4) |
| + DSS modification | 25 ± 12 (n=4) |

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Cetamide. After reduction and alkylation, Tg was dialyzed against PBS. The specific binding of 125I-TGF-β1 with 0.1 M Tris acetate, pH 7.4, 0.1 M acetate, pH 6.0, 0.1 M acetate, pH 5.0, and 0.1 M acetate, pH 4.2 were estimated to be 250 ± 50 nm (n = 4), 100 ± 20 nm (n = 4), 36 ± 6 nm (n = 4), and 25 ± 4 nm (n = 4), respectively. These results suggest that Tg can be activated by physiologically relevant acidic pH levels (pH 5–6), which are found in organelles such as endosomes, lysosomes, and the trans-Golgi network.
The underlined FBPM-52604 labeled with 0.1 nM 125I-TGF-β receptors in rat thyroid cells (FRTL-5 cells). Cells were affinity-labeled with 0.1 nM 125I-TGF-β receptors in human and bovine Tg (h-4, 5, and 6 (like growth factor-binding protein 3, h-IGFBP-3, h-IGFBP-4, h-IGFBP-5, h-IGFBP-6, and r-IGFBP-6). The underlined 8 amino acid residues are putative TGF-β active-site motifs. The numbers represent the positions in the amino acid sequence of the molecule.

### TABLE III

| Treatment                              | [methyl-3H]Thymidine incorporation (cpm/well) |
|----------------------------------------|---------------------------------------------|
| Control                                | 11,986 ± 259 (100%)                         |
| +150 nM untreated Tg                   | 12,456 ± 1,120 (104%)                       |
| +150 nM DSS-activated thyroglobulin    | 1,098 ± 320 (9%)                            |

Fig. 6. 125I-TGF-β, affinity labeling of cell-surface TGF-β receptors in rat thyroid cells (FRTL-5 cells). Cells were affinity-labeled with 0.1 nM 125I-TGF-β, in the presence and absence of 10 μM β125(41–65) or 0.1 μM DSS-activated Tg. 125I-TGF-β affinity-labeled TGF-β receptors were analyzed by 5% SDS-polyacrylamide gel electrophoresis and autoradiography. The brackets indicate the locations of the type I (TβR-I), type II (TβR-II), and type III (TβR-III) TGF-β receptors. The arrow indicates the location of the type V TGF-β receptor (TβR-V).

Fig. 7. Putative TGF-β active-site motifs in human and bovine Tg (h- and b-Tg) and human and rat insulin-like growth factor-binding protein 3, 4, 5, and 6 (h-IGFBP-3, r-IGFBP-3, h-IGFBP-4, r-IGFBP-4, h-IGFBP-5, r-IGFBP-5, h-IGFBP-6, and r-IGFBP-6).

The other prominent biological activity of TGF-β is transcriptional activation of fibronectin and PAI-1 (20–22). We therefore determined the effect of DSS-activated Tg on the transcriptional expression of PAI-1 in mink lung endothelial cells. As shown in Fig. 5C, DSS-activated Tg exhibited a small but significant activity in transcriptional activation of PAI-1 at 0.025, 0.05, and 0.1 μM, Tg exhibited 1.2 ± 0.05-fold (n = 6), 1.4 ± 0.1-fold (n = 6), and 1.5 ± 0.1-fold (n = 6) increase of the relative level of PAI-1/PDGF mRNA ratio, respectively. These results demonstrate that DSS-activated and untreated Tg can function as TGF-β agonist and antagonist, respectively.

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Growth Inhibition of Rat Thyroid Cells by DSS-activated Tg—As described previously, Tg may encounter intracellular acidic compartments during internalization and recycling in thyroid cells (2, 23). The thyroid cells are presumably the target cells for activated Tg. We, therefore, examined the effect of DSS-activated Tg on cell growth as measured by DNA synthesis of rat thyroid cells (FRTL-5 cells). FRTL-5 cells are a cell line of thyroid-stimulating hormone-dependent thyrocytes that exhibits two prominent biological activities of TGF-β (growth inhibition and transcription activation), although the transcription activation activity is relatively weak.

### FIG. 5

A. Growth inhibition of rat thyroid cells (FRTL-5 cells) by DSS-activated Tg. Untreated Tg alone did not affect DNA synthesis at concentrations up to 120 nM (Fig. 5A). Since untreated Tg inhibited 125I-TGF-β binding to TGF-β receptors (Fig. 1) but did not affect DNA synthesis, we determined the antagonist activity for untreated Tg toward TGF-β-induced inhibition of DNA synthesis. As shown in Fig. 5B, untreated Tg at 180 nM blocked the inhibition of DNA synthesis induced by 0.125 pm TGF-β1. Untreated Tg alone did not affect DNA synthesis. These results suggest that DSS-activated and untreated Tg can function as TGF-β agonist and antagonist, respectively.

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To determine how activated Tg exerts TGF-β, we identified naturally occurring, structurally unrelated TGF-β agonist. IGFBP-3 has a putative TGF-β binding site and inhibitory activity could not be segregated from Tg proteins by 5% SDS-polyacrylamide gel electrophoresis and by immunostaining inhibitory activity could not be segregated from Tg proteins heterodimerization or hetero-oligomerization of TGF-β antagonists. We speculate that untreated Tg functions as an antagonist and that untreated Tg antagonists contain this motif and also possess dimeric or oligomeric structures. Insulin-like growth factor-binding protein 3 (IGFBP-3) was the first to be identified as a TGF-β partial agonist. IGFBP-3 has a putative TGF-β active-site motif (WCVD) near its C terminus and forms a dimer in aqueous solution (13, 28). Human and bovine Tg contains 5–7 WCVD motifs, which are identical to those of IGFBP-3 and other IGFBPs (Fig. 7) (3, 4, 28). Since untreated and DSS-activated Tg can function as TGF-β antagonist and agonist, respectively, we hypothesize that the availability of the WCVD motifs in Tg molecules determines the function of Tg as antagonist or agonist. We speculate that untreated Tg functions as an antagonist when a few of the WCVD motifs are available for interaction with TGF-β receptor but unable to induce appropriate heterodimerization or hetero-oligomerization of TGF-β receptors. Upon activation by DSS modification or acidic pH treatment, most of the WCVD motifs in the Tg molecule become available and are capable of inducing appropriate heterodimerization or hetero-oligomerization of TGF-β receptors. Thus, activated Tg can function as an agonist.

**DISCUSSION**

In this report, the authenticity of TGF-β activities of untreated and activated Tg has been supported by several lines of evidence. These include the following: 1) The 125I-TGF-β 1 binding inhibitory activity could not be segregated from Tg proteins by 5% SDS-polyacrylamide gel electrophoresis and by immunnoprecipitation using specific antisum to Tg. 2) Reduced and alkylated Tg exhibited a TGF-β 1 binding inhibitory activity with an IC₅₀ close to that for Tg activated by DSS modification, 8 μM urea, or acidic pH treatments. 3) Both untreated and activated Tg were incapable of binding TGF-β 1. 4) The possibility that Tg obtained from commercial sources might contain activated Tg (potentially generated during purification), which might contribute to the TGF-β activity of untreated Tg, was ruled out by the observation that at 500 nM untreated Tg did not show any growth inhibitory activity whereas activated Tg inhibited growth at ~50 nM.

DSS-activated Tg shows TGF-β agonist activities in growth inhibition and transcriptional activation of PAI-1. The reason why DSS-activated Tg exerts a moderate effect on the transcriptional activation of PAI-1 is unknown. It would be interesting to determine qualitatively and quantitatively which types of TGF-β receptors are activated by DSS-activated Tg. Since the type I and type II TGF-β receptor heterodimerization is required for the transcriptional activation of PAI-1 (27), DSS-activated Tg would have a moderate effect on the type I/II receptor heterodimerization as demonstrated by its effect on the PAI-1 expression.

In the lumen of thyroid follicles, Tg can be found at a concentration up to 590 mg/ml (29). The levels of activated Tg which could be potentially generated during its receptor-mediated endocytosis and recycling are not known. It is possible that the steady-state activation of Tg may play a role in homeostasis of thyroid gland growth and regulation of immune status. Although the immunosuppressive activity of activated Tg has not been tested, activated Tg is presumed to possess such activity since TGF-β is known to be a potent immunosuppressor (30). We hypothesize that Tg may be involved in thyroid autoimmune diseases through the TGF-β antagonist activity of native Tg and/or through activation of its latent TGF-β agonist activity.

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