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TITLE: Bioavailability of TGF-Beta in Breast Cancer

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## 14. ABSTRACT
The Transforming Growth Factor beta (TGF-) superfamily includes three isoforms designated TGF-1, 2 and 3. All three isoforms are secreted as latent complex where the TGF- cytokine is non-covalently associated with an isoform specific latency-associated peptide (LAP). Mature cytokine binds cell surface receptors only after release from its LAP making extracellular activation a critical regulatory point for TGF-bioavailability. Proposed activation mechanisms include proteolysis and conformational changes. Previous work from our laboratory showed that latent TGF-1 (LTGF-1) is efficiently activated upon exposure to reactive oxygen species (ROS). ROS activation is restricted to the LTGF-1 isoform. Because of the amino acid sequence differences between the three LAPs, we postulate that the specificity of this activation mechanism lies within the LAP. Furthermore, we hypothesize that the presence of a metal in the latent complex could provide a redox active center for this process. Redox mediated activation provides a novel mechanism for TGF- participation in tissues undergoing oxidative stress. Moreover, this would allow TGF-1 to act both as a sensor of oxidative stress within tissues as well as a transducer of that signal by binding to its cellular receptors.

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Introduction

The Transforming Growth Factor beta (TGF-β) superfamily includes three isoforms designated TGF-β1, β2 and β3. All three isoforms are secreted as latent complex where the TGF-β cytokine is non-covalently associated with an isoform specific latency-associated peptide (LAP). Mature cytokine binds cell surface receptors only after release from its LAP making extracellular activation a critical regulatory point for TGF-β bioavailability. Proposed activation mechanisms include proteolysis and conformational changes. Previous work from our laboratory showed that latent TGF-β1 (LTGF-β1) is efficiently activated upon exposure to reactive oxygen species (ROS).

ROS activation is restricted to the LTGF-β1 isoform. Because of the amino acid sequence differences between the three LAPs, we postulate that the specificity of this activation mechanism lies within the LAP. Furthermore, we hypothesize that the presence of a metal in the latent complex could provide a redox active center for this process. Redox mediated activation provides a novel mechanism for TGF-β participation in tissues undergoing oxidative stress. Moreover, this would allow TGF-β1 to act both as a sensor of oxidative stress within tissues as well as a transducer of that signal by binding to its cellular receptors.
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Progress

We have characterized the effect of reactive oxygen species (ROS) on TGF-β. This research has discovered that LTGFβ-1 contains a redox switch centered at methionine 253, which provides the mechanism by which ROS mediates activation by the oxidation of LTGFβ-1. This mechanism of activation leads to the rapid and efficient release of TGF-β independent of a requirement for other proteins. Detailed information for this work is published in *Radiation Research* Jobling *et al* (2006), which summarizes the work until the 2006 annual report and has been attached into the appendix of this report to review further details.

To enable LTGFβ-1 to act as a redox switch, we postulate the presence of transition metals bound either adventitiously or specifically to LAP-β1. The combination of a transition metal with ROS would lead to site specific oxidation of methionine 253 and activation of the latent complex. The standard methods to determine the presence of metals in proteins, like atomic absorption spectrometry, inductively coupled plasma combined with atomic emission spectroscopy (ICP-AES), mass spectrometry (ICP-MS), synchrotron techniques such as x-ray fluorescence spectrometry, or extended x-ray absorption fine structure (EXAFS), require large amounts of protein. These tests are impracticable in the case of LTGFβ which is difficult and costly to synthesize and purify in quantity. Therefore, we worked on the optimization of a new chemiluminescent and colorimetric method described by Hogbom *et al* (2005). The advantage of this method is the low concentration of proteins used to determine the presence of transition metals.

The results obtained for these experiments are included in the 2007 annual report. The data showed that at least 1 nmol of metal is necessary to run the test with confidence. We therefore calculated the minimum amount of LTGF-β necessary for the assays according to its molecular weight (110KDa) and assuming one metal ion bound per molecule. The minimum amount of 1nmol of metal ion per well necessary as determined in the above standardization assays translates into 100µg of LTGF-β per well (8µl), which corresponds to a concentration of 12.5 µg/µl. This is still a very high concentration of LTGF-β especially because it is biologically functional on the order of picomolar concentrations. It is very difficult to obtain by synthesis or commercially such high quantities of LTGF-β, even more for all three different isoforms. In order to investigate further determination of transition metal binding to LTGF-β, we had been looking for different sources to obtain the necessary amount of protein to run the analysis but unfortunately it was impossible for us to get enough quantity of LTGF-β to run the test with confidence.

To further study the activation of LTGF-β1 by ROS, we transduced immortalized wild type or Tgfβ1 null murine embryonic fibroblasts (MEF) with cDNA encoding wild type LTGF-β1 or a mutant LTGF-β1 bearing a point mutation that substitutes methionine for alanine at position 253 (Jobling *et al*. 2006). As described before the M253A mutation leads to loss of response of LTGF-β1 to ROS.

Because transfection efficiency is very low for primary fibroblasts and because stable transfection is a long process, we followed a procedure (Jianming Xu, 2005) to obtain immortalized MEF cell lines prior to transfection. Several strategies were used to transfect the MEF cell lines with the WT and mutant LTGF-β1, although none of them was able to transfect with success. In the last two
month of project, we successfully stably transduced the LTGF-β1 and the three mutant versions (M112A, M132A or M253A) cDNAs using virus particles. The WT and mutant LTGF-β1 cDNA sequences were inserted into a pCDNA3 vector. The cDNAs sequences of interest were transferred from the pCDNA3 vector to a pMSCVpuro to generate viral particles used to transduce the WT and mutant LTGF-β1 cDNAs into the MEF cell lines.

The first step consisted of digestion with the restriction enzyme EcoRI to cut the cDNAs of interest from the vector pCDNA3. Then the cDNAs sequences were purified from the vector sequences using a high grade agarose gel. Once recuperated the cDNAs were ligated into the pMSCVpuro vector. Five different pMSCVpuro vectors were generated containing one of the corresponding LTGF-β1 cDNAs. Each one of those vectors was used to transfect PT67 cells. Transfected PT67 cells produced viral particles containing the sequences of interest. These viral particles were secreted to the culture media which was collected and incubated to transduce our MEF cell lines (MEF Tgfβ1 null or wildtype). Finally the MEF cell lines were grown with puromycin to select only the transduced MEF cells.

After selection with puromycin, the transduced MEF cells were split in two groups sham and treated with IR (1Gy). After the treatment, the media of both groups was switched to serum-replacement conditioned medium (SRM) and collected 24h later. Media subjected to heat, a treatment known to result in LTGFB-1 activation, will be used to measure the presence of TGF-β1 and compare the results obtained for non-transduced MEF cells between different mutant versions of LTGF-β1 transduced into MEF as well as sham and irradiated groups. Although we have finished collecting the media samples, the presence of LTGF-β1 will be measured on the following weeks after submit this report.

**Conclusions**

We identified LTGF-β1 as the unique LTGF-β isoform that is activated by ROS, and showed that the target for ROS-activation is located in the methionine 253 of the LAP-β1. The fact that the LAP-β1 oxidation is reversible indicates that LTGF-β1 could act as a redox switch. TGF-β is clearly implicated in the response to ROS, generating tissue response to inflammation (Wahl 1994), ischemia (Anscher et al. 1995), and radiation (Ewan K. et al. 2002), which could lead to a cascade were LTGF-β1 is activated by ROS and TGF-β1 stimulates cells to produce ROS. Overproduction of TGF-β is frequently a contributing factor to progressive fibrosis (Takehara 2003), autoimmunity and chronic inflammatory diseases (W. Chen and S. M. Wahl, (1999)) as well as cancer (Derynck, et al. (2001)).

Recent reports have confirmed in a large cohort that a TGFβ1 polymorphism that leads to increased synthesis associates with risk of breast cancer (Cox. DC. et al. 2007). Genetic polymorphisms are defined as small variation in the genetic sequence of different individuals can lead to diverse phenotypes. Our observations prove that only one (LTGF-β1) of the three LTGF-β isoforms could be activated with ROS and this characteristic is due to a single amino acid variation at position 253. These observations suggest that further investigation in humans might examine this region for polymorphisms that could affect the activation of TGFβ by ROS and thereby alter the response to oxidative stress.
References


**Cox, DG et all.** (2007) TGFβ1 and TGFBR1 polymorphisms and breast cancer risk in the Nurses' Health Study. *BMC Cancer* 7:175


Isoform-Specific Activation of Latent Transforming Growth Factor β (LTGF-β) by Reactive Oxygen Species

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INTRODUCTION

The transforming growth factor β (TGF-β) family consists of multifunctional cytokines that modulate myriad cellular and tissue processes, including cell growth, differentiation, apoptosis, inflammation and extracellular matrix deposition and composition. The three closely related isoforms, TGF-β1, 2 and 3, are the products of three different genes. All three isoforms are processed and secreted similarly in that intracellular proteolysis cleaves the N-terminal region to form an approximately 75-kDa homodimer called the latency-associated peptide (LAP), while the remaining C-terminal region forms a 24-kDa homodimer to produce the TGF-β cytokine (1). During protein processing, noncovalent association of the TGF-β cytokine with its respective LAP forms the latent TGF-β (LTGF-β) complex, which is secreted. In addition, LTGF-β frequently is covalently bound to latent TGF-β-binding protein (LTBP), which facilitates its sequestration within the extracellular matrix (2). The secretion and storage of TGF-β in such latent complexes restrict biological activity. Release of TGF-β from the latent complex, which is referred to as activation, permits TGF-β to be bound by its ubiquitously expressed cell surface tyrosine kinase type I and type II receptors that initiate signaling cascade (3). Thus activation of TGF-β is a major mode of biological regulation, and appropriate control of TGF-β activation is essential to maintain correct tissue homeostasis (4). Interestingly, although all three TGF-β isoforms bind to the same set of receptors, the phenotypes of isoform-specific knockout mice are distinct, suggesting that the biological roles of the three isoforms are different (5–7). One possible explanation for this observation is that the three LTGF-β isoforms are susceptible to different modes of activation during physiological processes.

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ROS ACTIVATION OF LTGF-β1

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Notes: Forward and reverse primers used to generate the three mutant LTGF-β1 proteins are shown. Nucleotides shown in bold resulted in the substitution of alanine for methionine.

Standard modes of activation of LTGF-β1 in solution use highly acidic or basic solutions or treatment with heat (8). Physiologically relevant modes of activation include proteolysis by direct cleavage of LAP or by proteolytic cascades (2, 9–11), binding to thrombospondin or integrins (12–15), and deglycosylation (16). However, these mechanisms require the participation of one or more additional proteins generally localized to the cell surface. Previously, we demonstrated that LTGF-β1 is activated rapidly and globally by ionizing radiation in vivo (17–19). Because reactive oxygen species (ROS) are a product of the interaction of ionizing radiation with water and biological materials (20), we postulated that the rapid activation of LTGF-β1 in vivo was due to ROS generated by ionizing radiation. Further studies using recombinant protein were conducted to show that a solution source of ROS efficiently activates LTGF-β1 in the absence of cells or other proteins (21).

In the current study, we determined the specific reactive oxygen radical and protein determinants of ROS-mediated activation of LTGF-β. Comparison of LTGF-β isoforms revealed that only LTGF-β1 is susceptible to ROS activation. We show that ROS activation depends on OH•, supporting oxidation-induced conformational change. We determined that the sensitivity to ROS activation lies within LAP-β1 and depends on a conserved methionine at amino acid position 253. Taken together, these observations suggest that ROS-mediated activation is due to a redox switch involving methionine 253 in LAP-β1. Oxidation of this switch is sufficient for rapid and efficient release of TGF-β1 independent of any other proteins, which allows ROS to elicit rapid activation of LTGF-β1, which in turn orchestrates multireceptor responses to oxidative stress.

MATERIALS AND METHODS

Reagents. Carrier-free recombinant human TGF-β1, TGF-β2, LAP1 and goat anti-TGF-β3 polyclonal antibody were purchased from R&D Systems (Minneapolis, MN). Wild-type LTGF-β1 was a kind gift from Dr. Monica Tsang (R&D Systems). Luciferin was purchased from Promega Corporation (Madison, WI). N-Acetyl-3,7-dihydroxphenoxazine (A-6559) was purchased from Molecular Probes (Eugene, OR). Coomassie 3-carboxylic acid was purchased from Aldrich Chemical Company Inc. (Milwaukee, WI). An ammonium sulfate suspension of superoxide dismutase (SOD) prepared from bovine liver was purchased from Sigma (St. Louis, MO). All solutions were prepared with sterile-filtered double distilled water. All other reagents were reagent grade and purchased from the Sigma Chemical Company.

Expression of LTGF-β3. Recombinant cDNA clone for murine LTGF-β3 was obtained from Dr. Lalage Wakefield (NCL, Bethesda, MD). The entire cDNA was excised from the plasmid pBluescript KMI (+) vector (Stratagene) using HindIII and BamHI, at the 5′ and 3′ ends, respectively. The fragment was inserted into the pCDNA3.1 (+) vector (Invitrogen) cleaved with the same enzymes. CHO-K1 (ATCC CCL-61) cells were maintained in F12 K medium (Invitrogen) containing 10% FBS, 1.5 g/liter sodium bicarbonate, and 2 mM L-glutamine. For mammalian expression of LTGF-β3, the pCDNA3.1 (+) vector containing murine LTGF-β3 was transfected into CHO-K1 cells using the Lipofectamine transfection reagent (Invitrogen), following the manufacturer's instructions. Stable cell lines were selected with G418 (800 μg/mL). Activity experiments were performed using serum-free medium.

Expression and purification of LTGF-β2, LTGF-β2 was produced at Genzyme (Cambridge, MA) from a transfected CHO cell line. The protein was captured on a SO₃⁻-Fractogel (EMD Chemicals Inc.) cation exchange column, eluted in 50 mM sodium phosphate, 500 mM sodium chloride, pH 7.0, and stored frozen at −40°C. LTGF-β2 was then passed through 10 mL of Sephacryl G-25 size-exclusion resin to remove low-molecular-weight impurities. Further purification was achieved using a Hitachi Packard 1100A HPLC. Protein was fractionated on a Zorbax C8 semi-preparative column using a gradient developed from 5% to 90% acetonitrile in water containing 0.1% trifluoroacetic acid for 45 min at a flow rate of 4 mL per minute. Protein was monitored at 220 nm. Eluted protein was concentrated under vacuum, resuspended in 10 mM phosphate-buffered saline, and stored at −30°C. Protein purity was visualized by Coomassie Brilliant Blue staining of SDS-PAGE.

Generation of LTGF-β1 mutants. The generation of methionine mutations at positions 112, 132 and 253 were performed using the QuickChange® mutagenesis kit (Stratagene) according to the manufacturer's protocol. Briefly, two primers were designed around each of the targeted methionines. The methionine was changed into alanine (methionine codon AUG, alanine codon GCG) by replacing the AT nucleotides found in the methionine coding sequence of TGF-β1 (NM000668) with GC codons (Table 1). The TGF-β1 gene, cloned into pCDNA3.2, was subjected to PCR amplification and selection. The presence of the mutation at each position was confirmed by sequencing. The mutated clones were used for transient transfection of HEK 293 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Approximately 18 h after transfection, the cells were washed twice with serum-free medium and left in a minimal volume of serum-free DMEM/F12 medium for approximately 30 h. This medium was collected for testing in the Fe3+/ascorbate activation assay. LTGF-β1 cysteine 33-serine mutant (22) was transfected into HEK-293 cells using Lipofectamine 2000, and a stable cell line expressing this mutant was
achieved by selection with G418 in DMEM supplemented with 5% FBS. To collect medium containing LTGF-B1 cysteine 36-serine mutant for testing, the cells were washed twice with PBS and incubated for approximately 48 h in serum-free DMEM. This medium was tested in the Fe(III)/ascorbate activation assay.

Quantification of LTGF-β. The biological activity of LTGF-β was measured by monitoring luciferase activity in mice lung epithelial cells transfected with a plasmid encoding luciferase reporter construct (23). Briefly, 50 μl of 3.2 X 10⁶ cells per milliliter was plated in 96-well plates in 0.5% fetal bovine serum in Dulbecco's modified Eagle medium. Samples and standards were assayed in triplicate using luciferase instrumentation. Three hours after cell plating, samples or standards were added to wells and incubated for 16–19 h at 37°C in 0.5% CO₂. The cells were washed with PBS and lysed in a 50 μl volume of a lysis buffer for 20 min. The luciferase activity was measured using an ECHOBiotherm Lumat LB 960 (Oak Ridge, TN) for 10 s immediately after manual injection of 50 μl of luciferase substrate and recorded as relative light units integrated over time. A standard curve using rTGFB1 was generated in every experiment. In some experiments, a tenfold excess of polyclonal TGF-β3 neutralizing antibody (20–80 μg/ml) was added to confirm specificity. DNA quantification was assayed to ensure that addition of experimental variables such as ROS scavengers did not affect cell viability.

Treatment of LTGF-β with ascorbic acid and Fe(III). All experiments were conducted in siliconized plastic tubes, and solutions were prepared with sterile, distilled water treated with the metal-chelating resin chel-100 (1 g/10 ml) by stirring overnight. Untreated LTGF-B1 was used to identify the amount of the binding material, and heat treatment of LTGF-B1 at 80°C for 5 min was used as a positive control for activity (6). The standard condition for ROS-mediated LTGF-β activation was incubation with ascorbic acid (200 μM) and ferric chloride (20 μM plus 200 μM EDTA) in metal-depleted saline at 37°C for 2 h with agitation (21). rTGFB1 was substituted for LTGF-B1 in some experiments. Substitution of dehydroascorbic acid (DHA; 200 μM) for ascorbic acid was carried out in the same manner. ROS scavengers, SOD (16 U), heat-denaturation of the binding material, and heat treatment of LTGF-B1 were added prior to addition of Fe(III)/ascorbate. Addition of Fe(III) (20 μM) and H₂O₂ (at indicated concentrations) to LTGF-B1 was carried out as described for the Fe(III)/ascorbic acid experiment. The total sample volume was 40–100 μl. After incubation, samples were diluted as necessary to the effective range of the bioassay. All experiment constituents were tested for bioactivity by performing the incubation without the additional purified LTGF-B1 and medium conditioned by CHO-K1 cells overexpressing LTGF-B3 were expressed under the same conditions described above for the LTGF-B1 studies.

Detection of H₂O₂ after ROS scavenger addition. The H₂O₂ assay was adapted from information provided by Molecular Probes (Eugene, OR). A 50 μM solution of A-5550 in PBS (196 μl) was mixed with an experimental sample (2 μl) at 0, 60 and 120 min after addition of ROS. Horseradish peroxidase was added to a final concentration of 75 μM. Each sample was scanned 30 min after addition of samples using a Fluoromax multiwell plate reader (Series 4000, PerSeptive Biosystems, Dublin, CA) at 530 nm excitation and 590 nm emission. A standard curve of H₂O₂ was prepared for each experiment and used to determine the amount of H₂O₂ in the experimental samples.

Detection of the hydroxyl radical. The HO· detection method was adapted from Maksimov et al. (24). Cuomann-3-carboxylic acid (CMA, 2 μM) was dissolved in buffer (10 mM sodium phosphate, pH 7.4, 0.09% NaCl) under continuous stirring and heating at 70°C for 60 min and allowed to cool to room temperature. The experimental sample (2 μl) was added to 198 μl of the CMA solution in a 96-well plate at 0, 60 and 120 min after addition of ROS. Each sample was measured as described above at 360 nm excitation and 460 nm emission.

Gel electrophoresis. Electrophoresis of LAP-B1 and LAP-B2 was performed on a Phastgel gel system (Amersham-Pharmacia) using 8-25% SDS-PAGE gels and was visualized by Cownamore Brilliant Blue. Circular dichroism. Circular dichroism was performed on untreated, heat-treated or Fe(III)/ascorbate-treated samples of LAP-B1 (carrier-free, from R&D Systems) using a Jasco J-810 system. Samples were diluted in 5 mM Hepes, pH 7.0, before scanning from 190–260 nm. The secondary structure content was calculated using Spectra Manager software.

Disulfide bond determination. The structural integrity of the disulfide bonds was determined by measuring the amount of reduced cysteines with the Ellman test (25). Briefly, a 3 ml solution of 0.1 μM LTGF-B3 was prepared in 10 mM potassium phosphate and mixed with 0.1 ml of 5.5'-dithiobis(2-nitrobenzoic) acid (4 mg/ml in 10 mM potassium phosphate). After 15 min the absorbance was measured at 410 nm and compared to a standard reference of DTNB (4 mg/ml in 10 mM potassium phosphate). The concentration of sulfhydryl groups was calculated from the equation: concentration of sulphydryl groups = [A₄₁₀nm] – [A₄₁₀nm]ref (1/360). This value allowed the percentage of reduced cysteines to be calculated based on TGF-β1 and TGF-β2 having 9, LAP-B1 having 3, and LAP-B2 having 5 cysteine residues.

LAP and TGF-B1 recombination. To determine which partner was functionally modified by ROS, TGF-B1 or LAP-B1 was pretreated with Fe(III)/ascorbate for 2 h at 37°C. In some experiments, this treatment was followed by exposure to 2 mM dichloroethylene (DTE) for 2 h prior to recombination. Untreated TGF-B1 and LAP-B1 were used as positive and negative controls, respectively, ROS-exposed and unexposed TGF-B1 (1 μg/ml) and LAP-B1 (5 μg/ml) were incubated together in various combinations for 2 h at 37°C. The solution was diluted into the effective range of the TGF-β bioassay, and inhibition of activity was measured using the bioassay as in the TGF-β experiments.

RESULTS

Activation of LTGF-β by ROS is Specific to the LTGF-B1 Isoform

Our previous studies demonstrated that LTGF-B1 was efficiently activated by ROS generated in solution by metal-catalyzed ascorbate oxidation (i.e., Fenton chemistry) (21). To determine whether ROS-mediated activation is common to all three LTGF-β isoforms, LTGF-B2 and LTGF-B3 were exposed to Fe(III)/ascorbate-generated ROS. After ROS exposure, TGF-β activity was measured using the PAI-1 luciferase assay. This assay serves as a sensitive biodetection system for active TGF-β regardless of isoform. Once activated, TGF-β binds receptors on the surface of cells transfected with a TGF-β-responsive reporter gene consisting of plasmogen activator inhibitor fused with luciferase (23). Heat treatment (80°C, 5 min) is the standard method for LTGF-β activation, which served as an activation control for subsequent experiments. Consistent with previous observations (21) and as shown in Fig. 1, ROS activation of LTGF-B1 produced a greater amount of TGF-B1 activity than did heat treatment. This phenomenon was observed consistently with purified recombinant LTGF-B1. As expected, purified recombinant LTGF-B2 was efficiently heat-activated to levels similar to that of LTGF-B1, but treatment with Fe(III)/ascorbate produced no significant activation, in direct contrast to the efficient LTGF-B1 activation observed by ROS treatment.

Because LTGF-B3 is not available commercially, it was overexpressed in CHO-K1 cells to evaluate whether LTGF-B3 was sensitive to ROS activation. Serum-free conditioned medium was collected, exposed to heat, and assessed for TGF-β activity. Heat treatment of the condi-
**ROS ACTIVATION OF LTGF-β1**

**FIG. 1.** Isoform specificity of LTGF-β activation exposed to ROS generated by Fe(III)/ascorbate. LTGF-β1 was substantially activated after treatment with the ROS-producing Fe(III)/ascorbate system. Neither LTGF-β2 nor LTGF-β3 was activated to the same extent by the ascorbate system. The values plotted are normalized to the amount of activation observed by heat treatment. ROS-activated recombinant LTGF-β1 consistently released more active TGF-β1 than heat-treated LTGF-β1. Using a two-tailed Student's t test, a P value of 2.6 × 10⁻⁴ was obtained for the comparison of heat and ROS activation of LTGF-β1; for the comparison of ROS activation of LTGF-β1 to LTGF-β2 or β3, the P value was 2.3 × 10⁻⁵ and 2.1 × 10⁻⁴, respectively.

**FIG. 2.** Activation of LTGF-β1 by the components of the Fe(III)/ascorbate system. Individual components, Fe(III) or ascorbic acid, did not activate LTGF-β1 to levels observed with the complete system. A byproduct of the Fe(III)/ascorbate Fenton chemistry, DHA, also did not activate LTGF-β1, nor did the combination of DHA and ascorbate. Comparison of Fe(III)/ascorbate activation to individual components and byproducts with a two-tailed Student's t test gave P values of 1.4 × 10⁻⁴ for Fe(III), 3.3 × 10⁻⁴ for ascorbate, 1.3 × 10⁻⁴ for DHA, and 1.5 × 10⁻⁴ for DHA and ascorbate.

Hydroxyl Radicals are Responsible for ROS Activation of LTGF-β1

The Fe(III)/ascorbate reaction generates a spectrum of ROS, including HO·, O₂⁻, and H₂O₂. To rule out a role of byproducts or reaction components, each was characterized for its role in the activation process. Individually, none of the reaction components or byproducts, such as dehydroascorbic acid (DHA), was capable of significantly activating LTGF-β1 (Fig. 2), which indicated that activation of LTGF-β1 was not the result of a direct effect from a component of the Fe(III)/ascorbate system but was most likely due to ROS generated during the reaction.

Analysis of ROS produced by the Fe(III)/ascorbate system confirmed that elevated levels of HO· and H₂O₂ were present throughout the 2-h course of the experiment (data not shown). To determine which ROS generated by Fe(III)/ascorbate system were required for LTGF-β1 activation, scavengers of O₂⁻, HO· and H₂O₂ were added to the reaction individually. Superoxide dismutase (SOD) efficiently removes O₂⁻ produced in vivo and in vitro (26). The presence of SOD in the reaction mixture did not significantly affect activation by Fe(III)/ascorbate, suggesting that O₂⁻ was not required for Fe(III)/ascorbate activation. Rather than blocking activation, SOD led to a moderate increase in LTGF-β1 activation (Fig. 3). Denatured SOD was used as a control to confirm that enzymatic activity of SOD was required for this phenomenon. Results of these experiments indicate that O₂⁻ was not responsible for Fe(III)/ascorbate activation of LTGF-β1, and these data suggest that it may be deleterious to the activation process or that SOD may affect the bioactivity of TGFB-1.

Other candidates for the ROS-mediated activation were H₂O₂ and HO·. Catalase promotes the dismutation of H₂O₂ to O₂ and H₂O. Thus its presence would reduce the amount of available H₂O₂, but because H₂O₂ can be an intermediate precursor to HO·, reducing H₂O₂ would also lead to a concomitant decrease in HO·. Addition of catalase to the Fe(III)/ascorbate reaction significantly reduced LTGF-β1 activation (Fig. 3B), whereas addition of denatured catalase did not. A parallel assay of ROS generated under these conditions showed that catalase reduced the H₂O₂ levels by 25% and HO· levels by 26% at 2 min (Table 2). Thus scavenging H₂O₂ or interfering with production of HO· reduced LTGF-β1 activation.
FIG. 3. Effect of scavenging by SOD and catalase on the activation of LTGF-β1 by ROS. Panel A: The presence of SOD, an enzymatic scavenger of superoxide, did not inhibit the ability of ROS to activate LTGF-β1. Denatured SOD was used as a negative control. Experimental values are expressed as the percentages of active TGF-β observed after heat activation. Panel B: Catalase, an enzymatic scavenger of H₂O₂, significantly inhibited the ability of Fe(III)/ascorbate-generated ROS to activate LTGF-β1. A two-tailed Student's t test comparing activation levels with and without catalase gave a P value of 0.01. Denatured catalase did not inhibit this process, suggesting that enzymatic activity of the catalase was necessary to block activation by the Fe(III)/ascorbate system.

Our previous studies suggested that H₂O₂ was not directly responsible for activation of LTGF-β1. Addition of H₂O₂ in concentrations ranging from 10–400 μM directly to solutions of LTGF-β1 was not sufficient for activation (27). Furthermore, addition of higher concentrations of H₂O₂ did not lead to activation LTGF-β1. Thus our attention turned to examination of HO\. If HO\ were responsible for the ROS-mediated activation of LTGF-β1, then specific scavengers would be expected to inhibit LTGF-β1 activation. Indeed, increasing concentrations of DMSO (0.5 mM to 500 mM) added to the Fe(III)/ascorbate system significantly reduced the amount of LTGF-β1 activation (Fig. 4). The greatest effect was observed at 50 mM DMSO. Parallel studies confirmed that addition of 500 mM DMSO reduced the amount of HO\ to 40% of control levels after 60 min. Similarly, addition of ethanol to the Fe(III)/ascorbate treatment of LTGF-β1 reduced activation (Fig. 4) and decreased the amounts of HO\ and H₂O₂ in a dose-dependent manner (data not shown). Although other ROS may also contribute to activation of LTGF-β1, the above results indicate that the presence of HO\ was critical for LTGF-β1 activation.

**LAP-β1 is the Target of ROS Activation**

The observation that HO\ was critical for ROS activation led to the hypothesis that oxidation of LTGF-β1 caused a

**TABLE 2**

<table>
<thead>
<tr>
<th>Amount of H₂O₂ and HO\ produced after LTGF-β1 Treatment with Fe(III)/Ascorbate and Fe(III)/Ascorbate + Catalase</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Time (min)</strong></td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td><strong>H₂O₂</strong></td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>60</td>
</tr>
<tr>
<td>120</td>
</tr>
<tr>
<td>*<em>HO*</em></td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>60</td>
</tr>
<tr>
<td>120</td>
</tr>
</tbody>
</table>

Notes. Values are expressed as percentages (±SE) of the Fe(III)/ascorbate sample at 2 min, which corresponds to 0.65 ± 0.06 mM H₂O₂. Detection was measured in relative units. Representative of n = 3 independent experiments.
conformational change in the latent complex, allowing release of active TGF-β1. In support of this hypothesis, we had noted that ROS-treated LAP-β1 migrated differently than untreated LAP-β1 in nonreducing SDS-PAGE. This did not occur with LAP-β2 or with TGF-β1 (data not shown). Treated and untreated LAP-β2 or TGF-β1 migrated similarly. This observation suggested that LAP-β1 may be the target for ROS-mediated activation via conformational changes induced by ROS. To test whether the conformation of LAP-β1 was indeed altered by Fe(III)/ascorbate treatment, circular dichroism was performed. Interestingly, this treatment caused changes that increased the alpha helix content from 12.9% in the untreated to 41.7% in the Fe(III)/ascorbate-treated sample. No increase in the beta sheet or random coil content was observed; neither treated nor untreated samples contained any beta sheet and random coil. However, heat treatment resulted in dramatic changes in the secondary structure of LAP-β1. The alpha helical content was completely abolished, beta sheet content was increased from zero to 54.7%, and random coil content was increased from zero to 13.5% by heat treatment. Thus treatment of LAP-β1 ROS induced restricted conformational changes, while heat caused changes consistent with denaturation.

To determine whether the ROS-induced changes in LAP-β1 were functionally significant, TGF-β1-LAP-β1 association experiments were conducted. TGF-β1 activity can be inhibited by LAP in vitro and in vivo by overexpressed LAP (27). Noncovalent association of LAP-β1 and TGF-β1 has a $K_d$ of approximately $10^{-9}$, indicating that reassociation is highly favored (28). However, latent complex reformation can be impaired by degradation or by protein modification; for example, nitric oxide treatment modifies LAP-β1 to prevent its association with TGF-β (29). Thus, if ROS treatment caused functional modifications in LAP-β1, it was likely that the LAP-β1 would fail to associate with TGF-β1 to form the latent complex and neutralize its activity; the same would be true if TGF-β1 was the target of modification. Incubation of equimolar amounts of TGF-β1 with biological activity LAP-β1 decreased the of TGF-β1 by approximately 60% (Fig. 5), suggesting formation of the latent complex. ROS treatment of TGF-β1 prior to incubation with untreated LAP-β1 did not hinder efficient neutralization of TGF-β1 activity. However, when LAP-β1 was treated with Fe(III)/ascorbate prior to incubation with TGF-β1, only a minimal reduction in biological activity of TGF-β1 was observed. This suggested that LAP-β1 was modified in a manner that prohibited the neutralization of the TGF-β1 activity. Either the conformational changes in LAP-β1 induced by ROS and observed by circular dichroism were sufficient to prohibit association with TGF-β1 or LAP associated with TGF-β1 but did not neutralize TGF-β1 activity. In favor of the former explanation, TGF-β1 activity was neutralized if the Fe(III)/ascorbate-treated LAP-β1 was subsequently exposed to a low concentration (2 mM) of a mild reducing agent, dithiothreitol (DTT). DTT at this low level is not detrimental to the activity of the TGF-β1 protein (not shown). This reversibility supports the specificity of the oxidation and is consistent with the hypothesis that LAP-β1 is the target of oxidation, which modifies the protein in a specific manner to cause release of TGF-β1.

Methionine 253 is Critical for Oxidative Activation of LTGF-β1

Carbohydrate moieties as well as amino acid side chains are potential sites for oxidation to occur within a glycoprotein such as LTGF-β1. To address the possibility of carbohydrates as targets of ROS, LTGF-β1 was carefully deglycosylated with a series of specific deglycosylases followed by evaluation of ROS activation. Interestingly, in contrast to previous reports (16), deglycosylation did not activate LTGF-β1, and more importantly deglycosylated LTGF-β1 responded to ROS activation in a similar manner to glycosylated LTGF-β1 (J. D. Mott and P. J. Walen, unpublished observations). Although it is possible that carbohydrate moieties may be oxidized during the ROS treatment, they were not targets within LAP-β1 responsible for ROS activation. Thus our attention focused on amino acid side chains as targets for ROS activation.

Several amino acids can undergo reversible biological oxidation, including cysteine and methionine. Each LAP-β1 monomer contains three cysteine residues, two of which are involved in the formation of disulfide bonds essential for dimer formation. Correct pairing of these cysteine res-
idues is critical for LAP-β1 to form the latent complex with TGF-β1 (30). Cysteine residues paired in disulfide bonds are in an oxidized state and are unlikely targets of the oxidation reaction leading to LTGF-β1 activation. The remaining cysteine, at position 33, is known to interact with cysteine residues with the LTBP (31). Because the LTGF-β1 protein used in the above experiments was expressed in the absence of the LTBP, it was possible that cysteine 33 was in a reduced state or that disulfide bonds were not correctly paired, leaving unpaired sulfhydryl groups available to participate in the ROS-mediated activation. The Ellman test (25) was used to evaluate the number of free thiol groups in the wild-type LTGF-β1 and resulted in the expected number of two free thiol groups. The cysteine residue at position 33 in LTGF-β1 is conserved between all three LTGF-β isoforms, and the observation that only LTGF-β1 was susceptible to ROS-mediated activation suggested that cysteine 33 was an unlikely participant in ROS-mediated activation. However, to directly test the participation of cysteine 33, LTGF-β1 with serine substituted for cysteine 33 was stably transfected in HEK-293 cells. Serum-free medium containing the expressed mutant was collected and subjected to activation by Fe3+/ascorbate. Results showed that LTGF-β1 Cys33Ser was activated by Fe3+/ascorbate or by heat identically to wild-type LTGF-β1 (data not shown). Taken together, these results indicate that cysteine 33 does not participate in the ROS-mediated activation of LTGF-β1.

Each monomer of LAP-β1 contains five methionine residues, which are also susceptible to oxidation. Of these five, two methionines (132 and 253) are unique to LAP-β1 (Fig. 6A). Because only LAP-β1 was susceptible to oxidative changes, we hypothesized that these two unique methionines played a role in the process of ROS activation. To examine this, single point mutations of methionine to alanine were made at position 132 or 253, methionines unique to LAP-β1. Additionally, a substitution at a conserved methionine, position 112, was also made to serve as a control. Mutants, as well as wild-type LTGF-β1, were transiently expressed in HEK-293 cells. Serum-free conditioned medium was collected and characterized for LTGF-β1 activation. Like the wild type, the mutants formed latent complexes that could be activated by heat treatment, which was used to normalize the ROS-mediated activation. Mutants M112A and M132A were efficiently activated by ROS treatment; indeed, these mutants were more susceptible to ROS activation than was the wild type (Fig. 6B). However, mutant M253A was resistant to ROS-mediated activation. These results indicated that the methionine at position 253 was critical for ROS-mediated activation.

In summary, this study provides a unique mechanism of LTGF-β1 activation involving ROS and a redox switch that requires the participation of methionine 253. This mechanism allows LAP-β1 to act as a sensor of ROS in tissues, resulting in rapid release of TGF-β1 that triggers cellular responses to oxidative stress.

**FIG. 6.** Susceptibility of LTGF-β1 mutants to ROS activation. Panel A. The LAP sequences of the three LTGF-β isoforms were aligned using Multiple Sequence Alignment (MAFFT) via the EMBL-EBI Proteomics Server at http://ca.expasy.org/. The sequences were analyzed with Bio-Edit using a gap opening penalty of 1.53 and an offset value of 0.123. Methionine positions of interest are shown in bold. Methionines at 132 and 253 are unique to LAP-β1. Panel B: Medium conditioned by HEK-293 cells transiently expressing wild-type or mutant LTGF-β1 was treated with heat or Fe3+/ascorbate followed by assay for TGF-β activity. To compare the amount of TGF-β1 activation induced by ROS, the amount of ROS activation was normalized to the amount of activity produced by heat treatment of an aliquot of the same conditioned medium. The values are expressed as percentages of ROS activation as a function of heat activation. Three independent experiments were performed. Mutation of methionine 253 was significantly less susceptible to ROS activation compared to wild-type LTGF-β1 (P value of 0.02 using a two-tailed Student's t test).

**DISCUSSION**

Biological activity of TGF-β is restrained by secretion as a latent complex, which makes activation a major regulatory point. Most modes of activation require the participation of one or more additional proteins (e.g., proteases, integrins). However, we have identified a unique mechanism of LTGF-β1 activation by ROS that is intrinsic to the protein, and importantly, to this isoform. Our previous studies have shown that LTGF-β can be activated rapidly in vivo by ionizing radiation, which is known to generate ROS. Our subsequent biochemical experiments showed that LTGF-β could indeed be activated by ROS in solution (27).
Exposing LTGF-β1 to ROS yielded more that 1.5 times as much TGF-β1 compared to heat or acid treatment, which makes this mechanism of activation a very efficient process. The wide range of tissue processes that generate ROS underscores its biological relevance. Here we have identified critical requirements for this activation mechanism, including the demonstration that it is unique to LTGF-β1. Exposure of the three isoforms of LTGF-β to oxidative activation led to activation of only LTGF-β1, indicating that ROS-mediated activation is isoform-specific. Although the three TGF-β cytokines have 75% identity, their respective LAPs exhibit only 34–38% identity. All three TGF-β cytokines are capable of binding to the same receptors, but null mutation in the three genes result in very different mouse phenotypes (5–7). Thus it is plausible that specificity of action resides within their respective LAP, allowing for differing susceptibilities to various modes of activation. To our knowledge, this is the first isoform-specific activation mechanism to be identified. Moreover, ROS activation was consistently more efficient than activation with heat, which is generally much more efficient than protein-mediated activation. Although other ROS species may also participate in this mechanism of activation of LTGF-β1, our studies indicate that H2O2 was critical for this process. Furthermore, functional and biochemical assays led us to conclude that LAP, rather than TGF-β1, was the target for ROS-mediated activation. Taken together, these results suggested the ROS-induced oxidation in LAP-β1 triggers a conformational change that release TGF-β1. Recent studies using ROS derived from asbestos also identified LAP as a target (32). Comparison of the non-conserved LAP isoform sequences provided target amino acids susceptible to oxidation. We conducted site-specific mutation of methionine to alanine at position 253 to generate a redox-insensitive LTGF-β1. Together these data define the chemical and structural determinants of a redox switch.

Examples of direct methionine oxidation acting as an oxidative sensor have been shown in thrombomodulin (33) and calmodulin (34). Because substitution of alanine for methionine at position 253 produced functional LTGF-β1 (i.e. still activated by heat) that was no longer susceptible to ROS, this suggests that methionine 253 may reside at or near an oxidative center and that its substitution with alanine disrupts the sensitivity of LAP-β1 to ROS activation. Alternatively, methionine 253 may be the direct target of the ROS where its side chain is oxidized, leading to conformational changes resulting in the activation of LTGF-β1. Interestingly, although substitution of alanine for methionine at position 132, a methionine unique to LAP-β1, did not confer resistance to ROS-mediated activation, nor did the substitution of a conserved methionine at position 112, these two mutants were activated more efficiently by ROS. Full understanding of the biochemical events triggering the oxidative switch in LAP-β1 and how methionine 253 participates in this switch requires further investigation. The observation that oxidation of LAP-β1 was reversible in a mild reducing environment has interesting biochemical and biological implications. Reversibility indicates that ROS modifies LAP-β1 in a manner that is flexible and not denaturing and that these modifications are restricted events. This hypothesis was supported by circular dichroism measurements. Currently, we are pursuing experiments to elucidate the detailed structural requirements of this oxidative switch.

When cellular production of ROS overwhelms antioxidan capacity, an "oxidative stress" state results, which is thought to contribute to a variety of diseases. Biologically, the potential activation of LTGF-β1 by ROS suggests a dual role in physiological and pathological processes that generate oxidative stress: LAP-β1 functions as a sensor, releasing TGF-β as a potent signal upon binding ubiquitous cell surface receptors (35). TGF-β is clearly implicated in the response to ROS, generating tissue response to inflammation (36), ischemia/reperfusion (37), and radiation (33, 38, 39). TGF-β signaling in certain cells also generates increased ROS production (40–42), which could lead to a cascade where LTGF-β1 is activated by ROS and TGF-β1 stimulates cells to produce ROS. TGF-β induces the production of H2O2 in bovine epithelial cells (43), and ROS is involved in TGF-β1-induced apoptosis of hepatocytes (44). It has been reported that in the presence of heme peroxidases, TGF-β1-induced H2O2 may mediate oxidative crosslinking of extracellular matrix proteins (45). It remains to be determined whether ROS-mediated injury is propagated by chronic ROS-mediated LTGF-β1 activation. Overproduction of TGF-β is frequently a contributing factor to progressive fibrosis (44, 46, 47), autoimmunity and chronic inflammatory diseases (48) as well as cancer (49). Our data provide insight into the structural and mechanistic basis of LTGF-β1 activation, the tissue conditions that may be present when LTGF-β1 is activated, and its prominence in mediating responses to tissue damage.

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