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The Transforming Growth Factor beta (TGF-b) superfamily includes three isofroms designated TGF-b1, b2 and b3. All three isoforms are secreted as latent complex where the TGF-b 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-b bioavailability. Proposed activation mechanisms include proteolysis and conformational changes. Previous work from our laboratory showed that latent TGF-b1 (LTGF-b1) is efficiently activated upon exposure to reactive oxygen species (ROS). ROS activation is restricted to the LTGF-b1 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-b participation in tissues undergoing oxidative stress. Moreover, this would allow TGF-b1 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.

# Specific Aims:

1. To characterize the interaction between reactive oxygen and latent TGF-β
2. To identify and localize the redox-metal center within the latent TGF-β
3. To determine the three-dimensional structure of latent TGF-β

# Progress:

### Aim 1. To characterize the interaction between reactive oxygen and latent TGF-β

We have collected data to show that activation by ROS is restricted to the LTGF-β1 isoform. Due to the high degree of similarity between the amino acid sequences of the three cytokines (TGFβ-1, β2 and β3), it is not likely that the redox center resides within the cytokine region. However, amino acid sequences of the three LAPs are not highly conserved suggesting that the redox center specific to LTGF-β1 may reside within LAP-β1. Methionine residues are particularly susceptible to oxidation, and there are two non-conserved methionine residues within LAP-β1. In collaboration with Dr. Dan Rifkin at NYU, we have developed a series of mutants where methionine residues in LTGF-β1 have been mutated to alanine. These mutants have been transiently expressed in mammalian cells and conditioned medium has been collected and characterized for ROS activation using the PAI-luciferase assay to measure the amount of active TGF-β. Results from these experiments suggest that methionine 253 is critical in the ROS activation mechanism. This work has recently been accepted for publication in *Radiation Research*.

### Aim 2. To identify and localize the redox-metal center within the latent TGF-β

We conducted site-specific mutation of methionine to alanine at position 253 to generate a redox-insensitive LTGF-β1 (see attached, Jobling et al. 2006). Together these data define the

chemical and structural determinants of a redox switch. Substitution of alanine for methionine at position 253 produced functional LTGF̃β1 (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 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 more efficiently activated 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.

These data supporting a redox switch provide further support for the potential role of transition metals in the latent complex. Future studies to Subject LTGF-β analogues to biophysical analysis to detect the presence of various metals, and degree of binding affinity, such as IMAC-HPLC and EPR to determine whether transition metals are involved will be carried out in collaboration here at LBNL and possibly with investigators at other institutions.

### Aim 3. To determine the three-dimensional structure of latent TGF-β

In collaboration with Dr. Peter Walian here at LBNL, the crystal structure of LTGF-β1 is being determined. Currently in the laboratory, we have a large amount of expressed and purified LTGF-β2. We have been using this material to test crystallization conditions. Because LTGF-βs are glycosylated proteins, the sugar moieties can interfere with crystallization. LTGF-β2 has been methodically deglycosylated in a manner that retains latency and native conformation as assessed by activation studies. Crystallization trials are underway to determine conditions necessary for crystal formation. Conditions determined for LTGF-β2 will be the starting conditions for crystallization of LTGF-β1. To begin characterization of steps needed for LTGF-β1 crystallization, we have determined that deglycosylation of LTGF-β1 does not induce activation, as has previously reported in the literature by others. Therefore, removal of the sugar moieties will be performed so that crystallization trials can begin when sufficient quantities of LTGF-β1 are available. In order to obtain the quantities of LTGF-β1 needed for crystallization studies, HEK-293 cells have been stably transfected with LTGF-β1 containing a substitution of cysteine 33 with alanine. This was required because LTGF-β1 can be covalently bound to latent TGF-β binding protein via Cys 33. Because we are expressing only LTGF-β1, this cysteine residue would be unpaired and potentially cause problems by forming inappropriate disulfide bonds. We have the capacity to scale up production of this protein to obtain the quantities needed for crystallization studies and we have devised a preliminary purification scheme.

## Key Research Accomplishments

1. Stable expression of LTGF-β1 and development of a preliminary purification scheme in order to obtain quantities of the protein needed for biochemical and structural studies.
2. Deglycosylation of LTGF-β2 and LTGF-β1 in a manner that retains the latency of the cytokine. This step is important in order to prepare for crystallization studies

## Reportable Outcomes

Jobling, MF, Mott, JD, Finnegan, M.T. Jurukovski, V., Erickson, A.C., Walian, P.J., Taylor, S.E., Ledbetter, S., Lawrence, C.M. Rifkin, D.B. and Barcellos-Hoff, M.H. Isoform-Specific Activation of Latent Transforming Growth Factor β (LTGF-β) by Reactive Oxygen Species. *Radiation Research* (in press) **Proof Attached**

## Additional Comments

Since departure of Dr. Michael Jobling, Dr. Joni Mott has been overseeing the continuation of these experiments. We requested a change in primary trainee, which was approved. In April, Dr Irineu Illa-Bochaca arrived and began required training to work on this project by learning TGFβ biology.

# Appendix

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

Michael F. Jobling,[a] Joni D. Mott,[a] Monica T. Finnegan,[a] Vladimir Jurukovski,[c,1] Anna C. Erickson,[a] Peter J. Walian,[a] Scott E. Taylor,[a] Steven Ledbetter,[b] Catherine M. Lawrence,[b] Daniel B. Rifkin[c] and Mary Helen Barcellos-Hoff[a,2]

*[a] Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, California, 94720; [b] Genzyme Corporation, Cambridge, Massachusetts, 02139; and [c] Departments of Cell Biology and Medicine, New York University School of Medicine, New York, New York 10016*

**Jobling, M. F., Mott, J. D., Finnegan, M. T., Jurukovski, V., Erickson, A. C., Walian, P. J., Taylor, S. E., Ledbetter, S., Lawrence, C. M., Rifkin, D. B. and Barcellos-Hoff, M. H. Isoform-Specific Activation of Latent Transforming Growth Factor β (LTGF-β) by Reactive Oxygen Species. *Radiat. Res.* 167, 000–000 (2006).**

**The three mammalian transforming growth factor β (TGF-β) isoforms are each secreted in a latent complex in which TGF-β homodimers are non-covalently associated with homodimers of their respective pro-peptide called the latency-associated peptide (LAP). Release of TGF-β from its LAP, called activation, is required for binding of TGF-β to cellular receptors, making extracellular activation a critical regulatory point for TGF-β bioavailability. Our previous work demonstrated that latent TGF-β1 (LTGF-β1) is efficiently activated by ionizing radiation *in vivo* and by reactive oxygen species (ROS) generated by Fenton chemistry *in vitro*. In the current study, we determined the specific ROS and protein target that render LTGF-β1 redox sensitive. First, we compared LTGF-β1, LTGF-β2 and LTGF-β3 to determine the generality of this mechanism of activation and found that redox-mediated activation is restricted to the LTGF-β1 isoform. Next, we used scavengers to determine that ROS activation was a function of OH· availability, confirming oxidation as the primary mechanism. To identify which partner of the LTGF-β1 complex was functionally modified, each was exposed to ROS and tested for the ability to form a latent complex. Exposure of TGF-β1 did not alter its ability to associate with LAP, but exposing LAP-β1 to ROS prohibited this phenomenon, while treatment of ROS-exposed LAP-β1 with a mild reducing agent restored its ability to neutralize TGF-β1 activity. Taken together, these results suggest that ROS-induced oxidation in LAP-β1 triggers a conformational change that releases TGF-β1. Using site-specific mutation, we identified a methionine residue at amino acid position 253 unique to LAP-β1 as critical to ROS-mediated activation. We propose that LTGF-β1 contains a redox switch centered at methionine 253, which allows LTGF-β1 to act uniquely as an extracellular sensor of oxidative stress in tissues.** 

## 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, non-covalent 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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**TABLE 1**
**Primers Used for Generation of Methionine Mutants**

| Mutation | Mutagenic primers |
|---|---|
| Met 112-Ala | Forward: 5′-gtcacccgcgtgctaGCggtggaaacccacaacg-3′ |
| | Reverse: 5′-cgttgtgggtttccaccGCtagcacgcgggtgac-3′ |
| Met 132-Ala | Forward: 5′-gtacacacagcatatatGCgttcttcaacacatcagagc-3′ |
| | Reverse: 5′-gctctgatgtgttgaagaacGCatatatgctgtgtgtac-3′ |
| Met 253-Ala | Forward: 5′-gccaccattcatggcGCgaaccggcctttcctgc-3′ |
| | Reverse: 5′-gcaggaaaggccggttcGCgccatgaatggtggc-3′ |

*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-β 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 by 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-β is activated rapidly and globally by ionizing radiation *in vivo*[3] (*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-β *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-β 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 $OH^{\bullet}$, 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 multicellular 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-dihydroxyphenoxazine (A-6550) was purchased from Molecular Probes (Eugene, OR). Coumarin-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-deionized water. All other materials 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 (NCI, Bethesda, MD). The entire cDNA was excised from the pBluescript KSII(+) vector (Stratagene) using *Hin*dIII and *Bam*HI, 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 m*M* 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 an $SO_3$-Fractogel (EMD Chemicals Inc.) cation exchange column, eluted in 50 m*M* sodium phosphate, 500 m*M* sodium chloride, pH 7.0, and stored frozen at −40°C. LTGF-β2 was then passed through 10 ml of Sephadex G-25 size-exclusion resin to remove low-molecular-weight impurities. Further purification was achieved using a Hewlett 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 230 nm. Eluted protein was concentrated under vacuum, resuspended in 10 m*M* 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 (NM_000660) with GC in the mutagenic primers (Table 1). The TGF-β1 gene, cloned into pcDNA3.1, 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 Fe(III)/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

[3] E. J. Ehrhart, HZE and Proton Induced Microenvironment Remodeling Mediated by Transforming Growth Factor-β1. Department of Radiological Health Sciences, Colorado State University, Ft. Collins, CO, 1996.

achieved by selection with G418 in DMEM supplemented with 5% FBS. To collect medium containing LTGF-β1 cysteine 33→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 TGF-β.* The biological activity of TGF-β was measured by monitoring luciferase activity in mink lung epithelial cells transfected with a plasminogen activator inhibitor 1 (PAI-1) promoter-luciferase reporter construct (*23*). Briefly, 50 μl of $3.2 \times 10^5$ 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 siliconized tubes. Three hours after cell plating, samples or standards were added to wells and incubated for 16–19 h at 37°C in 95% air/5% $CO_2$. 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 EG&G Berthold Microlumat LB 96P (Oak Ridge, TN) for 10 s immediately after autoinjection of 50 μl of luciferase substrate and recorded as relative light units integrated over time. A standard curve using rTGF-β1 was generated in every experiment. In some experiments, a tenfold excess of polyclonal TGF-β3 neutralizing antibody (20–80 ng/ml) was added to confirm isoform specificity. DNA quantification was assayed to ensure that addition of experimental variables such as ROS scavengers did not affect cell viability.

*Treatment of TGF-β with ascorbic acid and Fe(III).* All experiments were conducted in siliconized plastic tubes, and solutions were prepared with sterile, deionized water treated with the metal-chelating resin chelex-100 (1 g/10 ml) by stirring overnight. Untreated LTGF-β1 was used to identify the activity of the starting material, and heat treatment of LTGF-β1 at 80°C for 5 min was used as a positive control for activation (*8*). The standard condition for ROS-mediated LTGF-β activation was incubation with ascorbic acid (200 μ*M*) and ferric chloride (20 μ*M* plus 200 μM EDTA) in metal-depleted saline at 37°C for 2 h with agitation (*21*). rTGF-β was substituted for LTGF-β in some experiments. Substitution of dehydroascorbic acid (DHA; 200 μ*M*) for ascorbic acid was carried out in the same manner. ROS scavengers, SOD (16 U), heat-denatured SOD, catalase, heat-denatured catalase, and hydrogen peroxide were added prior to addition of Fe(III)/ascorbate. Addition of Fe(II) (20 μ*M*) and $H_2O_2$ (at indicated concentrations) to LTGF-β1 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 addition of the cytokine. Purified LTGF-β2 and medium conditioned by CHO-K1 cells overexpressing LTGF-β3 were exposed under the same conditions described above for the LTGF-β1 studies.

*Detection of $H_2O_2$ after ROS scavenger addition.* The $H_2O_2$ assay was adapted from information provided by Molecular Probes (Eugene, OR). A 50 μ*M* solution of A-6550 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 μ*M*. Each sample was scanned 30 min after addition of samples using a Fluorescence multi-well plate reader (Series 4000, PerSeptive Biosystems, Dublin, CA) at 530 nm excitation and 590 nm emission. A standard curve of $H_2O_2$ was prepared for each experiment and used to determine the amount of $H_2O_2$ in the experimental samples.

*Detection of the hydroxyl radical.* The $HO^{\cdot}$ detection method was adapted from Makrigiorgos *et al.* (*24*). Coumarin-3-carboxylic acid (CCA, 1 m*M*) was dissolved in buffer (10 m*M*, sodium phosphate, pH 7.4, 0.9% 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 CCA solution in a 96-well plate at 0, 60 and 120 min after generation of ROS. Each sample was measured as described above at 360 nm excitation and 460 nm emission.

*Gel electrophoresis.* Electrophoresis of LAP-β1 and LAP-β2 was performed on a Phastgel gel system (Amersham-Pharmacia) using 8–25% SDS-PAGE gels and was visualized by Coomassie Brilliant Blue.

*Circular dichrioism.* Circular dichroism was performed on untreated, heat-treated or Fe(III)/ascorbate-treated samples of LAP-β1 (carrier-free, from R&D Systems) using a Jasco J-810 system. Samples were diluted in 5 m*M* 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 μ*M* LTGF-β was prepared in 10 m*M* potassium phosphate and mixed with 0.1 ml of 5,5′-dithiobis(2-nitrobenzoic) acid (4 mg/ml in 10 m*M* 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 m*M* potassium phosphate). The concentration of sulfydryl groups was calculated from the equation: concentration of sulfydryl groups = $[A_{410}(\text{sample}) - A_{410}(\text{reference})]/13650$. This value allowed the percentage of reduced cysteines to be calculated based on TGF-β1 and TGF-β2 having 9, LAP-β1 having 3, and LAP-β2 having 5 cysteine residues.

*LAP and TGF-β1 recombination.* To determine which partner was functionally modified by ROS, TGF-β1 or LAP-β1 was pretreated with Fe(III)/ascorbate for 2 h at 37°C. In some experiments this treatment was followed by exposure to 2 m*M* dithiothreitol (DTT) for 2 h prior to recombination. Untreated TGF-β1 and LTGF-β1 were used as positive and negative controls, respectively. ROS-exposed and unexposed TGF-β1 (1 μg/ml) and LAP-β1 (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 LTGFβ-1 Isoform*

Our previous studies demonstrated that LTGFβ-1 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-β2 and LTGF-β3 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 plasminogen 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-β1 produced a greater amount of TGF-β1 activity than did heat treatment. This phenomenon was observed consistently with purified recombinant LTGF-β1. As expected, purified recombinant LTGF-β2 was efficiently heat-activated to levels similar to that of LTGF-β1, but treatment with Fe(III)/ascorbate produced no significant activation, in direct contrast to the efficient LTGF-β1 activation observed by ROS treatment.

Because LTGF-β3 is not available commercially, it was overexpressed in CHO-K1 cells to evaluate whether LTGF-β3 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-

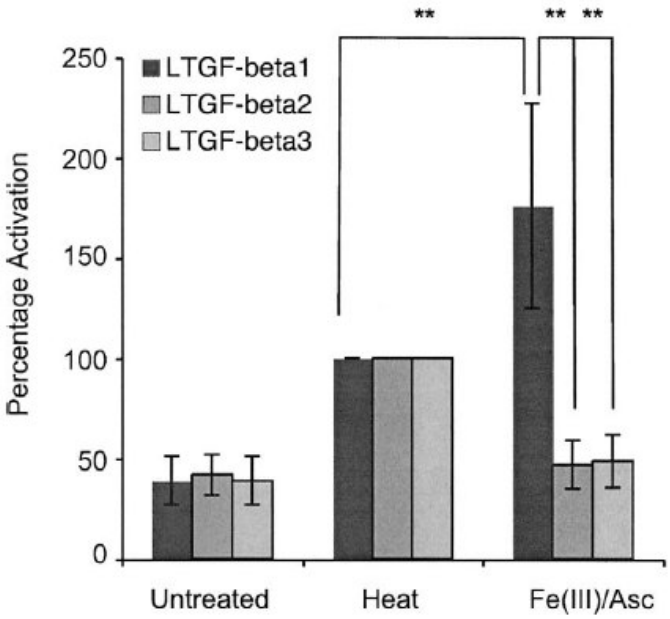


**FIG. 1.** Isoform specificity of LTGF-β activation exposed to ROS generated by Fe(III)/ascorbate. LTGF-β1 was substantially activated after treatment with the ROS-producing Fe(III)/ascorbate system. Neither LTGF-β2 nor LTGF-β3 was activated to the same extent by the ascorbate system. The values plotted 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 $2.6 \times 10^{-4}$ 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 $2.3 \times 10^{-6}$ and $2.1 \times 10^{-6}$, respectively.

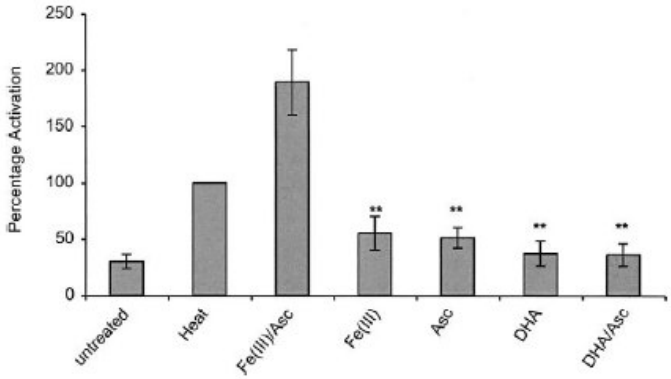


**FIG. 2.** Activation of LTGF-β1 by the components of the Fe(III)/ascorbate system. Individual components, Fe(III) or ascorbic acid, did not activate LTGF-β1 to levels observed with the complete system. A byproduct of the Fe(III)/ascorbate Fenton chemistry, DHA, also did not activate LTGF-β1, nor did 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 $1.4 \times 10^{-5}$ for Fe(III), $3.3 \times 10^{-5}$ for ascorbate, $1.3 \times 10^{-5}$ for DHA, and $1.5 \times 10^{-5}$ for DHA and ascorbate.

tioned medium resulted in significant TGF-β activity, which was abolished by the addition of TGF-β3-specific neutralizing antibody (data not shown). Fe(III)/ascorbate treatment of medium conditioned by the LTGF-β3-producing CHO-K1 cells did not generate significant TGF-β activity. Although unlikely, it was possible that the conditioned medium contained substances that interfered with ROS activation of the LTGF-β3. Therefore, to rule this out, the conditioned medium was spiked with LTGF-β1 and subjected to Fe(III)/ascorbate activation. Activation of LTGF-β1 was observed, suggesting that no substances were present in the conditioned medium that interfered with Fe(III)/ascorbate activation. These results indicate that ROS activation is isoform-specific and that only the LTGF-β1 isoform is susceptible.

### *Hydroxyl Radicals are Responsible for ROS Activation of LTGFβ-1*

The Fe(III)/ascorbate reaction generates a spectrum of ROS, including $HO^{\bullet}$, $O_2^{-\bullet}$ and $H_2O_2$. 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)/ascorbic 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 $HO^{\bullet}$ and $H_2O_2$ 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 $O_2^{-\bullet}$, $HO^{\bullet}$ and $H_2O_2$ were added to the reaction individually. Superoxide dismutase (SOD) efficiently removes $O_2^{-\bullet}$ 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 $O_2^{-\bullet}$ 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 $O_2^{-\bullet}$ 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 effect the bioactivity of TGF-β1.

Other candidates for the ROS-mediated activation were $H_2O_2$ and $HO^{\bullet}$. Catalase promotes the dismutation of $H_2O_2$ to $O_2$ and $H_2O$. Thus its presence would reduce the amount of available $H_2O_2$, but because $H_2O_2$ can be an intermediate precursor to $HO^{\bullet}$, reducing $H_2O_2$ would also lead to a concomitant decrease in $HO^{\bullet}$. 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 $H_2O_2$ levels by 25% and $HO^{\bullet}$ levels by 26% at 2 min (Table 2). Thus scavenging $H_2O_2$ or interfering with production of $HO^{\bullet}$ reduced LTFG-β1 activation.

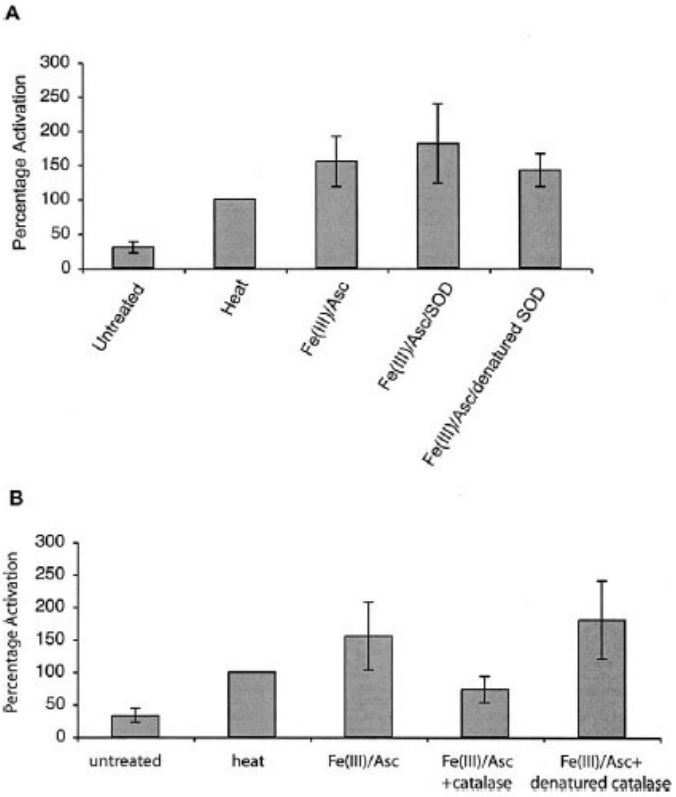


**FIG. 3.** Effect of scavenging by SOD and catalase on the activation of LTGF-β1 by ROS. Panel A: The presence of SOD, an enzymatic scavenger of superoxide, did not inhibit the ability of ROS to activate LTGF-β1. Denatured SOD was used as a negative control. Experimental values are expressed as the percentages of active TGF-β observed after heat activation. Panel B: Catalase, an enzymatic scavenger of $H_2O_2$, 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 $H_2O_2$ was not directly responsible for activation of LTGF-β1. Addition of $H_2O_2$ in concentrations ranging from 10–400 μ*M* directly to solutions of LTGF-β was not sufficient for activation (*21*). Furthermore, addition of higher concentrations of $H_2O_2$ did not lead to activation LTGF-β. Thus our attention

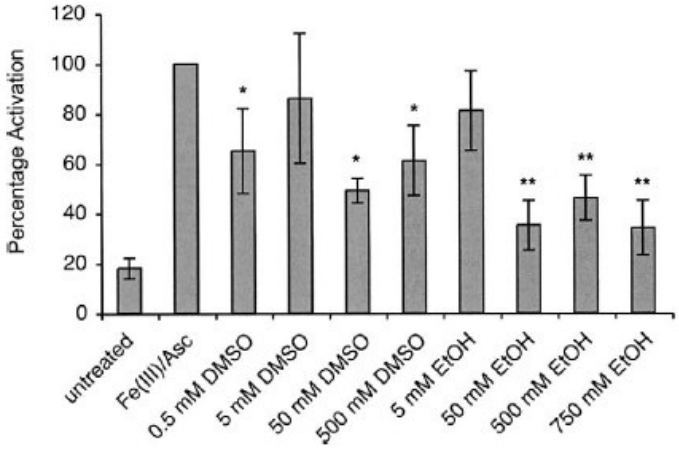


**FIG. 4.** Hydroxyl radical scavengers inhibited ROS-mediated activation of LTGF-β1. DMSO and ethanol (EtOH) scavenge hydroxyl radicals, and increasing concentrations of either reagent blocked activation of LTGF-β by ROS. Values are expressed as percentage of active TGF-β after Fe(III)/ascorbate activation. Ethanol blocked activation more efficiently, with *P* values of 0.003, 0.008 and 0.003 at 5, 500 and 750 m*M*, respectively, compared to Fe(III)/ascorbate activation, using a two-tailed Student's *t* test.

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 m*M* to 500 m*M*) added to the Fe(III)/ascorbate system significantly reduced the amount of LTGF-β1 activation (Fig. 4). The greatest effect was observed at 50 m*M* DMSO. Parallel studies confirmed that addition of 500 m*M* 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 $H_2O_2$ 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**
**Amount of $H_2O_2$ and HO• Produced after LTGF-β1 Treatment with Fe(III)/Ascorbate and Fe(III)/Ascorbate + Catalase**

| | Time (min) | Control | Fe(III)/ascorbate | Fe(III)/ascorbate + catalase | Fe(III)/ascorbate + denatured catalase |
|---|---|---|---|---|---|
| $H_2O_2$ | 2 | 50 | 100 | 76 (±3) | 72 (±4) |
| | 60 | 48 | 141 (±8) | 103 (±6) | 137 (±9) |
| | 120 | 48 | 183 (±10) | 128 (±8) | 189 (±7) |
| HO• | 2 | 35 | 100 | 74 (±3) | 100 (±5) |
| | 60 | 42 | 87 (±2) | 93 (±7) | 86 (±6) |
| | 120 | 29 | 111 (±18) | 100 (±9) | 114 (±7) |

*Notes.* Values are expressed as percentages (±SE) of the Fe(III)/ascorbate sample at 2 min, which corresponds to 0.65 ± 0.06 m*M* $H_2O_2$. 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-β/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 m*M*) of a mild reducing agent, dithiothreitol

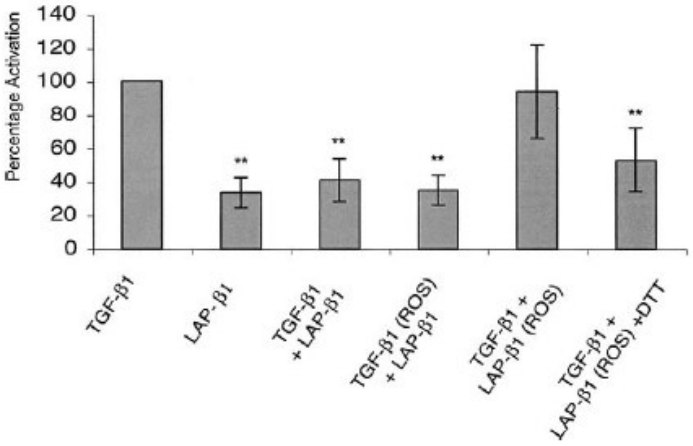


**FIG. 5.** ROS exposure of LAP-β1 prevented neutralization of TGF-β1 activity. Incubation of LAP-β1 with TGF-β1 in solution neutralizes the biological activity of TGF-β1 presumably by the formation of the latent complex. Treatment of TGF-β1 with ROS prior to co-incubation did not hinder neutralization of TGF-β1 activity. However, treatment of LAP-β1 with ROS prior to co-incubation did not neutralization TGF-β1 activity. Addition of DTT (2 m*M*) to reduce LAP-β1 after ROS treatment restored the neutralization of TGF-β1 activity by LAP-β1, suggesting that the oxidation events occurring in LAP-β1 are reversible under mild reducing conditions. Two-tailed Student's *t* test *P* values for LAP-β1, TGF-β1 + LAP-β1, ROS-treated TGF-β1 + LAP-β1, and TGF-β1 + ROS-treated LAP-β1 followed by reduction with DTT are $7 \times 10^{-12}$, $7 \times 10^{-9}$, $1 \times 10^{-11}$ and $4 \times 10^{-8}$, respectively.

(DTT). DTT at this low level is not detrimental to the activity of the TGF-β 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. Walien, 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 Fe(III)/ascorbate. Results showed that LTGF-β1 Cys33Ser was activated by Fe(III)/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.

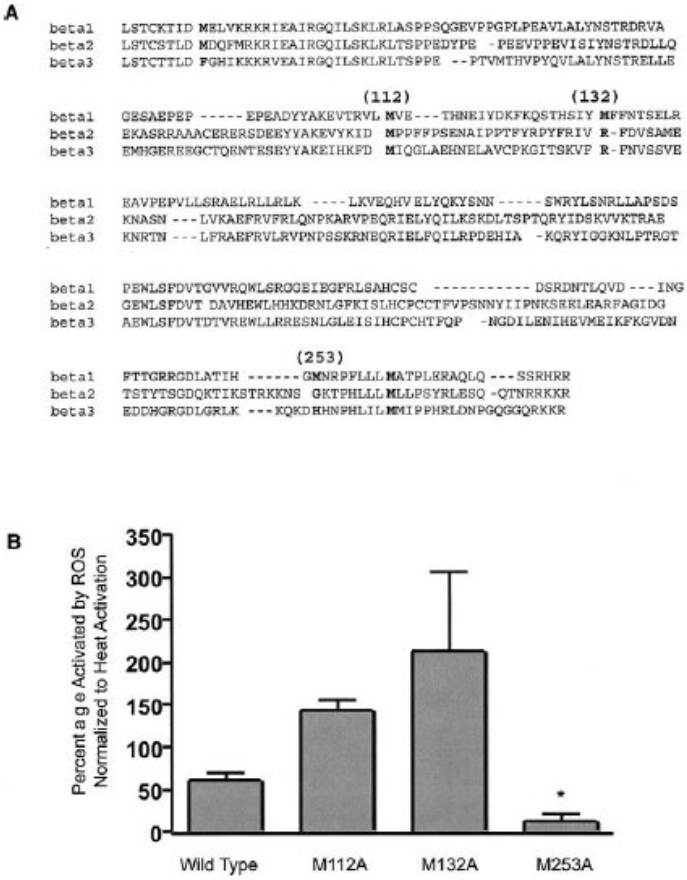


**FIG. 6.** Susceptibility of LTGF-β1 mutants to ROS activation. Panel A: The LAP sequences of the three LTGF-β isoforms were aligned using Multiple Sequence Alignment (MAFFT) via the ExPASy Proteomics Server at http://ca.expasy.org/. The sequences were analyzed with Blosum62 using a gap opening penalty 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 LAB-β1. Panel B: Medium conditioned by HEK-293 cells transiently expressing wild-type or mutant LTGF-β1 was treated with heat or Fe(III)/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 (*21*).

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 ranges of tissue processes that generate ROS underscore 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 $HO^{\bullet}$ 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 antioxidant 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 (*35, 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 $H_2O_2$ in bovine epithelial cells (*43*), and ROS is involved in TGF-β-induced apoptosis of hepatocytes (*44*). It has been reported that in the presence of heme peroxidases, TGF-β1-induced $H_2O_2$ 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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