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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Radiation elicits rapid and persistent changes in the mouse mammary microenvironment, and persistent transforming growth factor-b (TGF-b) activation. TGF-b has widely diverse functions in regulation of proliferation and cell fate-decisions, which contribute to a variety of tissue processes.

TGF-b superfamily includes three mammalian isoforms (b1, b2, and b3) that are secreted in a latent complex formed by non-covalent association with the relevant latency-associated peptide (LAP1, LAP2, or LAP3). Mouse models utilizing protein knockout protocols reveal that the three isoforms have distinctly different roles in the cellular pathway, as seen by the mouse phenotypes. Activation of TGF-b involves dissociation of this complex and perhaps degradation of LAP. However the mechanism and mode of activation is poorly understood.

We postulate that the presence of metals in the latent complex could provide a redox active center for this process. Given the LAP sequence differences we hypothesized that redox-mediated activation will be isoform specific.

Redox mediated activation offers a novel route for TGF-b1 involvement in chronic tissue processes in which oxidative stress is implicated and would endow LTGF-b1 with the ability to both sense extracellular oxidative stress and transduce the signal by eliciting changes in diverse cell types.

14. SUBJECT TERMS
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BODY

SPECIFIC AIMS

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

(a). To determine if amino acids specific to LAP1 are the underlying reason ROS can only activate LTGF-β1, we overexpressed mutant forms of LTGF-β1 containing altered susceptibility to oxidation.

(b). Using the established PAI-luciferase assay we tested to see if ROS activation of these mutated proteins had been modified.

(c). Identify specific amino acid modifications that occurred during activation.

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

(a). We have begun to develop a series of mutated latent TGF-β proteins containing altered affinity for metal interactions.

(b). 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.

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

(a). We have begun to design and develop a series of constructs, appropriate for LTGF-β expression in bacteria, insect and mammalian cells, and isolate overexpressed protein.

(b). Perform crystallization trials to grow suitable crystals for x-ray diffraction.

(c). Collect diffraction data from synchrotron sources, and structures solved by molecular replacement protocols and refined by standard software methods.

PROGRESS

To date, I have made significant headway towards completing Aim #1, and begun the first stages of Aim #2. In collaboration with Dr. Peter Walian of LBNL, we have discussed and explored the appropriate steps needed to accomplish Aim #3.

Along with the preliminary data outlined in the original fellowship proposal, the work to date was presented at the 10^{th} annual meeting of the Society for Free Radical Biology and Medicine held in Seattle, WA in November of 2003.

AIM 1

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

In order to determine if there are specific amino acids contained within LAP1 that facilitate ROS activation, we developed a series of LTGF-β1 mutant proteins. In collaboration with Dr. Dan Rifkin at NYU, we identified three methionine residues (at positions 253, 132 and 112) and mutated the protein sequence to alanine at the appropriate site. Three single methionine mutants, three double mutants, and one triple mutant were created. The constructs were incorporated into the mammalian cell lines, 293T and the TGF-β1 knockout liver fibroblast. Conditioned media was collected from the cell culture for use in the PAI-luciferase assay to measure TGF-β activity.

Each of the mutant proteins displayed biological activity after heat activation indicating that the methionine mutation had not adversely affect the manner in which LAP1 and TGF-β1 complex together (Figure 1). Following treatment with ROS, all proteins were activated to a similar degree as the wild-type LTGF-β1, except those mutants that contained the Met253Ala mutation. The activity of the single Met253 mutant (M253) after ROS was reduced by nearly 75%, and the two double mutants containing Ala253 (M253/M132 and M253/M112) by approximately 60%. These results implicate Met253 as having a key role in the ability of ROS to activate LTGF-β1. The fact that the M253/M132 and M253/M112 mutants have a lower activity than the single M253 mutant also may suggest that while Met132 and Met112 are not crucial for this mode of activation to proceed, these two sites may have some stabilization effect on the latent complex in the absence of Met253.

Initial experiments of the triple methionine mutant (MMM) also displayed a significantly reduced ROS susceptibility. However over time this affect, along with the increasing activity of the untreated sample, indicated that this protein was unstable and becoming constitutively active (data not shown).

As previously demonstrated, addition of 0.2mM DTT following ROS treatment is sufficient to reverse the oxidation event and allow recombination of the latent complex (Figure 2). Incubation with DTT following ROS treatment allowed all mutant TGF-β1 proteins to reform the latent complex as indicated by a significant decrease in TGF-β1 activity. These results demonstrate that the mutated proteins behave in a manner similar to the wild-type protein suggesting that the oxidative effect of ROS treatment is still reversible. As expected DTT treatment of the Met253Ala mutants did not alter the activity following ROS since the protein is resistant to oxidative activation.

Interestingly, when using the proteins expressed in the 293T cell system, the DTT treatment was not able to fully reduce the amount of TGF-β1 activity to baseline levels (data not shown). Addition of a TGF-β1 neutralizing antibody did lower the observed activity to baseline levels. It is hypothesized that the 293T cells produce a small amount of LTGF-β1 naturally that affected baseline levels, however this does not affect the results seen to date.

AIM 2

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

After identifying some promising regions to undergo oxidative modification, we will further explore these sites as a potential metal center. The mutated proteins described above will be the first series of proteins to be examined. At this stage all experiments have been using conditioned media from transient transfections of the two cell systems. To express the required amounts of protein to complete aim #2, stable transfections will be required. This work is currently in progress.

AIM 3

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

In a new collaboration with Dr. Peter Walian at LBNL, we have begun to address the need for significant quantities of the wild-type TGF-β1 protein. While suggested in the initial statement of work that bacteria expression systems would be used, it appears that such systems will not provide correctly folded LTGF-β1 and so we are exploring other options.

KEY RESEARCH ACCOMPLISHMENTS

- LTGF-β1 and 7 LTGF-β1 methionine mutants expressed in 293T and TGF-β1 knockout fibroblasts.
- Biological activity of mutant TGF-β1 determined. Methionine 253 mutant displays altered behavior to reactive oxygen mode of activation.

REPORTABLE OUTCOMES

1) Paper of preliminary data titled "Isoform specific regulation of latent transforming growth factor-β activation by reactive oxygen species" has been submitted to *Radiation Research.*
2) Abstract titled "Isoform specific regulation of latent transforming growth factor-β activation by reactive oxygen species" for a poster presented at the 10th annual meeting of the Society for Free Radical Biology and Medicine that took place in Seattle (USA) in November, 2003.

CONCLUSIONS

By examining the mutated proteins using the luciferase reporter assay we have determined methionine-253 to be critical in maintaining a LTGF-β1 complex that is susceptible to ROS activation. The other two methionines examined (Met132 and Met112) are not necessary for ROS to be effective although in combination with Met253 may enhance the stability of the latent TGF-β1 complex.

APPENDICES

Appendix I: Figure legends and figures
FIGURES

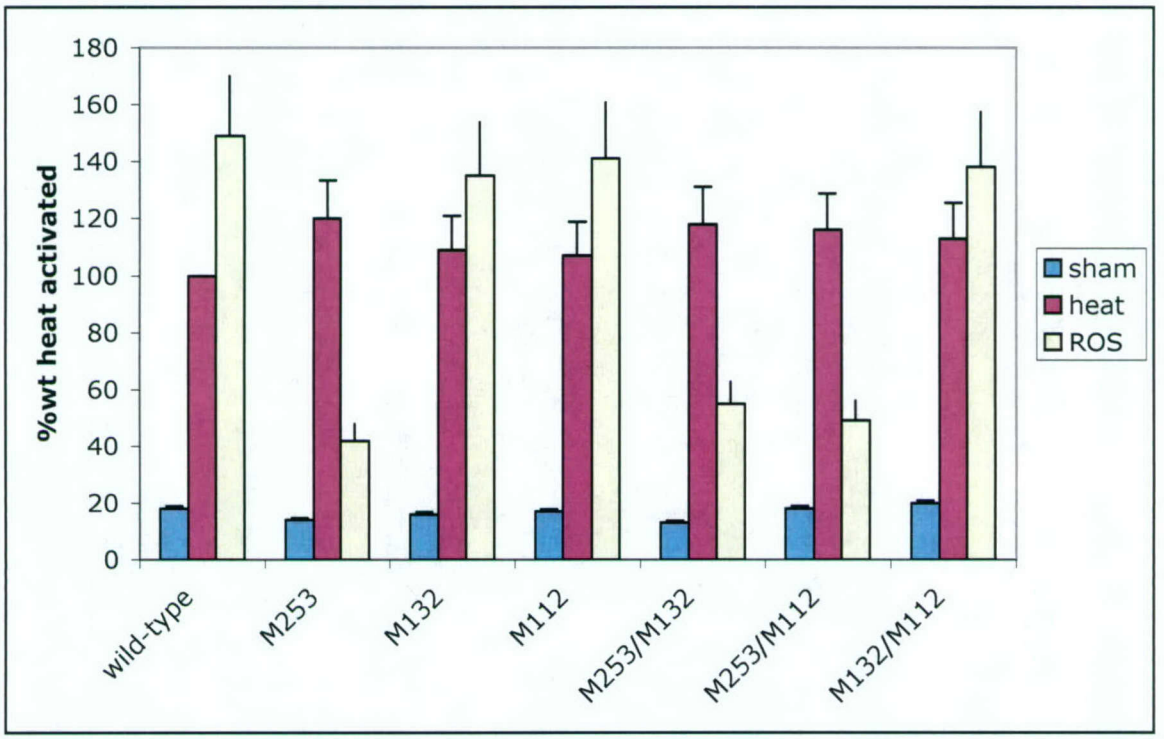

Figure 1. Effect of ROS treatment on wild-type LTGF-β1 and methionine mutants. All samples were treated with the standard Fe/asc system for 2 hours at 37°C prior to exposure to the reporter assay. Measurements are relative to the heat-treated (80°C 5 mins) wild-type sample. Following the trend of the wild-type protein, the M132, M112, and M132/M112 mutants are activated by ROS 20-40% more than is seen with heat activation. The M253 containing mutants show a significantly decreased amount of active TGF-β1 after ROS treatment. All samples demonstrate activity after heat activation, indicating biological relevance of the expressed protein.

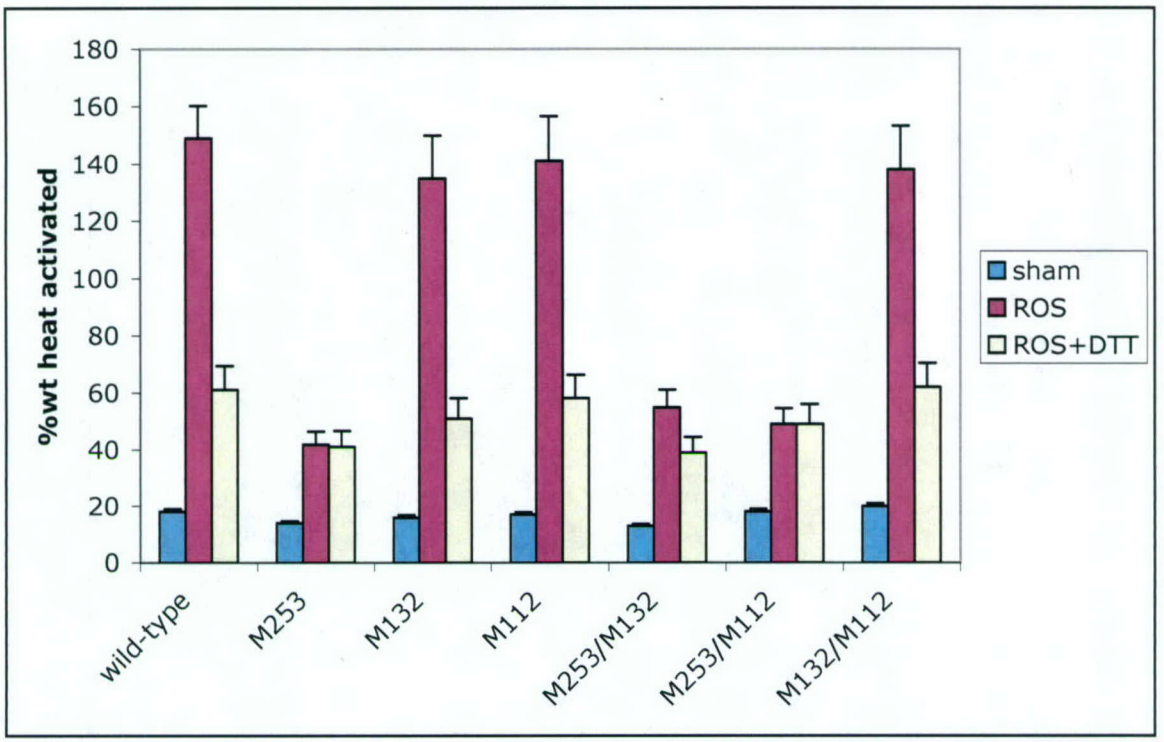

Figure 2. Effect of DTT on ROS treated LTGF-β1 protein. Samples were treated with 2mM DTT for 2 hours following ROS activation and incubated for a further 2 hours prior to the assay. The wild-type, M132, M112 and M132/M112 proteins display a dramatic drop in activity suggesting that the LTGF-β1 complex has reformed in solution. The M253 containing samples have no change or a smaller loss in activity indicating that there was no oxidative event to be reduced allowing the LAP1 and TGF-β1 interaction.