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13. ABSTRACT (Maximum 200 Words)

Reaper is a potent inducer of apoptotic cell death in the fruit fly *Drosoophila melanogaster*. Our lab unexpectedly discovered that Reaper also induces apoptosis in cultured human cells and cell free extracts prepared using eggs from the frog *Xenopus laevis*. In the *Xenopus* model system Reaper must bind another protein, Scythe, to induce apoptosis. Scythe forms a stable complex with the chaperone protein Hsp70 and inhibits its activity. Upon Reaper-Scythe binding, Hsp70 is released and free to refold protein substrates. Here we present evidence that the proapoptotic Bcl-2 family member BAX can be a target of the Reaper-Scythe-Hsp70 pathway. BAX is required for apoptosis mediated by mitochondrial cytochrome c release, and BAX is activated by regulated conformational change. BAX remains an attractive effector of the Reaper-Scythe proapoptotic pathway and further work will more completely characterize this novel mode of proapoptotic signaling.

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Table of Contents

Introduction:

The Drosophila melanogaster protein Reaper is a central regulator of apoptotic cell death. Reaper expression is required for programmed cell death in the fly, and its deletion prevents all developmental apoptosis. To date no vertebrate reaper homologs have been discovered. Several years ago our lab reported that recombinant Reaper causes apoptosis when added to Xenopus egg extracts; furthermore, apoptosis in the Xenopus system results from Reaper induced cytochrome c release from mitochondria into the cytosol [1]. Cytosolic cytochrome c serves as an essential cofactor for activation of caspases; the proteases responsible for the morphologic hallmarks of apoptosis [2]. A former graduate student, Ken Thress, purified a 150 kD Reaper-binding protein from Xenopus egg extracts that he named Scythe. Scythe is highly conserved in vertebrates, and its removal from egg extracts abrogates reaper induced caspase activation in the *Xenopus* system. Furthermore, a truncated Scythe protein induced apoptosis independently of reaper in Xenopus egg extracts [3]. The overall goal of this proposal was to elucidate the mechanism of Scythe/Reaper mediated release of cytochrome c from mitochondria.

Body of Report:

Although this grant was originally awarded to Ken Thress in our laboratory, I was given the opportunity to assume the remainder of the award after Dr. Thress graduated. During his tenure in the lab, Ken completed all of Technical Objective II, and his work established that Scythe is a suppressor of Hsp 70 mediated protein folding activity [4]. Ken also demonstrated that Reaper engagement of Scythe relieves Scythe-mediated Hsp 70 inhibition. Recent work in our laboratory has demonstrated that Reaper, Scythe, and Hsp 70 can act directly on mitochondria to promote cytochrome c release, and this observation will allow us to determine the precise mechanism of Reaper-Scythe mediated cytochrome c release during apoptosis. I have been funded by this agency for approximately two years, and I have continued the work outlined in Technical Onbjective I. In particular I have endeavored to characterize the human Scythe homolog, BAT3, in human cell lines. This work uncovered new roles for Scythe and the human Scythe homolog that I plan to more fully characterize as I complete my PhD. The following results correspond to the original technical objectives outlined in the statement of work:

Task 3: Cloning and characterization of the C-terminal region of BAT3. Although cloning and expression of the C-terminal 312 amino acids of BAT3 was originally proposed, expression of this construct has proved to be difficult. This C-terminal region of BAT3 contains a BAG domain responsible for suppression of Hsp 70 activity [5]. I have successfully expressed the BAT3 BAG domain in 293T cells and assayed its ability to modulate the proapoptotic activity of BAX, a protein known to be necessary for cytochrome c release in mammalian cells [6]. We hypothesize that BAX may be the downstream target of the Scythe/Hsp 70 complex such that Reaper may bind Sycthe

and derepress Hsp 70 allowing it to refold BAX into its active, ptoapoptotic conformation. Note that coexpression of the BAT3 BAG domain and BAX attenuates BAX induced caspase activation in human cells. These results support published observations that Scythe overexpression abrogates apoptosis in Xenopus egg extract, and suggest that Scythe regulates proapoptotic Bcl-2 family proteins, such as Bax, in mammalian cells. See Fig. 1 for documentation of BAT3 attenuation of BAX induced apoptosis.

To further characterize the role of Scythe during apoptosis our lab has developed *in vitro* caspase activation and cytochrome c release assays. I have used these assays to determine if *Xenopus* Scythe can regulate Bax mediated caspase activation and cytochrome c release in the egg extract model system. In particular, I have demonstrated that Scythe is able to suppress BAX mediated caspase activation and cytochrome c release in this context. Furthermore, reaper addition relieves Scythe-mediated suppression of BAX-induced caspase activity. We hypothesize that Scythe may act to inhibit Hsp 70 thereby suppressing the proapoptotic BAX conformation. Reaper presumably causes cytochrome c release in human cells by freeing Hsp 70 from BAT3 thus allowing Hsp 70 to refold BAX into its active, proapoptotic conformation. Experiments are underway to determine if Reaper mediated apoptosis requires BAX in human cells. See Fig. 2 for documentation of Scythe mediated suppression of BAX-induced caspase activity *in vitro*.

Task 4 is currently underway.

Task 5: Apoptotic analysis of human cells transfected with BAT3. Overexpression of full length Scythe in *Xenopus* egg extract suppresses reaper induced apoptosis. This observation suggests that human Scythe, BAT3, may inhibit reaper induced apoptosis in human cells; furthermore, BAT3 may be protect cells from a variety of apoptotic stimuli. To directly test the role of BAT3 during apoptosis of human cells, including breast cancer derived cell ines, I have designed RNA interference (RNAi) oligos to specifically knockdown BAT3 expression in human cells to more clearly elucidate its role during apoptosis. These studies are recently underway, and I anticipate that they will establish a role for BAT3 during human apoptotic cell death. See Fig. 3 for documentation of BAT3 knockdown in 293T cells.

Key Research Accomplishments:

- Demonstration that the BAG domain of BAT3 is able to suppress BAX mediated apoptosis in mammalian cells
- Demonstration that Scythe is able to suppress Hsp 70 mediated BAX refolding *in vitro*
- Successfully ablated BAT3 expression in human cells using RNAi

Reportable Outcomes: None

Conclusions:

The ultimate goal of this proposal is to elucidate the mechanism of Reaper-Scythe induced apoptosis. The work of Dr. Thress demonstrated that Hsp 70 inhibition through Scythe is necessary for Reaper induced apoptosis. Recent work described here strongly suggests that the proapoptotic protein BAX is the relevant target of Hsp 70 during Reaper-Scythe mediated apoptosis. Our work that employs both the *in vitro Xenopus* egg extract system and cultured human cells and will allow us to fully characterize the role of the Scythe-Reaper protein complex during apoptotic cell death.

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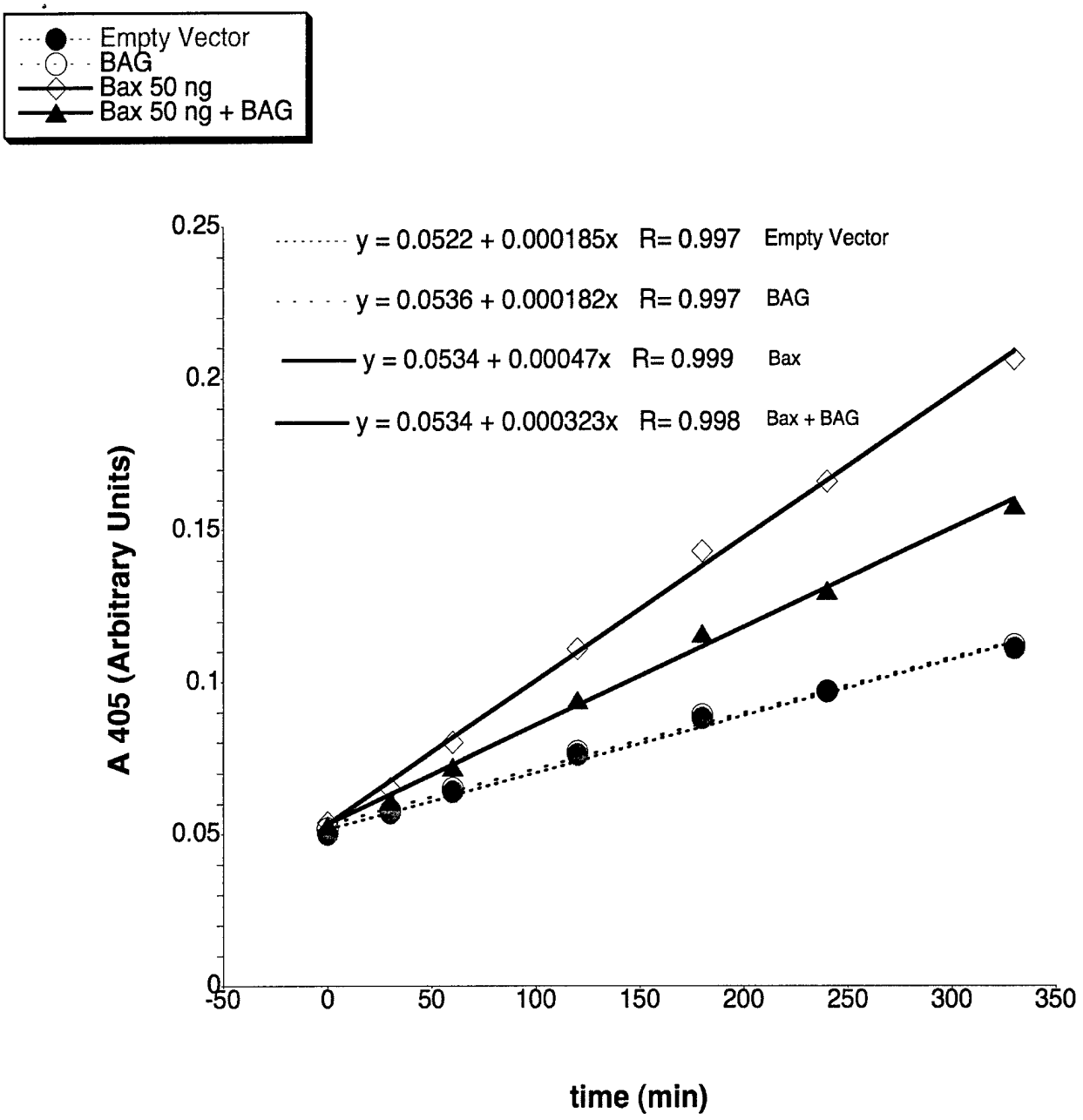
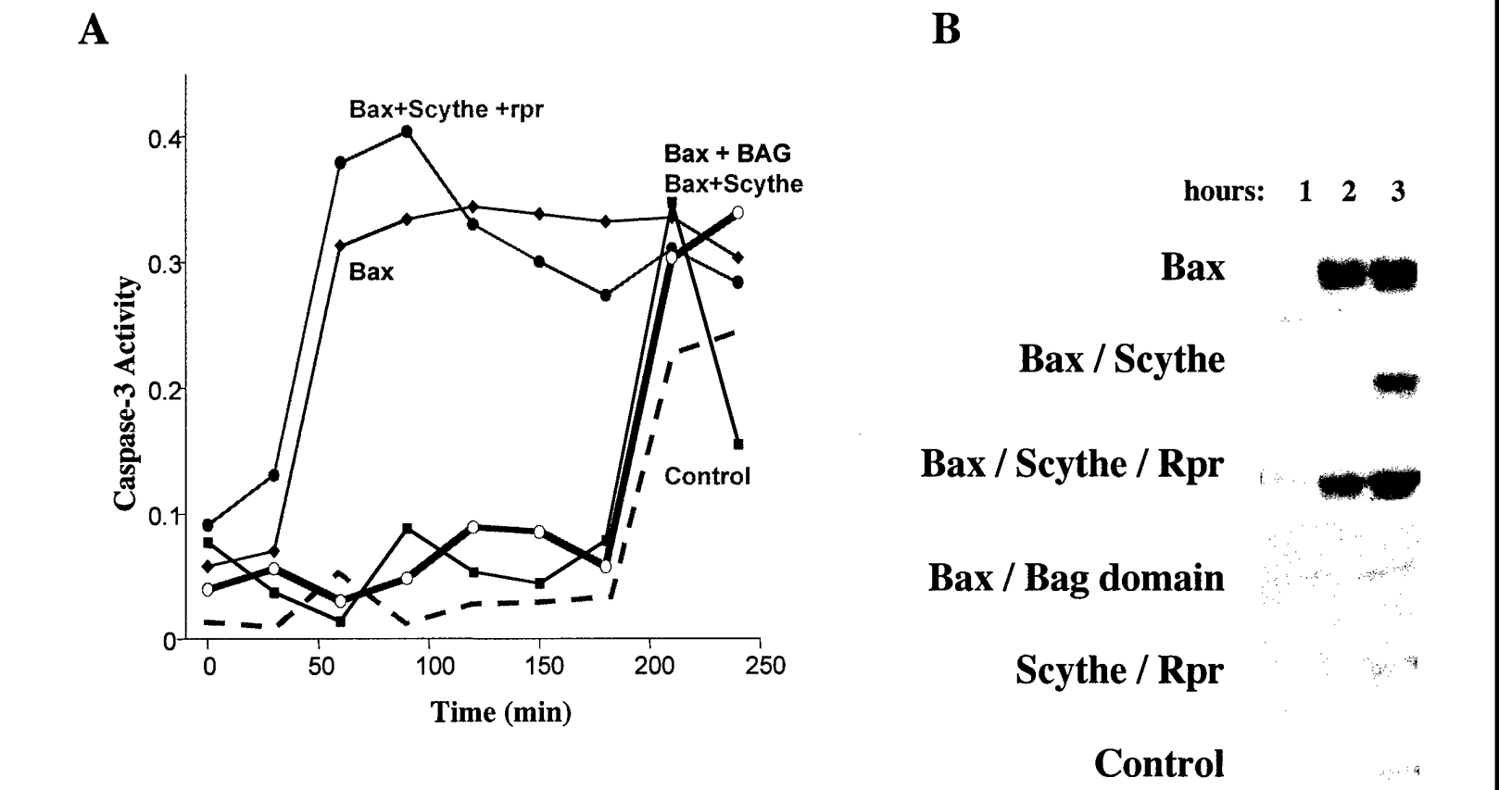


Fig. 1: The BAT3 BAG domain attenuates Bax induced caspase activation in 293T cells. 1.5X106 293T cells were seeded in 10 cm plates and cultured overnight at 37∞C in a humidified 5% CO2 atmosphere. Samples were transfected using standard calcium phosphate techniques with 500 ng BAG domain plasmid and the indicated amount of Bax. Cells were cultured for 24 hrs, lysed in buffer containing 1% CHAPS and assayed for caspase activity by incubation at 37∞C in the presence of a colorimetric pan-caspase DEVD substrate peptide. Increasing absorbance over time indicates caspase activity. The slopes of the linear curve fits reflect the rate of caspase activation.

Figure 2: Bax is a target of Scythe. Recombinant Bax ± (Scythe or Scythe's BAG domain ± Reaper was added to crude *Xednopus* egg extracts and caspase 3 activity was measured via colorimetric DEVDsubstrate **(A)**. Additionally, recombinant proteins were added to purified mitochondria and assays for Cytochrome c release were completed **(B)**. Both Scythe and the BAG domain of Scythe can inhibit Bax induced caspase activation and cytochrome c release. Inhibition by Scythe is reversed by Reaper.



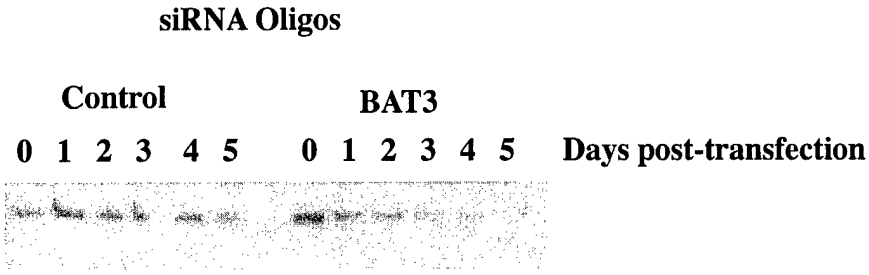


Figure 3: siRNA oligos specifically knockdown BAT3 expression.
RNA oligos were obtained from Dharmacon Inc. (Lafayette, CO), and annealed according to the manufacturer's protocol. HEK 293T cells were seeded in six-well plates at 125,000 cells per well. Each well was transfected with 1.68 μg of antisense control or BAT3 siRNA duplex using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Where indicated, cells were split 1:3 after two days in culture and retransfected with siRNA duplex as described above. At the indicated timepoints, cells were washed once in PBS and lysed in 150 μL lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 0.5% NP-40, 1 mM DTT, and 1X Complete Protease Inhibitor Cocktail (Roche)). BAT3 expression was determined by resolving 50 μg of cell lyste on SDS-PAGE and immunoblotting with antisera raised against the carboxy-terminus of human BAT3.