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PRINCIPAL INVESTIGATOR: James K. Foskett, Ph.D.

CONTRACTING ORGANIZATION: University of Pennsylvania
Philadelphia, Pennsylvania 19104-3246

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James K. Foskett, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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Philadelphia, Pennsylvania 19104-3246

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Apoptosis plays a critical role in growth and development of the mammary gland in normal and pathologic states. An important regulator of apoptosis is the bcl-2 oncogene, whose expression prevents apoptosis and is associated with poor responses to cancer therapies. Other bcl-2-related genes have been identified, defining a gene family with anti- and pro-apoptotic members. The molecular mechanisms which link bcl proteins to apoptosis are unclear. bcl-X_L forms ion channels in artificial membranes. To determine whether these proteins form or regulate ion channels in the endoplasmic reticulum in vivo, we have employed a novel nuclear envelope patch-clamp technique. During the first funding period, we have cloned bcl-X_L and bax into vectors for transient expression in *Xenopus* oocytes. We injected in vitro-transcribed mRNA into oocytes, and have achieved expression as evidenced by Western blotting. Bax expression caused oocyte cell death, as expected based on its pro-apoptotic activity, indicating functional activity. A series of patch clamp of nuclei from injected oocytes has been initiated, although novel ion channel activity has not been detected to date. These results suggest that we have not yet identified the proper conditons for observing channel activity, and that further studies as well as initiation of the other specific aims are necessary.

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FOREWORD

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INTRODUCTION

Apoptosis, the morphological and biochemical manifestation of programmed cell death, plays a critical role in maintaining homeostasis of tissue and organ cell number, and is involved in differentiation, growth and development. Mammary gland physiology is strongly influenced by apoptosis in both normal and pathologic states. Involution of the lactating gland is due to apoptosis of differentiated epithelial cells, and an emerging hypothesis is that dysfunction of the apoptotic pathways in mammary gland is significantly involved in the causes and progression of breast cancer. Thus, definition of the biochemical pathways involved in mammary gland apoptosis is an important goal in breast cancer research. An important regulator of apoptosis is the bcl-2 oncogene. Bcl-2 expression prevents apoptosis in several cell types and is associated with a poor prognosis in response to various cancer therapies in patients. Bcl-2 is normally expressed at high levels in some tissues, including mammary gland. More recently, other bcl-2-related genes have been identified, defining a gene family. Like bcl-2, some are anti-apoptotic, whereas others promote apoptosis. It is likely that the pro:anti -apoptotic expression level ratio regulates sensitivity to apoptosis. Breast cancer is associated with an altered ratio, which correlates with failure to respond to therapy and poor survival. Thus, many breast cancers may be diseases of apoptosis. The molecular mechanisms which link bcl proteins to apoptosis are undefined, although bcl proteins act at a critical juncture which integrates different death signals and activates a single death pathway. Intracellular [Ca^{2+}] and intracellular Ca^{2+} stores may be involved in regulating apoptosis, and expression of bcl-2 has been linked to alterations in Ca^{2+} signaling and in the handling of Ca^{2+} by intracellular stores, including the endoplasmic reticulum (ER) and mitochondria. The bcl proteins are localized to the outer mitochondrial membrane, ER membrane and outer membrane of the nuclear envelope. Recent studies suggest that bcl-related proteins are closely associated with permeability pathways in membranes. Cyotchrome c (CytC) release from mitochondria *in vitro* could be blocked by bcl-2. In addition, bcl-xL was demonstrated to form ion channels in artificial membranes. These data suggest that bcl proteins can form and/or regulate channels, perhaps for organic (e.g. CytC) and well as for inorganic (e.g. Ca^{2+}) molecules. Nevertheless, the physiological relevance of these data are questionable without measurements of channel activity in the membranes in which these proteins normally reside in cells. This has not been possible because the intracellular location of the membranes has prevented use of rigorous electrophysiological approaches, in particular the single-channel patch clamp technique. My laboratory recently developed novel technology for measuring ER- and nuclear envelope-localized ion channel activities. We proposed to employ this approach, together with recombinant bcl proteins, stably-expressing cell lines and expression systems, in a novel series of experiments designed to determine whether bcl-related proteins form ion channels in the ER and nuclear envelope, and whether these proteins regulate the activities and regulation of other permeability pathways which exist in these membranes. The specific aims are to: 1. Determine whether recombinant bcl-related proteins can form functional ion channels in the outer membrane of the nuclear envelope; 2. Determine whether expression of bcl-related proteins confers novel ion channel activities in the outer nuclear

membrane; 3. Determine the role of bcl-like proteins in influencing the activities of resident ion channels in the nuclear envelope and the permeability of the nuclear pore. These studies may provide direct evidence for a biochemical function of proteins critically involved in apoptosis, mammary gland biology and breast cancer.

BODY

Accomplishments during the first year

We proposed to undertake 3 specific aims during the 3 year granting period:

1. Determine whether recombinant bcl-related proteins can form functional ion channels in the outer membrane of the nuclear envelope.

2. Determine whether expression of bcl-related proteins confers novel ion channel activities in the outer nuclear membrane.

3. Determine the role of bcl-like proteins in influencing the activities of resident ion channels in the nuclear envelope and the permeability of the nuclear pore.

Our efforts during the first year had as their focus specific aim 2, with some attention also directed to specific aim 3. As proposed, we initiated experiments to determine whether heterologous expression of bcl-related proteins would result in novel ion channel activities in the outer membrane of the nuclear envelope. Our focus thus far has been on the *Xenopus* oocyte system, because of our familiarity with the procedures involved in the isolation of intact nuclei, patch clamp electrophysiology of the outer membrane, and expression of recombinant ion channels in this system. Because the *Xenopus* oocyte can express recombinant proteins, we reasoned that bcl-related proteins could be expressed and localized to the nuclear envelope, as in mammalian cells, and that patch clamp of the ioslated nucleus could provide an opportunity to record ion channel activities which they might possess. Because recombinant protein expression in oocytes is facilitated by mRNA injection, we first attempted to clone bcl-X_L and bax into the pSP64 oocyte expression vector, to ensure consistent and optimal, high-level expression . This project was undertaken by a

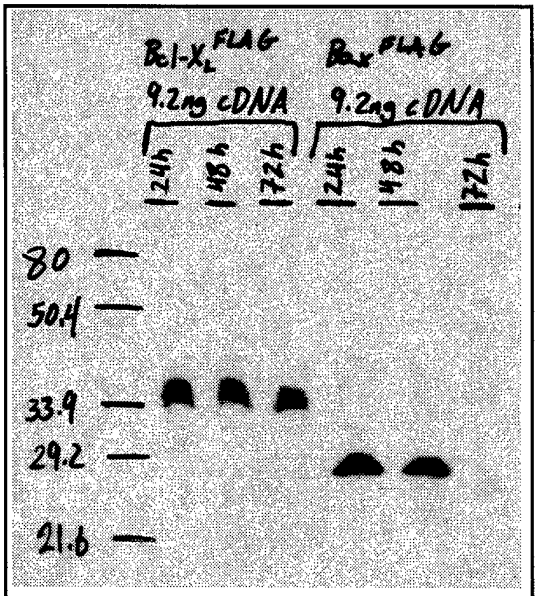

Figure 1. Western blot of lysates from FLAG-tagged Bcl-X_L (left 3 lanes) and Bax (lanes 4 and 5) cDNA-injected oocytes 24-72 hrs after injection blotted with polyclonal anti-FLAG antibody.

rotation graduate student. After a considerable effort without success, the goal was abandoned, and intranuclear injections of cDNAs were performed instead. We utilized FLAG-tagged cDNAs (kindly provided by Dr. Z. Yang at Penn), and harvested oocytes 24-72 hrs post-injection and performed Western blot analysis. As shown in Fig. 1, this method worked well to express both bcl-X_L (left lanes) as well as bax (right lanes). Death of the bax-expressing oocytes by 72 hrs did not permit detection at that time. However, the expression for both proteins was maximal at 24 hrs post-injection, and expression was constant for 48 hrs. This time-frame therefore provided the window in which to sample oocytes for patch-clamp electrophysiology.

Nuclei were isolated and gently immobilized, and a patch pipette (5-20 MΩ when filled with 140 mM KCl) formed gigaseals (>80% success rate) when gentle suction was applied. The standard solution contained 140 mM KCl, 10 mM HEPES, 3 mM $MgCl_2$, 1 mM MgATP, 1 mM Bapta, 0.543 mM $CaCl_2$ at pH 7.3 with calculated (Maxchelator software; C. Patton, Stanford University, CA) free [Ca^{2+}] of 200 nM. Experiments were performed at room temperature. Nuclei from control as well as injected oocytes were studied. We focussed our attentions for the initial experiments on the control nuclei. Our reasoning was that expression of bcl-related proteins would confer novel ion channel activity, requiring some knowledge of the ion channel activity endogenous to the oocyte nuclei. A variety of ion channel activities were observed, as evidenced by channel conductance and gating behavior, although most patches were electrically silent. A similar variety and incidence of channel activities were observed in bcl-X_L expressing oocytes. However, we are currently in the process of analyzing all the data more rigorously to verify this early impression.

Proposed studies during the second year

We are encouraged that we have been able to express recombinant bcl-related proteins in *Xenopus* oocytes and subsequently isolated their nuclei and achieve giga-ohm seals. We are a little disappointed that novel ion channel activities were not immediately obvious in these experiments, but we are also not too surprised. We have recorded under only a very limited range of conditions. We are now extending these experiments to include different recording conditions which might be more favorable for observing ion channel activities. In particular, we will investigate the effects of pH and [Mg^{2+}] in the pipette (cytoplasmic) solution, as these variables have been reported to be important in lipid bilayer reconstitution experiments. We will also explore the effects of membrane potential, since our initial experiments were performed under a limited range of positive potentials. We will also begin to undertake experiments outlined in Aim 3 during these studies.

We will also undertake the experiments outlined in Specific Aim 1, to determine whether recombinant bcl-like proteins can form functional ion channels in the outer membrane of the nuclear envelope. Nuclei will be isolated from *Xenopus* oocytes and CHO and MCF-7 cells, as described. Recombinant bcl proteins (provided by C. Thompson) will be included in the patch pipette filling solution. Of note, Dr. Thompson, one of the leaders in this field, recently (July 1, 1999) moved from the University of Chicago to join the Penn faculty as

Director of the new Abramson Cancer Center. This is a particularly exciting development for both our labs, and I anticipate that the new proximity of our labs will greatly facilitate our proposed studies.

KEY RESEARCH ACCOMPLISHMENTS during the first granting period.

- Acquisition and development of reagents for expression of bcl-related proteins in *Xenopus* oocytes.
- Successful expression by cDNA injections of recombinant bcl-X_L and bax in oocytes.
- Successful patch clamping of nuclei isolated from oocytes engineered to express Bcl-X_L.

CONCLUSIONS

Our initial results, related to our second specific aim, do not support our hypothesis that expression of bcl-related proteins confers novel ion channel activities in endoplasmic reticulum membranes. Nevertheless, these results are still preliminary, and further work under a variety of conditions are still required to test this hypothesis. Although we consider the experiments to be too preliminary to justify changes in the original goals of the specific aims, we are aware of emerging data suggesting that bcl-related proteins may interact with VDAC and the ATP-translocase as their mechanism of apoptotic actions (C. Thompson, personal comm.). We will therefore consider the possibly of expressing these proteins as well in oocytes, and then examing the effects of bcl expression on ion channel activities.