Activation of the ERK1/2 Signaling Pathway Promotes Phosphorylation and Proteasome-dependent Degradation of the BH3-only Protein, Bim

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Both the ERK and phosphatidylinositol 3’-kinase (PI3K) signaling pathways can protect cells from apoptosis following withdrawal of survival factors. We have previously shown that the ERK1/2 pathway acts independently of PI3K to block expression of the BH3-only protein, BimEL, and prevent serum withdrawal-induced cell death, although the precise mechanism by which ERK reduced BimEL levels was unclear. By comparing Bim mRNA and Bim protein, expression we now show that the rapid expression of BimEL following serum withdrawal cannot be accounted for simply by increases in mRNA following inhibition of PI3K. In cells maintained in serum BimEL is a phosphoprotein. We show that activation of the ERK1/2 pathway is both necessary and sufficient to promote BimEL phosphorylation and that this leads to a substantial increase in turnover of the BimEL protein. ERK1/2-dependent degradation of BimEL proceeds via the proteasome pathway because it is blocked by proteasome inhibitors and is defective at the restrictive temperature in cells with a temperature-sensitive mutation in the E1 component of the ubiquitin-conjugating system. Finally, co-transfection of BimEL and FLAG-ubiquitin causes the accumulation of polyubiquitinated forms of Bim, and this requires the ERK1/2 pathway. Our findings provide new insights into the regulation of Bim and the role of the ERK pathway in cell survival.

Higher eukaryotes possess two major pathways for initiating programmed cell death or apoptosis: the cell-extrinsic death pathway, involving death receptors, or the cell-intrinsic pathway (1). Apoptosis following withdrawal of survival factors typically proceeds via the cell-intrinsic pathway because it is regulated by Bcl-2 proteins. Pro-apoptotic proteins such as Bax and Bak can disrupt the outer mitochondrial membrane and promote the release of apoptogenic factors such as cytochrome c (1), but in viable cells they are normally repressed by binding to pro-survival proteins such as Bcl-2 and Bcl-xL. The “BH3-only” proteins respond to stresses by binding to Bcl-2 or Bcl-xL, thereby releasing Bax or Bak, which undergo conformational changes before oligomerizing at the mitochondria to promote cell death (1, 2). Thus, BH3-only proteins act at the interface between stress signaling pathways and the core apoptotic machinery and are subject to many forms of regulation (2). Some BH3-only genes are transcriptionally regulated; for example, Puma is up-regulated by p53 in response to DNA damage (3), whereas Hrk/DP5 is up-regulated by NGF withdrawal (4). Other BH3-only proteins are regulated by post-translational modifications; for example, caspase-8 cleaves Bid to yield an “activated” version called tBid (5).

Withdrawal of serum or defined survival factors results in the inactivation of the Raf-MEK-ERK and phosphatidylinositol 3’-kinase (PI3K)/PKB survival signaling pathways. Two BH3-only proteins, Bad and Bim, have been implicated particularly in apoptosis following withdrawal of survival factors, and both are regulated by these signaling pathways. Bad is phosphorylated by PKB (6) and RSK1 (7), which facilitates its sequestration by 14-3-3 proteins thereby preventing it from binding to Bcl-2. Withdrawal of survival factors results in the dephosphorylation of Bad, allowing it to bind to Bcl-xL and release Bax. In contrast, Bim is expressed de novo following withdrawal of survival factors or inactivation of the PI3K pathway (8–11). In neurons the withdrawal of NGF results in activation of the c-Jun N-terminal kinase (JNK)-c-Jun pathway; this is required for increases in Bim mRNA (10). In contrast, in lymphocytes withdrawal of cytokines causes the inactivation of PKB, dephosphorylation of the forkhead transcription factor FKHL1/FOXO-3A, and transcription of Bim (8).

In CCI39 cells, we have previously shown that, following serum withdrawal, the initiation of Bax activation and subsequent activation of caspases are both strongly inhibited by actinomycin D and cycloheximide (11). This finding suggests that de novo expression of Bim rather than dephosphorylation of pre-existing Bad is the most likely mechanism by which serum withdrawal-induced death is initiated. In these cells the (extracellular signal-related kinase 1 and 2 (ERK1/2) pathway

* This work was supported by a grant from Cancer Research UK (SP2458/0201), a project grant from the Biotechnology and Biological Sciences Research Council (BBSRC) (BB/D015785), and a competitive strategic grant from the BBSRC to the Babraham Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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This paper is available on line at http://www.jbc.org

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The abbreviations and trivial names used are: BH3, Bcl-2 homology domain 3; CHO, Chinese hamster ovary; HEK, human embryonic kidney; ERK, extracellular signal-regulated kinase; PBS, fetal bovine serum; RT-PCR, reverse transcriptase-PCR; HA, hemagglutinin; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MKK, MEK kinase; NGF, nerve growth factor; PI3K, phosphatidylinositol 3’-kinase; PKB, protein kinase B; SAPO, stress-activated protein kinase; Ub, ubiquitin; 14-3-3, 14-3-3 protein; 2-methoxy-4-cyclopropylmethoxy-3,4-difluorobenzamide.
acts independently of PI3K to prevent Bim expression and serum withdrawal-induced cell death, although the precise mechanism by which ERK reduces Bim levels is unclear (11).

By comparing Bim mRNA and Bim protein expression, we now show that the rapid expression of BimEL following serum withdrawal cannot be accounted for simply by increases in mRNA following down-regulation of the PI3K/ERKβ pathway. We show that BimEL is phosphorylated in response to activation of the ERK/2 pathway and that this targets BimEL for degradation by the proteasome pathway. Our findings provide new insights into the regulation of Bim and the role of the ERK pathway in cell survival.

Experimental Procedures

Materials—Cell culture reagents were purchased from Invitrogen. U0126 was purchased from Promega. LY294002, MG132, and zVAD-fmk were from Calbiochem. The following antibodies were used throughout this study. Phospho-ERK1/2, total ERK1/2, phospho-PKB (Ser-473), and total PKB were from Cell Signaling Technology/New England Biolabs; Bim was from Chemicon; Bad and rabbit anti-HA were from Santa Cruz Biotechnology; anti-FLAG was from Sigma; and mouse anti-PI3K was from the Babraham Institute Monoclonal Antibody Facility. Horseradish peroxidase-conjugated secondary antibodies were from Bio-Rad. All other chemicals were purchased from Sigma, unless otherwise stated in the text, and were of the highest grade available.

Cell Culture—Culture of CCl39, CR1–11, and CM3 cells has been described previously (11–13). The ts20 CHO cells (14) were maintained in minimal Eagle’s medium containing a HEPES buffer. For serum starvation, cells judged to be 50–60% confluent were washed once in serum-free medium and then placed in fresh serum-free medium with the indicated dose of 4-hydroxytamoxifen (4-HT), FBS, inhibitors, or the relevant vehicle control for the times indicated in the figure legends. For emetine chase experiments, cells were starved for 18 h and then treated with emetine (10 μM) to block protein synthesis prior to further treatment. Cells were harvested at the indicated times for further analysis.

Real-time RT-PCR—Preparation of total RNA was performed as described previously (11). RT-PCR was performed according to the protocol supplied with the Taqman® Reverse Transcription reagents (Applied Biosystems) as described previously (11). For hamster Bim we used 5′-TAAGGCCAATCTCAAGGAGAACTT-3′ as the forward primer and 5′-AGATTCTGGAACTCCTGTCCTCA-3′ as the reverse primer. These primers are common to BimS, BimL, and BimEL. For hamster β-actin we used 5′-CCAGATGAAGGAGATCA-3′ as the forward primer and 5′-GCCACCATCCCACACAGTA-3′ as the reverse primer.

Western Blot Analysis—Cells were lysed and analyzed by Western blot exactly as described previously (11, 12). For treatment with calf intestinal phosphatase, cells were lysed in lysis buffer without phosphatase inhibitors and then treated with 10 units of calf intestinal phosphatase in the presence of the appropriate buffer for 2 h at 37 °C.

Plasmids and Transfections—HA-tagged BimEl was constructed by subcloning rat BimEl from the plasmid pDNA-FLAG-BimEl (provided by Drs. Stephen Neame and Jonathan Ham) into the pcDNA3-HA plasmid. In-frame fusion to the HA sequence was confirmed by sequencing and by transient expression and immunoblotting.2 pcDNA-FLAG-ubiquitin was kindly provided by Dr. Paul Evans (The Babraham Institute). HEK293 cells were transfected by the calcium phosphate precipitation technique (15). HA-tagged BimEl was immunoprecipitated from cell lysates using either mouse anti-HA antibodies conjugated to protein G-Sepharose beads or rabbit anti-HA antibodies conjugated to protein A-Sepharose.

Results

Expression of BimEL Cannot Be Accounted for by Increases in mRNA Following Inhibition of PI3K—Bim is an important mediator of apoptosis in response to loss of survival signals (8–11). There are three major splice variants of Bim, short, long, and extra long (BimS, BimL, and BimEL, respectively) (16–18), although other forms have recently been reported (19, 20). In most cell types, including CCl39 cells (11), BimEL is the major species expressed, with some cell types expressing little or none of the other forms (9). In hematopoietic cell lines, such as Ba/F3, removal of cytokines results in loss of PI3K activity and dephosphorylation of PKB, leading to activation of the forkhead transcription factor (FKHR-L1/FOXO-3A) and an increase in expression of Bim (8). In CCl39 fibroblasts, BimEl expression is substantially increased following serum withdrawal (11); therefore we compared this response with that seen following inhibition of PI3K.

Using a real-time RT-PCR protocol we observed that the PI3K inhibitor LY294002 caused a striking 25-fold induction of Bim RNA levels, compared with a β-actin control, when added to cells in 10% FBS (Fig. 1A). This was accompanied by a substantial increase in expression of BimEL protein (Fig. 1B). Surprisingly, although serum starvation caused a much smaller (5-fold) increase in Bim mRNA (Fig. 1A), it actually caused a greater increase in BimEL protein expression compared with that seen following addition of LY294002 (Fig. 1B). Analysis of PKB phosphorylation (Fig. 1B) confirmed that serum withdrawal and LY294002 were equally effective in reducing the activity of the PI3K/PI3Kβ pathway. This disparity indicated that the increase in BimEL expression following serum withdrawal could not be accounted for simply by increases in Bim mRNA following loss of PI3K activity and the resultant dephosphorylation of PKB. Because BimEL was by far the major form of Bim expressed under these conditions, with only low levels of BimL seen, further analysis focused on the regulation of BimEL.

Activation of the ERK1/2 Pathway Is Necessary and Sufficient to Promote Phosphorylation of BimEL—We (11) and others (21, 22) have previously noted that in viable cells the Bim

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2 Details are available from authors upon request.
Cells with 4-HT caused further phosphorylation of Bim EL, but might play a role in regulating Bim EL expression, perhaps by increased, and the BimEL protein resolved as a monomeric form phosphorylation and inactivation of ERK1/2 as well as PKB plays no role in the serum-stimulated degradation of BimEL. We therefore examined the expression and dephosphorylation of BimEL. Subsequent activation of ΔMEKK3:ER* stimulated the phosphorylation of BimEL, and this was abolished by U0126 (Fig. 2B). These results indicate that selective activation of the ERK1/2 pathway is sufficient to induce substantial phosphorylation of BimEL.

Serum Stimulation Increases the Turnover of BimEL, and This Requires the ERK1/2 Pathway—Stimulus-dependent phosphorylation can serve to “flag” proteins for destruction (24), and so we examined the turnover of BimEL. CCl39 cells were serum-starved to induce the expression of the hypophosphorylated form of BimEL. These cells were then “chased” with emetine for 4 or 8 h, to prevent new protein synthesis, in the presence or absence of 10% FBS. When cell lysates were immunoblotted for Bim, it was apparent that BimEL protein levels decayed relatively slowly in serum-free medium with a half-life (t1/2) in excess of 8 h. Serum stimulation accelerated the decay of BimEL, with a t1/2 of ~3 h, and this was associated with the phosphorylation of the protein. If cells were treated with PD184352, which inhibits the ERK1/2 pathway but not the PI3K pathway (13), the FBS-stimulated phosphorylation of BimEL was abolished and FBS no longer increased the turnover of BimEL (t1/2 > 8 h). As a control, the turnover of the BH3-only protein, Bad, was unaffected by FBS or PD184352 (Fig. 3A).

In common with the ERK1/2 pathway, the PI3K/PKB pathway is also inactivated upon withdrawal of serum and strongly reactivated when quiescent cells are stimulated with FBS. We therefore examined the effect of inhibiting the PI3K pathway on FBS-stimulated BimEL turnover. The PI3K inhibitor, LY294002, prevented PKB phosphorylation in response to serum stimulation but had no effect on ERK1/2 phosphorylation (Fig. 3B). Under these conditions LY294002 had no effect on FBS-stimulated turnover of BimEL, indicating that the PI3K/PKB plays no role in the serum-stimulated degradation of BimEL.

Selective Activation of the ERK1/2 Pathway Is Sufficient to Stimulate the Turnover of BimEL.—To determine whether activation of the ERK1/2 pathway alone was sufficient to promote turnover of BimEL, we performed emetine chase experiments in CR1–11 cells expressing ΔRaf-1:ER* (23), which selectively activates the ERK1/2 pathway. Serum withdrawal again promoted the expression and dephosphorylation of BimEL. Subsequent activation of ΔRaf-1:ER* stimulated the phosphorylation of BimEL, and this was abolished by U0126 (Fig. 2C). These results indicate that selective activation of the ERK1/2 pathway is sufficient to induce substantial phosphorylation of BimEL.
in Figs. 3 and 4 indicate that activation of the ERK pathway is necessary and sufficient to stimulate the turnover of pre-existing BimEL protein.

**ERK1/2-dependent Degradation of BimEL Proceeds via the Proteasome Pathway**—The regulated degradation of proteins proceeds, in most cases, via the proteasome pathway. Proteins marked for destruction by the covalent attachment of ubiquitin moieties to lysine residues are rapidly degraded by the 26 S proteasome (24). To determine whether serum-stimulated degradation of BimEL was proceeding via a proteasome-dependent pathway, we repeated the emetine chase experiments in the presence of the cell-permeant proteasome inhibitor, MG132. In these experiments we again observed that FBS stimulation caused the phosphorylation and degradation of BimEL (Fig. 5A). In contrast, MG132 completely blocked the FBS-stimulated degradation of BimEL without blocking phosphorylation, indicating that MG132 did not simply prevent activation of ERK1/2 (Fig. 5A).

As a specificity control, CCl39 cells were serum-starved and restimulated with FBS and emetine in the absence or presence of various protease inhibitors. Neither the lysosomal inhibitor chloroquine nor the caspase inhibitor zVAD.fmk had any effect on the serum-stimulated degradation of BimEL. In contrast, both proteasome inhibitors, MG132 and lactacystin, blocked the turnover of BimEL, without blocking its phosphorylation (Fig. 5B). We also observed that the proteasome inhibitor LLnL (N-acetyl-Leu-Leu-norleucinal) inhibited turnover of BimEL.

These results suggest that serum-stimulated, ERK-dependent BimEL degradation proceeds via the proteasome.

As an independent confirmation of these results, we assessed the accumulation of BimEL protein in the ts20 CHO cell line, which harbors a temperature-sensitive mutation in the E1 component of the ubiquitin-conjugating system (14) and thus fails to support ubiquitination when switched from the permissive (32 °C) to the restrictive temperature (39 °C). First we noted that when ts20 CHO cells were serum-starved overnight at 32 or 39 °C, the shift to the restrictive temperature was sufficient to cause an increase in the level of BimEL.

**Fig. 3.** FBS stimulation increases the turnover of BimEL via activation of the ERK1/2 pathway. A, CCl39 cells were serum-starved for 18 h (t = 0) and then chased for 4 or 8 h with 10 μM emetine in serum-free medium (SF) with fresh 10% FBS alone or 10% FBS in the presence of PD184352 (PD; 5 μM). B, emetine chase experiments were repeated in FBS-stimulated cells in the presence or absence of LY294002 (LY) for the indicated times. Cell lysates were subjected to Western blot with antibodies to Bim, Bad, phospho-ERK1/2, total ERK1/2, and phospho-PKB (Ser-473) as indicated. Expression of BimEL protein was quantified by scanning densitometry and normalized to expression levels at t = 0 h.

**Fig. 4.** Selective activation of the ERK1/2 pathway is sufficient to promote the turnover of BimEL. CR1–11 cells were serum-starved (SF, serum-free) for 18 h (t = 0). Cells were treated with emetine (10 μM) and chased for the indicated times in the absence or presence of 100 nM 4-HT to activate Raf-1:ER*. Cell lysates were subjected to Western blot with antibodies to Bim, Bad, phospho-ERK1/2, and total ERK1/2. Expression of BimEL protein was quantified by scanning densitometry and normalized to expression levels at t = 0 h. Data are taken from single experiments representative of at least three others giving similar results.
inhibitor MG132, we were able to clearly detect poly-Ub conjugates associated with Bim, resolving as a smear up the gel. These were not seen when HA-BimEL was expressed alone in the presence of MG132 (Fig. 6B, lane 2 versus 6). The appearance of this smear of poly-Ub-conjugated Bim was partially reduced by treatment of cells with PD184352 (Fig. 6B, lane 1 versus 2), which also caused the dephosphorylation of BimEL (Fig. 6A, lane 1 versus 2). These results confirm in a heterologous system that transiently over-expressed BimEL is ubiquitinated; this is enhanced by over-expression of FLAG-Ub (allowing detection of FLAG-Ub/Bim conjugates) and is dependent, in large part, on the ERK1/2 pathway. Taken together the results shown in Figs. 3–6 indicate that BimEL is degraded by the ubiquitin proteasome system in response to activation of the ERK1/2 pathway.

**DISCUSSION**

It is of utmost importance for the survival of multicellular organisms that the execution of cell death is kept under stringent control. In the mitochondrial death pathway, the fine balance between pro-death and pro-survival Bcl-2 proteins is regulated by the BH3-only proteins (1, 2). Higher eukaryotes possess a variety of BH3-only proteins. Each responds to a discrete set of stress or survival signals, with the result that each is subject to different forms of regulation. Some BH3-only genes such as Puma (3, 25), Noxa (26), and Hrk/DP5 (4) are regulated transcriptionally. Other BH3-only proteins are regulated by post-translational modifications; for example, caspase-8 cleaves Bid to yield an activated version called tBid (5), whereas Bad is phosphorylated by PKB (6) and RSK1 (7), which facilitates its sequestration by 14-3-3 proteins.

Bim has previously been shown to be regulated by two discrete mechanisms. First, BimL and BimEL interact with 8-kDa dynein light chain 1 (27), providing a mechanism to sequester Bim at microtubules away from the mitochondria. This spatial segregation of Bim may be regulated in response to some stresses, although it’s mechanism is unclear.Second, the de novo expression of Bim is a major mechanism of regulation. Bim mRNA and Bim protein are expressed at very low levels in most viable cells but are rapidly and substantially up-regulated following withdrawal of survival factors from lymphocytes (8, 21), neurons (9, 10), and fibroblasts (11). In addition, previous studies have noted that BimEL is a phosphoprotein (11, 21, 28), although the role of this phosphorylation and the signaling pathway responsible were not resolved. Here we have shown for the first time that the ERK1/2 pathway is the major pathway promoting BimEL phosphorylation and that activation of this pathway targets BimEL for degradation via the proteasome.

Our analysis of Bim phosphorylation was facilitated by the use of conditional MAPK kinase kinases, which allow rapid and selective activation of defined signaling pathways (12, 23). ΔMEKK3:ER*, which activates the ERK, JNK, and p38 pathways (12), promoted phosphorylation of BimEL implicating the MAPK and/or SAPK pathways. The ability of U0126 to block this ΔMEKK3:ER*-induced phosphorylation further focused our attention on the ERK1/2 or ERK5 pathways, whereas the ability of PD184352 to block BimEL phosphorylation strongly suggested that the ERK1/2 pathway was responsible, because 5 μM PD184352 completely inhibits the ERK1/2 pathway without impacting on ERK5 (13). Finally, the ability of ΔRaf-1:ER* to selectively activate the ERK1/2 pathway (23) and induce BimEL phosphorylation confirmed that the ERK1/2 pathway was responsible.

A recent study in PC12 cells showed that NGF could promote BimEL phosphorylation and cause a slow decrease in BimEL protein levels over a period of several days (28). However, the
authors did not examine whether this decline was due to changes in Bim mRNA or in protein stability, which is an important distinction because both the ERK and PI3K pathways can regulate Bim mRNA levels in some cells (11). Furthermore, the study relied solely on the use of 50 µM U0126, a dose that completely inhibits both the ERK1/2 and ERK5 pathways (22). Because NGF activates both ERK1/2 and ERK5 (22, 29) it was unclear which pathway was responsible for the slow decline in BimEL. In contrast, our results, using PD184352 to selectively inhibit and ΔRaf-1:ER* to selectively activate the ERK1/2 pathway, clearly define the ERK1/2 pathway as being responsible for promoting BimEL phosphorylation. In addition, it is not clear that we are examining the same mechanism for down-regulation of BimEL as the study in PC12 cells (28). For example, the effects we observed on Bim turnover were rapid, with ΔRaf-1:ER* reducing Bim levels substantially within a few hours in the presence of emetine, whereas the down-regulation of BimEL described for NGF required several days (28). Although activation of ERK1/2 is necessary and sufficient for phosphorylation and degradation of BimEL, it is not yet clear that ERK1/2 are the kinases responsible for phosphorylating BimEL. Certainly there are other kinases downstream of ERK1/2, such as RSK1 (7), that have been implicated in cell survival, and future studies should be directed toward defining the identity of the kinase and the sites of phosphorylation.

Activation of the ERK1/2 pathway, either by FBS or ΔRaf-1:ER*, was sufficient to greatly accelerate the turnover of BimEL from > 8 h to ~3 h, indicating that activation of this pathway targets BimEL for degradation. Three independent lines of evidence lead us to conclude that this ERK1/2-dependent degradation proceeds via the ubiquitin-proteasome pathway. First, cell-permeant, small molecule inhibitors of the proteasome prevented ERK-dependent degradation of BimEL. Second, serum-stimulated degradation of BimEL in ts20 CHO cells was blocked at the restrictive temperature at which the E1 ubiquitin-activating enzyme is inactive (14). Importantly, in neither case did these interventions prevent BimEL phosphorylation, suggesting that inhibition of the ubiquitin-conjugating system or the proteasome was not simply blocking ERK activation. In these experiments we analyzed the expression of Bad as an internal control, as it is another BH3-only protein, it has also been implicated in death following withdrawal of survival factors and is regulated in part by the ERK1/2 pathway (7). We found that the expression of Bad remained constant despite manipulation of the ERK, PI3K, or ubiquitin-proteasome pathways, which is consistent with other analysis indicating that Bad is a stably expressed protein that is relatively refractory to inhibition of de novo protein synthesis. Third, and finally, when BimEL was over-expressed in HEK293 cells, MG132 or co-expression of FLAG-UB allowed us to detect Bim-FLAG-poly-UB conjugates; this ubiquitination was reduced by PD184352. Taken as a whole these results provide very strong evidence to support a model in which activation of the ERK1/2 pathway promotes the phosphorylation of BimEL, which serves to flag BimEL for ubiquitination and destruction by the proteasome.

Our results suggest that the rapid expression of BimEL following withdrawal of survival factors proceeds via at least two discrete mechanisms. The rapid inactivation of the ERK1/2 pathway will result in dephosphorylation and substantial stabilization of pre-existing and newly synthesized BimEL, which will be accompanied by increases in Bim mRNA because of activation of JNK (10) or loss of PI3K (8) or ERK1/2 activity (11), depending on the cell type. Thus, we provide a new insight into how the ERK1/2 survival pathway can “neutralize” BimEL and provide a novel mechanism by which the ERK1/2 pathway can contribute to cell survival following withdrawal of survival factors. This may be relevant to the survival of lymphocytes (8, 21) or neurons (9, 10) following withdrawal of the relevant pro-survival cytokines. In addition, because tumor cells are characterized, in part, by their ability to survive at reduced levels of growth factors (30), this finding may be relevant to tumor cells that harbor activated mutants of Ras or Raf.

Acknowledgments—We thank members of the Cook Group and the Inositol laboratory for discussion and comments on the study and Len Stephens for advice and critical reading of the manuscript. We also thank Stephen Neame and Jonathan Ham for provision of the rat BimEL cDNA, Paul Evans for provision of the pcDNA-FLAG-UB plasmid, Maureen Mee and John Mayer for provision of the ts20 CHO cell line, and Martin McMahon and Ron Hay for useful discussions.

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* C. J. Chalmers, K. Balmanov, K. Hadfield, R. Ley, and S. Cook, manuscript submitted.
