Restraint of apoptosis during mitosis via interdomain phosphorylation of caspase-2

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1st Editorial Decision 06 May 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments to the authors are shown below. As you will see all three referees consider the study as highly interesting and important. Referees 2 and 3 offer strong and enthusiastic support for publication of the work here after minor revisions whereas referee 1 is more critical and raises a number of detailed issues/concerns (see below). Taking together all three reports we will be happy to consider a revised manuscript in which the referees concerns need to be addressed (or responded to) in an adequate manner. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The Kornbluth laboratory has previously reported that under conditions of nutrient abundance (or
more specifically high NADPH levels). Xenopus procaspase-2 is phosphorylated by CaMKII in oocyte extracts within its prodomain at Ser135, which in turn inhibits the activation of caspase-2 by preventing its association with the adapter protein RAIDD. They further demonstrated that caspase-2 activation was essential for mitochondrial outer membrane permeabilization (MOMP), cytochrome c release, and caspase-3 activation. Similarly, in this manuscript by Andersen et al., they now propose that caspase-2 is phosphorylated by Cdk1/Cyclin B1 during mitosis at a conserved serine residue, located within the linker region separating its large and small subunits (Ser308 in Xenopus; Ser340 in humans), and that this phosphorylation inhibits caspase-2 activation and suppresses apoptosis, not by disrupting its interaction with RAIDD but by inhibiting interchain cleavage. In addition, Ser308/Ser340 was found to be susceptible to dephosphorylation by PP1 during interphase, but not during mitosis when PP1 is inhibited. Thus, collectively, they propose that an increase in Cdk1/Cyclin B1 activity and a decrease in PP1 activity promotes stable caspase-2 phosphorylation. The paper is well written, and for the most part, the experiments are well done. I also find the authors' arguments to be logical. There are however several specific experimental and conceptual concerns (detailed below), including precisely how Cdk1/Cyclin B1-dependent phosphorylation of caspase-2 prevents its activation.

Major concerns:

1. In Fig. 1C, does Roscovotine promote MOMP in mitotic extracts containing mitochondria? In Fig. 1D, the authors argue that mitochondria in mitotic and interphase extracts are equally susceptible to tBid-induced MOMP. However, the concentration of tBid (1 μM) is very high. Is this true at more physiological concentrations of tBid, and equally important, does Roscovotine promote Bid cleavage in mitotic extracts?

2. In Fig. 2A, the authors demonstrate that [35S]caspase-2 is processed in interphase but not in mitotic extracts. Does Roscovotine promote caspase-2 cleavage in the latter extracts? Additionally, numerous studies have shown that caspase-2 cleavage is generally absent in cells (or cell extracts) deficient in Apaf-1, caspase-9, or caspase-3. Are the authors certain that caspase-2 cleavage in the interphase extracts does not occur downstream of the apoptosome? For example, does addition of the caspase-3/7 inhibitors, DEVD-cho or XIAP (Bir1-Bir2), have any effect on caspase-2 cleavage? This is an important point because it seems plausible that caspase-2 cleavage by caspase-3 could serve to promote MOMP via a caspase amplification loop.

3. In Fig. 3B, if CaMKII can phosphorylate caspase-2 at Ser135 in interphase and mitotic extracts, why isn't there incorporation of [32P] into both "WT C2" and "C2 308A"? Is it because the levels of NADPH have already been depleted in these extracts?

4. The data presented in Fig. 3E, F, and H are of poor quality and unconvincing. In Fig. 3I, one assumes that "lane 3" does not contain I2, but in any event the conclusion that I2 inhibits PP1-dependent dephosphorylation of caspase-2 in "lane 4" is unconvincing. How much Cdk1/Cyclin B1 and PP1 were added to these incubations? Though it might be atypical, can the authors rule out that PP1 does not bind to caspase-2 independent of phosphorylation?

5. While procaspase-2 phosphorylation at Ser135 inhibits its activation by preventing its interaction with RAIDD, it remains unclear precisely how phosphorylation at Ser308 prevents caspase-2 activation? The authors argue, based in large part on previous in vitro studies by Sharad Kumar's laboratory, that dimerization of procaspase-2 is sufficient for its activation, but that interchain cleavage is required for stable formation of the active caspase-2 dimer. If so, does phosphorylation at Ser308 prevent the initial dimerization of procaspase-2 or the subsequent interchain cleavage? If the latter is true, given that Ser308 is located equidistant between the autocatalytic cleavage site and the caspase-3 cleavage site in the activation loop, does phosphorylation of Ser308 also prevent caspase-3-dependent cleavage of procaspase-2? If so, again, how do the authors rule out that phosphorylation merely disrupts a caspase amplification loop (see concern #2)? The data in Fig. 4B, using recombinant RAIDD and wild-type caspase-2 or the phosphomimetic caspase-2 mutant (S308E) are not convincing (as the processing of wild-type caspase-2 is barely detectable), nor do they address at which step caspase-2 activation is inhibited.

6. In Fig. 5A, the authors argue that loss of phosphorylation at Ser135 further enhances caspase-2 processing in mitotic extracts (when both Ser135 and Ser308 are mutated to alanines), because it
relieves the inhibition normally mediated by NADPH. However, since the kinetics of caspase-2 cleavage differ slightly from one incubation to the next, dependent upon how fast NADPH is depleted, "C2 S135A/S308A" should be compared with "C2 S308A" (rather than "WT C2") within the same experiment.

7. In Fig. 6, the authors have sought to confirm the importance of human caspase-2 phosphorylation at Ser340 in U2OS cells by using an RNAi-based replacement strategy with wild-type or mutant nonphosphorylatable caspase-2. Though not discussed, previous reports by Allan and Clark suggest that Cdk1/Cyclin B1 also phosphorylates procaspase-9, and using a virtually identical approach, they demonstrated that cells expressing nonphosphorylatable procaspase-9 were also more susceptible to nocodazole. If Cdk1/Cyclin B1 suppresses apoptosis by phosphorylating caspase-2 and preventing MOMP/apoptosome formation, it is unclear why procaspase-9 phosphorylation would have any effect? This should, at the very least, be discussed.

8. In Fig. 7, the authors suggest that evolutionarily conserved Cdk1 phosphorylation sites may exist within the linker regions of other caspases, including caspases-8 and -9. It should be noted, however, that Ser307 in human caspase-9 is not conserved in the rat or mouse.

9. Finally, based upon their previous and current work, it would appear that caspase-2 is exquisitely regulated, undergoing reversible phosphorylation at two distinct sites in response to nutrient and cell cycle status, resulting in inhibition through two completely different mechanisms. Nevertheless, as the authors are aware, the role of caspase-2 in apoptosis is highly controversial, in large part because mice deficient in PIDD, RAIDD, and caspase-2 (all components of the PIDDosome) exhibit virtually no developmental phenotype, outside of the previously reported effects on oocyte number in the caspase-2 knockout mice. It would be helpful if the authors could try and reconcile this apparent paradox.

Minor comments:

1. In the introduction, the authors note that apoptosis induced by caspase-2 overexpression requires Bid cleavage, and they cite Bonzon et al., Mol. Biol. Cell (2006). To be fair, however, Emad Alnemri's group was the first to show that caspase-2 can cleave Bid and that the resulting tBid could induce MOMP. Therefore, Guo et al., J. Biol. Chem. (2002) should also be cited.

2. In Fig. 2D, it is unclear how much RAIDD was used. The concentration of RAIDD should be included in the figure legend.

3. Fig. 3A needs to be enlarged.

4. In Fig. 7B, it is unclear how the data presented in the bar graph were "normalized". Do these values represent GFP+/PI+ cells?

Referee #2 (Remarks to the Author):

With characteristic rigor, the Kornbluth laboratory defines a mitosis-specific event, phosphorylation of Caspase-2, as being critical for inhibition of Caspase-2 activation and apoptosis during mitotic division. Moreover, the authors show that Caspase-2 is subject to regulation both by the mechanism they previously identified, based on the pentose phosphate metabolic pathway, and this novel cyclin-dependent event. Finally, the authors identify Cdk consensus phosphorylation sites in similar locations near the linker domains of several apical procaspases, raising an interesting hypothesis concerning mitotic regulation of other caspase-dependent mechanisms of apoptosis induction during mitosis. This is important work that I believe should be published by EMBO J.

There are some minor points that should be addressed:
1. The authors might mention a recent report of a RAIDD/PIDD-independent caspase-2 activation pathway.
2. The restoration of C2 phosphorylation by the inhibitor I2 in Fig. 3I is not terribly clear, and there should be quantitation of these data.
3. Fig. 3A is too small to see clearly.
4. In Fig. 6B, was there a reason the investigators did not gate on the cells expressing GFP (i.e. the infected cells), rather than correcting for % of infection? How was the calculation performed?

Referee #3 (Remarks to the Author):
The authors have succeeded in something remarkable: uncovering a mechanistic link between the cell cycle and apoptosis that is both novel and informative, in addition to being quite exciting. They convincingly show that phosphorylation of S318 in xenopus caspase-2 (and S340 in human) by cdk1 prevents its activation by RAIDD and in response to microtubule inhibitors. Overall, the work is interesting, important, and convincing.

I have a few small concerns that I think the authors can easily address. Some are very minor.

1. In several figures, cytochrome c release is shown by western blot, but we have no way of knowing how much of this is mitochondrial contamination. While I do NOT suggest that all of these be repeated, it might be helpful if in one case (perhaps the first?), they show pellets and supernatants to give an idea of the extent of release. Since this is very well established, I am not sure it is necessary, but it would help some readers in their interpretations.

2. In Figure 3D, there appears to be a mistake in the numbering (332 is listed at the start of each, even when the final is 319).

3. In Figure 4E they show oocytes, some of which are undergoing apoptosis. While to an experienced eye the effects are clear, they might consider providing some guidance to readers with less knowledge of oocyte apoptosis in evaluating what we are seeing. Perhaps a supplemental figure showing more examples of the differences might help?

4. There are some concerns about the experiment in Figure 6. First, they do not describe the siRNA they have used. Since there is a problem with one of the siRNAs for caspase-2 (the one used by Lazebnik, et al and since then many other labs has been demonstrated to act off target), it is important that we know that this is not the one they used (or if it is, see #5).

5. The problem in 4 is a bit compounded due to a missing control in 6B. The cells treated with siRNA and transfected with wt or mutant caspase-2 should be examined for apoptosis without nocZ treatment. If, indeed, the restoration of caspase-2 does not kill the cells but makes them susceptible to drug treatment, the concerns about the siRNA sequence are less important.

This really is a terrific paper, and the results are clear overall. I hope that the changes I have suggested do not cause any substantial delays.

1st Revision - authors’ response 22 July 2009

We thank the reviewers for their thoughtful critiques of our manuscript. We have provided new and revised data and have altered the text of the manuscript where recommended. We hope that these changes satisfactorily address the concerns of the reviewers. Below you will find a point-by-point response to each of the reviewers’ critiques.

Reviewer 1

1. Does roscovitine promote MOMP in mitotic extracts?
The experiment in figure 1C has been redone with roscovitine as a control. We found that adding roscovitine to mitotic extract completely reversed the mitotic suppression of cytochrome c release. These data are shown in figure 1D.

2. The concentration of tBid is very high.
The concentration of tBid that we reported in the materials and methods (1 µM) is incorrect (it was a typographical error). It should have read ‘1 nM’. We apologize for this mistake. In previous studies, we had used tBid at a concentration of 25 nM in the extract (Nutt et al. 2005 Cell 123: 89-103). In our current study, for the express purpose given in the critique (to achieve a more physiological, less overwhelming concentration of tBid), we had titrated the amount of tBid down to 1 nM.
3. Does roscovitine promote bid cleavage in mitotic extracts?
To address this question, we incubated $^{35}$S-labeled Bid in interphase extract and mitotic extract with and without roscovitine. In agreement with our previous data, processing of Bid was suppressed in mitosis and this suppression was reversed by the addition of roscovitine. The data are shown in figure 2B.

4. Does roscovitine promote caspase-2 cleavage in mitotic extract?
As in figure 1C, we repeated the experiment in figure 2A with roscovitine as a control. In agreement with the experiments mentioned above, roscovitine completely reversed the mitotic suppression of caspase-2 processing. Taken together, roscovitine was very effective at lifting the mitotic suppression of MOMP, bid and caspase-2 processing.

5. Does caspase-2 cleavage in the extract happen downstream of the apoptosome?
We refer the reviewer to our previous publication (Cell, Vol. 123, 89-103, October 7, 2005,) in which we showed that Bcl-xL prevented mitochondrial cytochrome c release, but not caspase 2 processing and that zVAD-fmk, which inhibited caspase 9 and caspase 3 activity did not block caspase 2 processing. Therefore caspase-2 cleavage can occur independently of apoptosome activation in the egg extract.

6. With CaMKII active, why is there no phosphorylation of C2 S308A (at S135)?
The phosphorylation by Cdk1 is more rapid and robust. We have observed that appreciable phosphorylation of GST-caspase-2 at S135 requires longer incubation in the extract (typically 1 hour versus 15-30 minutes for S308) in comparison with phosphorylation at S308 due to the relative kinase activities in the extract. Thus a longer incubation would reveal S135 phosphorylation.

7. Poor quality of data in Figure 3
We purified new phospho-antibody to more clearly see phosphorylation of human caspase-2 at S340 (shown in figure 3F). The 2D gel data in figure 3E were repeated several times and, although consistent, were not cleaner than the data presented in the paper. We stuck with our current 2D gel images but cropped the image to focus on the bands of interest. We repeated the experiment in figure 3H (now figure 4B) and now show a clearer image of suppression of caspase-2 processing by I2. In addition we’ve included new data to show in vitro dephosphorylation of caspase-2 by PP1 with I2 as a control (previously figure 3I, now figure 4C). We have also included the amounts of recombinant proteins used (added to figure legends).

8. Can the authors rule out that PP1 does not bind to caspase-2 independent of phosphorylation?
We have a very recent report (Nutt et al., 2009 Dev. Cell 16(6): 856-866) in which we show that phosphorylation of S135 on caspase-2 is also controlled by PP1, but that PP1 binding to caspase-2 is not governed by phosphorylation at or near this site. In addition, our lab has just published a report (Wu et al., 2009 Nature Cell Bio. 11:644-651) showing that PP1 is suppressed in mitosis through the dual action of Cdk1 phosphorylation and binding of inhibitor 1. It is not known whether this inhibition of PP1 is catalytic, interferes with targeting of PP1 to its substrates, or both. However, based on these data, we favor a model in which PP1 binding to caspase-2 is not dependent on caspase-2 phosphorylation at any site and, conversely, the lack of PP1 binding to caspase-2 in mitosis is a result of the mitotic regulation of PP1 but not caspase-2. This work is currently ongoing.

9. How does phosphorylation at S308 inhibit caspase-2 activation?
We added new data (supplementary figure 2) showing that mitotic phosphorylation of caspase-2 does not affect caspase-2 binding to XRAIDD. We also included new data showing that XRAIDD-induced processing of the phospho-mimic caspase-2 S308E is suppressed compared to wild type (figure 5B). This experiment was done in vitro with recombinant XRAIDD and $^{35}$S-labeled caspase-2 and therefore focuses specifically on caspase-2 itself, ruling out contributions of other caspases or extraneous factors. In addition, we examined the possibility that mitotic phosphorylation of caspase-2 blocks the ability of caspase-3 to ‘feedback’ on caspase-2. We found that cdk1/cyclin B1-mediated phosphorylation at S308 had no effect on the ability of caspase-3 to cleave caspase-2. Based on these data and given the position of the phospho site (between both autoprocessing sites), we propose that mitotic phosphorylation of caspase-2 affects its autocatalytic processing, which has been shown to stabilize the active form of caspase 2.
10. Data in figure 4B (RAIDD-induced processing of the caspase-2 S308E) are not convincing. The original experiment in figure 4B was performed in interphase extract spiked with radiolabeled caspase-2 and the effect of the E mutant was not dramatic. We chose to repeat this experiment in vitro, reasoning that endogenous competing non-phosphorylated caspase-2 or other factors might have contributed to the unclear results. We were able to achieve more robust inhibition of caspase-2 E mutant processing in vitro. The results are now shown in figure 5B.

11. Processing if the S135A/S308A mutant should be compared to S308A in mitotic extracts. We have repeated this experiment as recommended and the results are shown in figure 4A.

12. If Cdk1 suppresses apoptosis upstream of cytochrome c release, it is unclear why procaspase-9 phosphorylation would have any effect. We have included a paragraph in the discussion that addresses this issue (2nd paragraph of the discussion). In short, we have also observed mitotic suppression of apoptosis (in extract) at the level of the apoptosome. Although delayed, cytochrome c is eventually released in mitotic extract. In agreement with previous reports, initially the mitotic apoptosome is very resistant to this release of cytochrome c but the block is eventually overcome and caspase-3 becomes fully active. In this scenario, either relieving the mitotic suppression of cytochrome c release or caspase-9 is sufficient to ultimately accelerate the activation of caspase-3.

13. Ser307 of caspase-9 is not conserved in rat or mouse. We have now made note of this in the manuscript (second to last paragraph of the discussion).

14. Mice deficient in RAIDD, PIDD, Caspase-2 show virtually no developmental phenotype. We added new discussion in which we attempt to reconcile this issue (4th paragraph of the discussion). It may well be that caspase 2 is important for death in response to particular stressors (heat shock, nutrient depletion), but that it does not play a critical role in normal developmental pathways (except in oocytes, perhaps due to the unique metabolism of the oocyte).

Minor comments:
1. We added the recommended reference (Gue et al., J. Biol. Chem. 2002) to our introduction with regard to bid cleavage by caspase-2.

2. We have included the amount of XRAIDD used in the legend of figure 2D (now figure 2E).

3. We split the original figure 3 into two figures (figures 3 and 4) so that panel 3A could be enlarged.

4. The experiment in figure 7B was set up as follows: One well of cells was infected with the caspase-2-expressing virus (for the ‘addback’ of caspase-2 into the siRNA-treated cells) then split into two wells. One of the two wells was treated with nocodazole then analyzed by flow cytometry for PI staining, and the other was analyzed by flow cytometry to determine the percentage of cells infected with the virus (by measuring virally expressed GFP). We measured GFP and PI staining separately because we found that doing a two color analysis of the nocodazole-treated cells greatly underestimated the number of infected cells (based on our analysis of the parallel sample not treated with nocodazole), as much of the GFP, which was somewhat weak to begin with, appeared to be lost in the dying cells. In the revised figure, we have eliminated the bar graph with the normalized data, and instead have included the raw flow cytometry data from the PI analysis (figure 7C) and, additionally, the raw flow cytometry data showing our measurement of the percentage of cells infected as determined by gating around GFP+ cells (figure 7B). We think this is a more straightforward way to present the data.

Reviewer 2

1. Include reference for a recent report showing RAIDD/PIDD-independent caspase-2 activation pathway. We have included this point in our discussion of caspase-2 activation in the introduction (4th paragraph).
2. *The restoration of C2 phosphorylation by I2 is not clear.*
We repeated this experiment with new preparations of reagents and achieved a much clearer result.
The data is now shown in figure 4C of the revised manuscript.

3. *Figure 3A is too small.*
We split figure 3 into two figures so that figure 3A could be enlarged.

4. *Figure 6B: why not gait GFP cells rather than correcting for the % of infected cells?*
See the discussion above for point 4 under minor comments of reviewer 1.

Reviewer 3

1. *Control for mitochondrial contamination in the cytochrome c blots.*
We repeated the experiment showing suppression of cytochrome c release in mitosis (figure 1C) and
have included a panel in which we blotted for VDAC to measure contamination of our cytosolic
supernatants with mitochondria.

2. *Mistake in numbering of figure 3D.*
The original figure should have shown the xenopus and zebrafish sequences starting at amino acid
300. This mistake has been corrected in the figure.

3. *Oocyte images are not clear in showing death.*
We replaced the original images with photos from the 6.5 hour time point where the difference in
death between the two groups can be seen more clearly.

4. *The siRNA used for caspase-2 is not described in the text.*
We have included the sequence information for our caspase-2 siRNA in materials and methods. We
custom designed the oligo to recognize the 3' UTR of caspase-2 and it is not the same sequence
used in previous caspase-2 papers.

5. *Cells treated with siRNA and transfected with wt or mutant caspase-2 should be examined for apoptotic without nocodazole treatment.*
These data have been included in figure 7B.

2nd Editorial Decision 29 July 2009

Thank you for sending us your revised manuscript. Our original referee 1 has now seen it again, and
you will be pleased to learn that in his/her view you have addressed all criticisms in a satisfactory
manner. The paper will now be publishable in The EMBO Journal and you will receive a formal
acceptance letter shortly.

Thank you very much again for considering our journal for publication of your work.

Yours sincerely,

Editor
The EMBO Journal