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Viral apoptosis is induced by IRF-3 mediated activation of Bax

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 30 November 2009

Thank you very much for submitting your research manuscript for consideration to The EMBO Journal editorial office. The enclosed comments indicate a clear interest in your study that proposes a direct role for IRF-3 in apoptotic induction, independent of transcription mechanistically via Bax-binding. As you will see all referee's request revisions that are aimed to increase experimental support and impact of the study. The most critical one (ref#2) asks for a lot of further detailed binding characteristics and exploration of Traf2/6 contributions. Given the rather straightforward message of the paper, we would not ultimately insist on the Traf part. We will however demand additional data on IRF-3/Bax-binding and testing for the specificity.
All in all, I am happy to invite submission of a modified version of your work that would attend to the critical concerns raised by the referees. I also have to remind you that it is EMBO_J policy to allow a single round of revisions only, which means that the final decision on your work depends on the content within its final version.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website:
http://www.nature.com/emboj/about/process.html

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

Outside the Bcl-2 family members Bid, Bim and Puma, only the transcription factor p53 has been (somewhat controversially) suggested to interact with and directly activate Bax (Science. 2004 Feb 13;303(5660):1010-4). It is amazing that the work presented here now describes that another transcription factor, IRF-3, can induce apoptosis independent of its transcriptional activity by its direct interaction with Bax. The model put forward in this work is tested in a suitable number of experimental settings to warrant publication, and overall, the findings will be of immense interest to the cell death and immune signaling fields. However, a few additional experiments, explanations and controls, would significantly enhance the conclusions and physiological relevance of the current manuscript. These include:

i) The authors have recently published that the initiating caspase for SeV and IRF3 dependent death occurs by caspase-8 activation and does not require caspase-9 (Journal of Virology, Apr. 2008, p. 3500-3508), the opposite of the data presented in the current manuscript, and is conveniently ignored (even though referenced). The authors should clarify their position for readers in light of their current results.

ii) Most readers will want to know the % of cells undergoing apoptosis (ie. AnnexinV and/or propidium iodide staining), especially for several key experiments where only caspase activity assays or PARP cleavage are presented (ie. Fig 1D, 2B, 4C, S3C, 8C). The arbitrary units provided by measuring caspase activity and examining PARP cleavage, although suitable when used in conjunction with quantitatively measuring apoptotic death, do not alone provide sufficient information to judge whether a significant population of cells are undergoing, or are protected from, cell death.

iii) Quantitative measurements of viral induction of apoptosis in the various knockout MEFs used would strengthen the findings made used by poly(I:C) transfection alone (ie. Fig.2C, 3A, 8C).

iv) The caspase inhibitors used need to be clearly defined in the text.

v) More of the PARP blots should be shown so both full length and cleaved PARP can be observed, and their relative intensities compared.

vi) Fig 5E. Cytochrome C levels in the mitochondria fraction should be shown for a direct comparison to the cytoplasmic cytochrome C levels.

vii) Fig 5F. IRF3 levels in the nuclear fraction should be provided as a direct comparison to the amount translocating to the mitochondrial fraction.

viii) Fig. 6A and B. Bax levels in the cell lysates (input) used for the IPs should be shown.

ix) Fig. 6D. The mitochondria Bax levels should be shown in direct comparison to those found in the cytoplasm.

Referee #2 (Remarks to the Author):

Manuscript by Chattopadhyay et al describes the regulation of viral apoptosis by IRF-3 mediated activation of Bax. The authors claim that IRF-3 mediated signaling proceeds by two pathways, one leading to gene expression and the other to apoptosis. The apoptotic pathway supposedly involves IRF-3 mediated activation of Bax.

The authors have demonstrated that IRF-3 activation by cytoplasmic dsRNA signaling causes apoptosis, as was shown before. They also confirm that there are shared signaling proteins for gene induction and apoptosis downstream of IRF-3. However, it is not clear at all, and there is no

apparent link between the investigation of IRF-3 mediated apoptotic signaling and Bax activation. The authors never checked any other Bcl-2 family members; an issue that is critical for understanding of mechanism and specificity of IRF-3 mediated Bax activation. Thus, the authors should test if IRF-3 binds any other Bcl-2 family members. The most critical member is obviously Bak, a pro-apoptotic Bcl-2 family member that shares great functional and structural similarity with Bax. The authors should test if Bak knockdown or Bak -/- MEFs affect IRF-3 mediated apoptosis. They should also investigate if IRF-3 has a Bcl-2 homology (BH) region that is critical for activation of Bax and Bak. If IRF-3 has a BH(3) domain, it needs to be mutated to check for binding to Bcl-2 proteins and mediation of apoptosis. If IRF-3 does not have a BH(3) domain, the authors should explore which part of the molecule binds Bax so selectively.

Second, it is not clear what role(s) Traf2 and Traf6 have in IRF-3 mediated apoptosis. Unlike Traf3, these Traf proteins appear to be involved in IRF-3 mediated apoptotic signaling and not gene expression, but how and why are they important is not explained at all.

Specific points:
1. Why do the authors use IRF-3 isolated from dsRNA-stimulated cells for binding assays? What post-translational changes in IRF-3 are expected to be important for putative binding to Bax? It would be much better to generate recombinant purified IRF-3 and test it for binding to Bax using Bak and other Bcl-2 proteins as control, not just GST protein.
2. In figure 4A the authors should treat the cells with CHX and ActD alone, without poly(I:C) to investigate what effect would those chemicals have on the caspase activation and cell death.
3. In figure 7B the authors should use stimuli that kill cells in Bax-dependent (chemotherapeutics) and Bax-independent fashion (Fas in Jurkat cells for example) as positive and negative controls to verify their experimental system.

Referee #3 (Remarks to the Author):

This an interesting manuscript that describes a pro-apototic activity of IRF3 that is independent of its transcriptional activation function. The authors demonstrate that its is due to binding to Bax and localization to the mitochondria.

A few minor comments.

1. The authors should be explicit throughout the manuscript in describing the experiments which are are done with transient transfections and which are done in cell lines or pools of cells that harbor mutant or variant forms of IRF3. For some of the experiments it was difficult/impossible to determine.

2. In figure 4, it would be good to show the levels of the various IRF3 molecules in the actaul figure and not in the supplemental materials.

3. Was the experiment shown in figure 7Brepeated? If so how reproducible was the experiment. A close examination of the IRF-3 minus cells also reveals some of the higher molecular weight complex. Is the difference real or due to different exposures. how is protein being normalized in this experiment?

4. Minor typo in Heading title on page 8.

1st Revision - Authors' Response 23 February 2010

Referee #1

"The authors have recently published that the initiating caspase for SeV and IRF3 dependent death occurs by caspase-8 activation and does not require caspase-9 (Journal of Virology, Apr. 2008, p. 3500-3508), the opposite of the data presented in the current manuscript, and is conveniently

ignored (even though referenced). The authors should clarify their position for readers in light of their current results."

In the *Journal of Virology* paper we studied rapid (4-6h) apoptosis in SeV infected cells that had also been treated with a PI3 kinase inhibitor, whereas here we study slow (12-48h) apoptosis in response to only SeV infection. Thus, there is no obvious discrepancy; Caspase 8 could be the primary initiating enzyme in the rapid pathway and Caspase 9 could be the primary enzyme in the slow pathway. Moreover, we used only chemical inhibitors in the previous study, but because we used here siRNA-mediated gene ablation as well, the drawn conclusions are more definitive.

"Most readers will want to know the % of cells undergoing apoptosis (ie. AnnexinV and/or propidium iodide staining), especially for several key experiments where only caspase activity assays or PARP cleavage are presented (ie. Fig 1D, 2B, 4C, S3C, 8C). The arbitrary units provided by measuring caspase activity and examining PARP cleavage, although suitable when used in conjunction with quantitatively measuring apoptotic death, do not alone provide sufficient information to judge whether a significant population of cells are undergoing, or are protected from, cell death."

The quantitative apoptosis information wanted by this reviewer is provided in 2C, 3B, S1D and S1E

"Quantitative measurements of viral induction of apoptosis in the various knockout MEFs used would strengthen the findings made used by poly(I:C) transfection alone (ie. Fig.2C, 3A, 8C)."

New results from virus infection of primary knock-out cells are shown in Fig 9E

"The caspase inhibitors used need to be clearly defined in the text."

Done, in the Fig legend

"More of the PARP blots should be shown so both full length and cleaved PARP can be observed, and their relative intensities compared."

Done, see Fig 1E, 4A

"Fig 5E. Cytochrome C levels in the mitochondria fraction should be shown for a direct comparison to the cytoplasmic cytochrome C levels."

This comparison is shown in Fig 7C

"Fig 5F. IRF3 levels in the nuclear fraction should be provided as a direct comparison to the amount translocating to the mitochondrial fraction."

Done, see Fig 5F

"Fig. 6A and B. Bax levels in the cell lysates (input) used for the IPs should be shown."

Done, see Fig 6A, Fig S4B

"Fig. 6D. The mitochondria Bax levels should be shown in direct comparison to those found in the cytoplasm."

Done, see Fig 7A, 7C

Referee #2

"The authors have demonstrated that IRF-3 activation by cytoplasmic dsRNA signaling causes apoptosis, as was shown before. They also confirm that there are shared signaling proteins for gene induction and apoptosis downstream of IRF-3. However, it is not clear at all, and there is no apparent link between the investigation of IRF-3 mediated apoptotic signaling and Bax activation. The authors never checked any other Bcl-2 family members; an issue that is critical for understanding of mechanism and specificity of IRF-3 mediated Bax activation. Thus, the authors should test if IRF-3 binds any other Bcl-2 family members. The most critical member is obviously Bak, a pro-apoptotic Bcl-2 family member that shares great functional and structural similarity with Bax. The authors should test if Bak knockdown or Bak -/- MEFs affect IRF-3 mediated apoptosis. They should also investigate if IRF-3 has a Bcl-2 homology (BH) region that is critical for activation of Bax and Bak. If IRF-3 has a BH(3) domain, it needs to be mutated to check for binding to Bcl-2 proteins and mediation of apoptosis. If IRF-3 does not have a BH(3) domain, the authors should explore which part of the molecule binds Bax so selectively."

As suggested by this reviewer, we have tested possible interaction of IRF-3 with Bak, Bcl-xL and Bcl2. None of these proteins co-immunopricipitated with IRF-3, but Bax did (Fig 6B). Since we did not observe any interaction with Bak, we did not test any functional effect of Bak ablation.

As suggested, we delineated the domain of IRF-3 (residue 366 to 427) that is required for Bax interaction and apoptosis (Fig 6D). Moreover, we identified a BH3 domain within this region (Fig 6E). When appropriate point mutations were introduced to this BH3 domain, Bax interaction (Fig 6F) and apoptosis (Fig 9F) were abolished. These results clearly established the nature of interaction between IRF-3 and Bax.

"Second, it is not clear what role(s) Traf2 and Traf6 have in IRF-3 mediated apoptosis. Unlike Traf3, these Traf proteins appear to be involved in IRF-3 mediated apoptotic signaling and not gene expression, but how and why are they important is not explained at all."

We agree that this is an interesting issue. However we believe that the investigation required to explore it, is open-ended and well beyond the scope of this paper; this conclusion was supported by the Editor's comments.

Specific points

"Why do the authors use IRF-3 isolated from dsRNA-stimulated cells for binding assays? What post-translational changes in IRF-3 are expected to be important for putative binding to Bax? It would be much better to generate recombinant purified IRF-3 and test it for binding to Bax using Bak and other Bcl-2 proteins as control, not just GST protein."

Our in vivo results show that IRF-3 needs to be activated by RLH signaling to enable its interaction with Bax. Moreover, the protein kinase TBK-1 was needed for this activation, indicating that phosphorylation of unknown residues of IRF-3 might be required. For these reasons, we used IRF-3 purified from stimulated cells in our in vitro Cyt C release assays.

"In figure 4A the authors should treat the cells with CHX and ActD alone, without poly(I:C) to investigate what effect would those chemicals have on the caspase activation and cell death."

The suggested experiments have been presented in Fig 4A; see lanes 3 and 5

"In figure 7B the authors should use stimuli that kill cells in Bax-dependent (chemotherapeutics) and Bax-independent fashion (Fas in Jurkat cells for example) as positive and negative controls to verify their experimental system."

The suggested agents can be used only in whole cell experiments, not in the in vitro experiments shown in old Fig 7 (new Fig 8). Instead, we use activated Bid as the positive control for activating Bax in this experiment.

Referee #3

"The authors should be explicit throughout the manuscript in describing the experiments which are done with transient transfections and which are done in cell lines or pools of cells that harbor mutant or variant forms of IRF3. For some of the experiments it was difficult/impossible to determine."

No result is from transient transfection. All cells were stably expressing Wt IRF-3 or its mutants.

"In figure 4, it would be good to show the levels of the various IRF3 molecules in the actual figure and not in the supplemental materials."

Done, see Fig 4C

"Was the experiment shown in figure 7Brepeated? If so how reproducible was the experiment. A close examination of the IRF-3 minus cells also reveals some of the higher molecular weight complex. Is the difference real or due to different exposures. how is protein being normalized in this experiment?"

Yes, the experiment shown in old Fig 7B (new Fig 8B) was repeated four times and produced essentially the same results. The image in the figure is from one experiment and both panels were handled similarly and exposed for the same period of time. Same amount of Bax was used with and without IRF-3 and the same volume of each fraction was analyzed by Western Blot. No protein normalization was involved in the analysis.

"Minor typo in Heading title on page 8."

Typo corrected.

