Increased Levels of Inositol Hexakisphosphate (InsP$_6$) Protect HEK293 Cells from Tumor Necrosis Factor α- and Fas-induced Apoptosis*

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The overexpression of inositol 1,3,4-trisphosphate 5/6-kinase has recently been shown to protect HEK293 cells from tumor necrosis factor α (TNFα)-induced apoptosis. This overexpression leads to an increase in the levels of both inositol 1,3,4,5,6-pentakisphosphate (InsP$_5$) and inositol 1,2,3,4,5,6-hexakisphosphate (InsP$_6$). Cells that overexpress InsP$_5$ 2-kinase have increased levels of InsP$_5$, and are also protected from TNFα-induced apoptosis; furthermore, cells that express an RNA interference construct to the 2-kinase are deficient in InsP$_5$ and are sensitized to TNFα-induced apoptosis. Therefore the protective effect of 5/6-kinase on TNFα-mediated apoptosis is due to an increase of InsP$_6$ or to a metabolite derived from InsP$_6$. Furthermore, we find that the InsP$_6$ also protects from Fas-mediated apoptosis. No effect was seen in the endocytic rate of transferrin receptor, caspase 8 activity, or TNF receptor number at the cell surface. Cells that overexpress 2-kinase do show an increase in the amount of receptor-interacting protein (RIP), while cells with reduced InsP$_6$ levels show relatively less RIP, providing a possible mechanism for the effect on apoptosis.

The pathways that produce the higher soluble inositol phosphates in human cells have been elucidated recently (1–3). The action of phospholipase C on phosphatidylinositol 4,5-bisphosphate, yields inositol 1,4,5-trisphosphate (InsP$_3$) and diacetyl glycerol. In mammalian cells, InsP$_3$ is phosphorylated to inositol 1,3,4,5-tetrakisphosphate by an InsP$_3$ 3-kinase and dephosphorylated to inositol 1,3,4-trisphosphate (Ins(1,3,4)P$_3$) by a 5-kinase (4). Ins(1,3,4)P$_3$ is then phosphorylated to inositol 1,3,4,6-tetrakisphosphate (Ins(1,3,4,6)P$_4$) by the Ins(1,3,4)P$_3$ 5/6-kinase, to inositol 1,3,4,5,6-pentakisphosphate (InsP$_6$) by Ins(1,3,4,6)P$_4$-kinase, to inositol 1,2,3,4,5,6-hexakisphosphate (InsP$_6$) by InsP$_6$ 2-kinase (1, 5). In cell culture, production of InsP$_6$ is regulated by the activity of 5/6-kinase, which is rate-limiting (6) producing the committed isomer in the synthesis of InsP$_6$. Ins(1,3,4,6)P$_4$. Overexpression of 5/6-kinase results in an increase in InsP$_6$ and InsP$_6$, while depletion of 5/6-kinase results in the loss of InsP$_5$ and InsP$_6$.

Another function for the inositol (1,3,4)P$_3$ 5/6-kinase other than phosphorylating inositol phosphates was recently discovered. Wilson et al. (7) have shown that 5/6-kinase co-purifies with the COP9 signalosome from cow brain. This complex of nine proteins has been shown to have the ability to phosphorylate c-Jun, IxBα, and p53 (8–10). Wilson et al. (7) subsequently showed that 5/6-kinase purified from insect cells also phosphorylates c-Jun, p53, and IxBα, making it likely that in part the protein kinase activity of the COP9 signalosome may be attributed to 5/6-kinase.

c-Jun and IxB are both involved in TNFα signaling and apoptosis. TNFα is involved in numerous processes including cell death and development and oncogenesis and immune, inflammatory, and stress responses (11). TNFα acts in opposing ways with regards to apoptosis. Through one arm of the pathway it can activate transcription by NFκB; TRADD is recruited to the TNF receptor through its death domains, and it in turn recruits RIP, a death-domain-containing kinase, and TRAF2. Together TRAF2 and RIP recruit and activate IκB kinase complex, which phosphorylates IκBα, signaling it for ubiquitination and destruction. NFκB is then translocated to the nucleus to stimulate transcription. Two of the many transcriptional targets of NFκB are FLIP and cIAP, proteins that inhibit apoptosis, and thus NFκB activity is considered anti-apoptotic. The second of the pathways it shares with Fas; this pathway stimulates apoptosis by recruiting FADD and caspase 8, which results in the cleavage and activation of caspase 8, initiating the caspase cascade. The pro-apoptotic action of TNFα cannot overcome its anti-apoptotic activity through NFκB in most cells; thus, to induce apoptosis in cells, protein synthesis has to be inhibited or NFκB signaling has to be blocked.

The ability of 5/6-kinase to phosphorylate proteins involved in TNFα-mediated apoptosis led Sun et al. (12) to determine whether the overexpression of 5/6-kinase had an effect on apoptosis. They found that HEK293 cells were protected from TNFα-induced apoptosis when there were elevated amounts of 5/6-kinase. They investigated whether this protection was due to increased NFκB signaling, but they found no difference in IκBα stability or NFκB activity by gel shifts of 5/6-kinase-expressing lines. This led to the possibility that the protection from apoptosis was due to inhibition of caspase activation rather than NFκB stimulation. It also suggested that the protection from apoptosis may not be due to the associated protein kinase activity of 5/6-kinase but rather through its inositol phosphate kinase activity and thus to the soluble, more highly phosphorylated inositol phosphates.
We recently have shown that an increase in 5/6-kinase activity in cells results in a concomitant increase of InsP₆ and InsP₅. Thus activities ascribed to the inositol phosphate kinase function of 5/6-kinase may be due to actions of InsP₅ or InsP₆ or to another downstream metabolite. Here we show that expression of 2-kinase and an increase of InsP₆ can protect cells from TNFα-mediated apoptosis, and we attribute the protective effect of 5/6-kinase on TNFα-mediated apoptosis to the production of InsP₆ itself. In addition, we find that expression of 2-kinase also results in a protection from Fas mediated apoptosis, and thus InsP₆ may be a general regulator of apoptosis. Furthermore, we notice that altered InsP₆ levels in cells result in altered levels of the protein RIP, which may provide a mechanism for the role of InsP₆ in apoptosis.

MATERIALS AND METHODS

All chemicals were reagent grade or better. Restriction endonucleases, DNA modifying enzymes, and general reagents were from Amer sham Biosciences, Roche Applied Sciences, Fisher, Invitrogen, New England Biolabs (NEB), Promega Corp., Sigma, Stratagene, and Bio-Rad, unless stated otherwise. Acrylamide solution, Bio-Safe Coomassie Blue stain, and Bradford protein assay kit used for protein work were purchased from Bio-Rad. The SuperSignal West Pico kit used for detection of Western blots was from Pierce. Radiolabeled [³H]inositol and [³H]InsP₆ were purchased from American Radiolabeled Chemicals (St. Louis, MO) and Amersham Biosciences, respectively. TNFα was obtained from Peprotech and activating Fas antibody from BD Biosciences. Anti-caspase 8 (e20) goat polyclonal antibody was obtained from Santa Cruz Biotechnology, anti-caspase 8 monoclonal antibody (mAb) from Cell Signaling, and anti-RIP mAb from BD Biosciences. Protein G was obtained from Sigma.

Strains, Plasmids, and Growth Conditions—Methods for Escherichia coli growth and selection were described previously (13, 14). E. coli strain DH5α (lacZΔM15, thi-1, hsdR17 (rK-,M-) strain XLI-Blue (Stratagene) was used as the bacterial host for all plasmids unless stated otherwise. Bacterial strains were cultured in LB (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.4) medium supplemented with ampicillin (100 μg/ml) where appropriate and transformed by standard methods. All bacterial strains were grown at 37°C.

Cloning, Production, and Maintenance of Cell Lines—Stable cell lines expressing 2-kinase were reported previously (6). The stably transfected 2-kinase RNAi line was produced as follows. Oligonucleotides containing the antisense target to the 2-kinase (5′-GAAGAGCTGGAAAGGAGATA-3′) or to luciferase (5′-CTTACGCTGAGTACTTCGA-3′) were annealed and ligated in the pSUPER vector (a gift from Dr. Reuven Agami). Equal amounts of either the 2-kinase or the luciferase RNAi construct were mixed 7:1 with pBabe containing a puromycin resistance gene and transfected into the same parent cells as the overexpression vector pEmptyTrex (pSUPER-EH3 tetracycline inducible vector, Invitrogen) (5′-TTATGCACCTTGAACTACAGC-3′). Cells were selected with 1 μg/ml puromycin and then grown for 24 h. Western analysis was conducted using anti-RIP mAb for 1 h. The complexes were precipitated with 2 μg of anti-caspase 8 antibody and 50 μl of protein G-agarose at 4°C overnight. Beads were recovered by centrifugation, washed twice with lysis buffer, and subjected to Western analysis as above. To ensure that equal amounts of caspase 8 were being precipitated, 5% of the precipitated protein was run on a separate gel and blotted with mAb against caspase 8. Total cellular RIP was immunoprecipitated as follows: 0.5 × 10⁶ cells were plated on 6-well plates and grown for 24 h. Cells from each well were lysed for the caspase 8 immunoprecipitations, and RIP was precipitated with 1 μg of anti-RIP mAb for 1 h.

RESULTS

HEK293 Cells Expressing a Stably Transfected RNAi Construct to the 2-Kinase Result in Altered InsP₆ Profiles—HEK293 cell lines constructed with a tetracycline inducible 2-kinase gene show an altered inositol phosphate profile as reported previously; specifically, expression of the 2-kinase results in an increase in InsP₆ and a loss of InsP₅ (3). Total cellular RIP was immunoprecipitated as follows: 0.5 × 10⁶ cells were plated on 6-well plates and grown for 24 h. Cells from each well were lysed as for the caspase 8 immunoprecipitations, and RIP was precipitated with 1 μg of anti-RIP mAb for 1 h. The complexes were precipitated with 25 μl of protein G-agarose for 30 min and washed three times with PBS, and the pellets were subjected to Western blot analysis as above.

High Performance Liquid Chromatography (HPLC)—HPLC was conducted as follows. Cells were grown in the presence of [³H]inositol (10 μCi/ml) for 4 days. Cells were lysed in methanol/0.5 M HCl (2:1) and extracted with chloroform. The aqueous phase was separated, dried, and resuspended in distilled water. Samples were applied to a Whatman TLC plate and chromatographed using a solvent system consisting of 50% methanol/0.5 M NaCl at 4°C. The acid wash was combined with a short wash in the same buffer and used to determine the amount of surface-bound [³H]-lignand. The cells were lysed in 1 N NaOH to determine the intracellular (internalized) radioactivity. The ratio of internalized to surface radioactivity was plotted against time. At 10 minutes, a 100-fold molar excess of unlabeled lignand was added, and the cells were treated as before to determine the background binding.

Immunoprecipitation—For caspase 8 co-immunoprecipitations, 90% confluent p150 tissue culture plates treated as indicated were used for each immunoprecipitation. Cells were washed in PBS and lysed in 1.5 ml of lysis buffer (20 mM HEPES, pH 7.6, 140 mM NaCl, 10% glycerol, 0.2% Nonidet P-40 plus protease inhibitors (Complete Mini EDTA-free, Roche)) for 30 min on ice. An equal mass of each lysate was precleared on protein G-agarose for 1 h, and the caspase 8 complex was precipitated with 2 μg of anti-caspase 8 antibody and 50 μl of protein G-agarose at 4°C overnight. Beads were recovered by centrifugation, washed twice with lysis buffer, and subjected to Western analysis as above. To ensure that equal amounts of caspase 8 were being precipitated, 5% of the precipitated protein was run on a separate gel and blotted with mAb against caspase 8. Total cellular RIP was immunoprecipitated as follows: 0.5 × 10⁶ cells were plated on 6-well plates and grown for 24 h. Cells from each well were lysed as for the caspase 8 immunoprecipitations, and RIP was precipitated with 1 μg of anti-RIP mAb for 1 h. The complexes were precipitated with 25 μl of protein G-agarose for 30 min and washed three times with PBS, and the pellets were subjected to Western blot analysis as above.
2-Kinase-expressing Cells Are Resistant to TNFα-mediated Apoptosis, whereas 2-Kinase RNAi Lines Are More Susceptible to Apoptosis—5/6-Kinase-overexpressing cells show an increase of InsP₆ and a protection from TNFα-induced apoptosis. 2-Kinase-overexpressing cells show increased InsP₆ and a loss of InsP₅. When treated with TNFα 2-kinase-overexpressing cells (Fig. 2B) show a relative decrease in the number of apoptotic cells, as determined by APOPercentage staining, when compared with vector cells (Fig. 2A). Similarly, relative to the luciferase lines (Fig. 2C) the 2-kinase RNAi lines (Fig. 2D) showed more apoptotic staining when treated with TNFα.

The results shown in Fig. 2 were confirmed by Western blot analysis. Cells expressing 2-kinase and the vector control were treated with TNFα and cycloheximide for 7 and 24 h and their extracts blotted with a monoclonal antibody against PARP, a target of caspase 3. Consistent with the results from the cell staining, cells overexpressing 2-kinase showed a decrease in the amount of apoptosis as determined by the amount of cleaved PARP relative to the vector lines at both time points (Fig. 3A). We also looked at cleaved PARP accumulation in 2-kinase RNAi lines. Compared with the luciferase lines, the 2-kinase RNAi line showed more cleaved PARP at 7 and 24 h, and is therefore more susceptible to TNFα-mediated apoptosis (Fig. 3B).

In both sets of experiments, the amount of apoptosis seen in the tetracycline-induced vector lines was greater than that seen in the luciferase RNAi control lines. Since the vector lines were treated with tetracycline, the transcription machinery was induced by the addition of tetracycline, as it is in the 2-kinase-expressing lines. The RNAi lines were not treated with tetracycline. This may account for the difference between the control lines. Therefore, it is necessary to consider the increase or decrease of apoptosis relative to their respective control.

The 2-Kinase RNAi Construct Can Overcome the Protection from Apoptosis of 5/6-Kinase Expression—To address whether the protective effect was solely due to the presence of InsP₆ and does not involve other products resulting from 5/6-kinase and its associated protein kinase activity, we expressed the 2-kinase RNAi construct in cells overexpressing 5/6-kinase. There was an increase in apoptosis in the cells transfected with the 2-kinase RNAi relative to those transfected with the luciferase RNAi (Fig. 4). Taken with the above results, we conclude that the protective effect from TNFα-mediated apoptosis is due to the presence of InsP₆ or a downstream metabolite.

Increased InsP₆ Protects against FAS-mediated Apoptosis, while Decreased InsP₆ Levels Render Cells More Susceptible to FAS-mediated Apoptosis—In addition to protection from TNFα-mediated apoptosis, we also found that 2-kinase overexpression could protect cells from Fas-induced apoptosis as shown in Fig. 5. Cell lines were treated with increasing amounts of Fas antibody for 20 h, and their cell extracts were analyzed by Western blotting for PARP. Cells that were overexpressing the 2-kinase showed less PARP cleavage at 1 and 3 μg/ml as compared with the vector control lines (Fig. 5A). Furthermore, the 2-kinase RNAi line showed more apoptosis as compared with the luciferase control (Fig. 5B). The results of PARP cleavage for the 6 μg/ml Fas treatment were analyzed by densitometry to normalize loading. Whereas the ratio of full-length PARP to tubulin were similar for RNAi and Luciferase lines (1.7 to 1.6, respectively), the ratio of cleaved PARP to tubulin was almost three times greater in the RNAi line than luciferase line (0.41 to 0.15, respectively). Therefore, relatively more PARP is cleaved in the RNAi line than the luciferase line.

Alterations in InsP₆ Levels Do Not Affect Receptor Internalization, Caspase 8 Activity, or TNF Receptor Number—Since it is unlikely that the protective effect of the 2-kinase on apoptosis is due to NFαB activation, we looked for other cellular alterations that would explain the protective effect. Receptor internalization is required for TNF induced apoptosis and is mediated by a canonical XXXX motif known to mediate receptor internalization by clathrin-coated vesicles (15); when mutated, TNF receptor internalization is inhibited, and the death inducing signaling complex is not formed, thus blocking apoptosis. Since InsP₆ has been implicated in endocytosis, we measured transferrin internalization in cells overexpressing the 2-kinase. As seen in Fig. 6, 2-kinase overexpression does not affect transferrin internalization compared with vector control. The regression coefficient of four experiments, denoting the internalization constant (kₑ), was averaged and showed no difference between the 2-kinase or vector lines. Furthermore, we saw no difference in internalization rates between 2-kinase and luciferase lines (data not shown). We also measured the TNF and Fas receptor number at the cell surface by FACs analysis and found no difference between the cell lines containing increased amounts of InsP₆ and those with depleted levels of InsP₆. Finally, caspase 8 activity was assayed with increasing amounts of InsP₆ or inositol hexakissulfate to control for
nonspecific charge effect, and no effect was seen on caspase 8 activity up to 100 μM InsP₆ or inositol hexakisulfate.

**2-Kinase-overexpressing Cells and 2-Kinase RNAi Lines Showed Altered Levels of the Anti-apoptotic Protein RIP**—Western blotting of 25 μg of cell lysate showed an increase in the level of RIP in the 2-kinase-expressing lines as compared with the vector control and a decrease in the RNAi stable lines as compared with the luciferase control (Fig. 7). When normalized to the tubulin control, RIP levels were increased by about 50% in the 2-kinase-expressing lines and decreased by about 50% in the RNAi lines.

The elevated amount of RIP in cellular extracts is reflected in an increase in co-immunoprecipitations of RIP with anti-caspase 8. The method of Micheau and Tschopp (16) was employed to address the effect of the 2-kinase on the formation of the caspase 8/TRADD/RIP complex with the TNF receptor. Interestingly, it was unnecessary to stimulate cells with TNF to co-immunoprecipitate RIP with caspase 8, suggesting that...
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**DISCUSSION**

The overexpression of 5/6-kinase has been shown by Sun *et al.* (12) to protect against TNFα-mediated apoptosis, although the mechanism was unknown. Since the overexpression of 5/6-kinase also results in an elevation in the amounts of InsP₆ and InsP₇ (1), it is possible that this protective effect is due to one or more of these isomers of inositol phosphate. Cells overexpressing 2-kinase accumulate InsP₆, deplete InsP₅, and show protection from apoptosis relative to vector controls. Furthermore, HEK293 cells stably transfected with an RNAi construct to 2-kinase overexpressing cell lines. These results would suggest that in these cells 2-kinase overexpression affects the turnover rate of the caspase 8, FADD, TRADD, and RIP co-complex.

As mentioned above, our results do not conclusively implicate InsP₆ itself in the protection from apoptosis; we can only say that the isomer involved has to lie downstream of InsP₆. InsP₆ is metabolized to the higher inositol pyrophosphates InsP₇ and InsP₈. We, though, have seen no increase in an InsP₇ isomer when expressing the 2-kinase in metabolically labeled cells (data not shown), and there is some evidence that the inositol pyrophosphates may stimulate apoptosis. Morrison *et al.* (18) used an antisense technique to identify genes that are involved in interferon β-induced apoptosis in an ovarian carcinoma cell line. One gene that was shown to sensitize these cells to interferon β-induced apoptosis was the inositol hexakisphosphate kinase 2. This protein converts InsP₆ to the pyrophosphate InsP₇. Interferon β acts by stimulating transcription through the JAK/STAT pathway. Interestingly, the transcription of a number of targets stimulated by INFβ are involved in TNFα-mediated apoptosis (e.g., caspases 8) or are analogous to TNFα apoptosis, e.g., TRAIL (TNF-related apoptosis-inducing ligand). Since InsP₆ kinases phosphorylate InsP₆, their expression may result in the depletion of cellular levels of InsP₆, which we show here to sensitize cells to apoptosis. Thus the balance between InsP₆ and the inositol pyrophosphates may act as a switch controlling apoptosis. Nonetheless, such speculation requires experiments with the InsP₆ kinases to determine definitively the inositol isomer involved.

The protection from apoptosis afforded by expression of the 2-kinase probably is not due to the known anti-apoptotic consequences of TNFα, namely through NFκB activation. No decrease in IκB stability or increase in NFκB activity is seen in 5/6-kinase-overexpressing cells. The protection from Fas-mediated apoptosis would argue against the role of NFκB activation. Also, the TNFα assays are done in the presence of cycloheximide, where protein synthesis is inhibited. The protective effect may work on the apoptotic branch of TNFα signaling.

We addressed a number of the possible steps in the activation of the apoptotic branch of TNFα signaling. We looked at cell surface expression of the TNF receptor between lines that had altered levels of InsP₆ and saw no difference nor did we see an effect of InsP₆ on caspase 8 activity in *vitro*. TNF activation has recently been shown to require the internalization of the TNF receptor (19). InsP₆ has been implicated in endocytosis and could delay activation of caspase 8 by altering the endocytic rate of the receptor. Yet cells with altered levels of InsP₆ showed no defect in the uptake of transferrin, and it is unlikely that this would provide the mechanism for the protection from apoptosis. Furthermore, our results provide the first *in vivo* description of the effect of altered InsP₆ levels on endocytosis. Although it has been implicated in endocytosis by a number of studies, we did not see an effect on endocytosis when InsP₆ levels were elevated (Fig. 6) or depleted (data not shown).

The increase in InsP₆ is correlated with an increase in RIP and may provide a mechanism for the protection afforded by an increase in InsP₆ levels. RIP−/− mouse embryonic fibroblasts are sensitive to TNFα-mediated apoptosis (20), likely due to its role recruiting and activating the IκB kinase complex, resulting in the activation of NFκB. Since this does not seem to be
involved in the current study, other roles for RIP must be considered. RIP is cleaved by caspase 8 upon TNFα engagement of its receptor, resulting in a C-terminal and N-terminal fragment, and consequently RIP levels drop. Expression of the C-terminal fragment of RIP in cells stimulates the FADD and TRADD interaction, while expression of the full-length RIP inhibits the FADD to TRADD interaction (21). If full-length RIP can compete for binding with cRIP to TRADD, this may result in a delay of recruitment of FADD to TRADD and thus delay caspase activation. Our results are consistent with the work by Sun et al. (12) that suggested that the protection from TNFα-mediated apoptosis was due to an inhibition of the recruitment of FADD to TRADD.

It is not clear how InsP₆ affects RIP levels. Preliminary experiments to determine the effect of InsP₆ itself on the half-life of RIP did show that cells deficient in InsP₆ lost RIP more quickly when treated with cycloheximide; at 3 h after treatment, RIP levels dropped to 40% of the original level of RIP in the 2-kinase RNAi lines, while they remained at about 90% of the original levels in the luciferase control lines (data not shown). The role of InsP₆ is well known in mRNA export. Since RIP is a protein with a relatively short half-life, it may require more efficient export of mRNAs to maintain a level of message for efficient translation of protein. Alternatively, InsP₆ may affect its turnover rate by affecting the proteosome or its targeting for degradation by ubiquitination.

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