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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 $InsP_6$ and are also protected from TNFα-induced apoptosis; furthermore, cells that express an RNA interference construct to the 2-kinase are deficient in $InsP_6$ 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 ($InsP_3$)[1] and diacylglycerol. 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-phosphatase (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 ($InsP_5$) by $Ins(1,3,4,6)P_4$ 5-kinase, and to inositol 1,2,3,4,5,6-hexakisphosphate ($InsP_6$) by $InsP_5$ 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 $InsP_5$ 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, IκBα, and p53 (8–10). Wilson *et al.* (7) subsequently showed that 5/6-kinase purified from insect cells also phosphorylates c-Jun, p53, and IκBα, making it likely that in part the protein kinase activity of the COP9 signalosome may be attributed to 5/6-kinase.

c-Jun and IκB 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.

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[1] The abbreviations used are: $InsP_3$, inositol 1,4,5-trisphosphate; $Ins(1,3,4)P_3$, inositol 1,3,4-trisphosphate; $Ins(1,3,4,6)P_4$, inositol 1,3,4,6-tetrakisphosphate; $InsP_5$, inositol 1,3,4,5,6-pentakisphosphate; $InsP_6$, inositol 1,2,3,4,5,6-hexakisphosphate; HPLC, high performance liquid chromatography; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PARP, poly(ADP-ribose) polymerase; cPARP, cleaved PARP; TNF, tumor necrosis factor; RIP, receptor-interacting protein; RNAi, RNA interference.

We recently have shown that an increase in 5/6-kinase activity in cells results in a concomitant increase of $InsP_5$ and $InsP_6$. Thus activities ascribed to the inositol phosphate kinase function of 5/6-kinase may be due to actions of $InsP_5$ or $InsP_6$ or to another downstream metabolite. Here we show that expression of 2-kinase and an increase of $InsP_6$ 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 $InsP_6$ itself. In addition, we find that expression of 2-kinase also results in a protection from Fas mediated apoptosis, and thus $InsP_6$ may be a general regulator of apoptosis. Furthermore, we notice that altered $InsP_6$ levels in cells result in altered levels of the protein RIP, which may provide a mechanism for the role of $InsP_6$ in apoptosis.

MATERIALS AND METHODS

All chemicals were reagent grade or better. Restriction endonucleases, DNA modifying enzymes, and general reagents were from Amersham Biosciences, Roche Applied Sciences, Fisher, Invitrogen, New England Biolabs, Promega Corp., Sigma, and Stratagene 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 transfer blots was from Pierce. Radiolabeled [^{3}H]inositol and [^{3}H]$InsP_6$ 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 (c20) 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 XL-1Blue (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′-GAAGACCTCGGAAGAGATA-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 lines, TRex HEK293(Invitrogen), using Lipofectamine as per the manufacturer's protocol. Cells were allowed to recover for 1 day and serially diluted, and clones were selected with 1 μg/ml puromycin. Individual clones were obtained and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1 μg/ml puromycin.

High Performance Liquid Chromatography (HPLC)—HPLC was conducted as follows. Cell were grown in the presence of [^{3}H]inositol (10 μCi/ml) for 4 days. Cells were lysed in methanol/0.5 N HCl (2:1) and extracted with chloroform. The aqueous phase was separated, dried, and resuspended in distilled water. Samples were applied to a Whatman Partisphere SAX strong anion exchange column (4.6 mm x125 mm) running a 30 min gradient from 0 to 1.7 M ammonium phosphate, pH 3.5, followed by a 30-min isocratic elution with 1.7 M ammonium phosphate, pH 3.5. Radioactivity was measured using the inline detector β-RAM (IN/US System Inc.), and the identity of the individual inositol phosphates was assigned on the basis of co-elution with known standards.

Apoptotic Cell Staining—Cells were grown on polylysine-treated coverslips to ~80% confluence, then treated with 1 ng/ml TNFα (Peprotech) and cycloheximide (1 μg/ml) for 18 h. Apoptotic cells were detected using the APOPercentage apoptotic kit (Accurate Chemical, Westbury, NY).

Western Blot Analysis—Specified tissue culture cells were removed from the plates by gentle aspiration, washed with PBS, and lysed in 20 mM HEPES, pH 7.6, 140 mM NaCl, 10% glycerol, 0.2% Nonidet P-40 plus protease inhibitors (Complete Mini EDTA-free, Roche Applied Science). Cells were treated to two freeze-thaw cycles in an ethanol-dry ice bath, and particulate debris was removed by centrifugation at 10,000 × *g* in an Eppendorf centrifuge at 4 °C. The protein concentration of the clarified lysate was determined using the Bradford assay (Bio-Rad protein assay) as per the manufacturer's protocol. Using standard techniques, 25 μg of protein of each sample was loaded onto a 10% gel for SDS-PAGE and subsequently electroblotted onto polyvinylidene difluoride membrane (Immobilon-P, Millipore). Western analysis was conducted using anti-PARP polyclonal antibody (Cell Signaling) using standard techniques. The appropriate horseradish peroxidase-conjugated secondary antibody and the SuperSignal West Pico chemiluminescent substrate (Pierce) were used to visualize the appropriate bands. Where indicated, bands were compared by densitometry of Western blots using an Eastman Kodak Co. Image Station 440 CF, and the data were analyzed using Kodak 1D V.3.5.4 (Scientific Imaging System).

TNFα or Fas treatment of HEK293 Cells—2×10^5 cells were plated in 12-well plates in the presence (for the 2-kinase-expressing lines or vector control) or absence (for 2-kinase RNAi or luciferase control lines) of tetracycline for 24 h and then treated with TNFα (1 ng/ml) plus cycloheximide (1 μg/ml) or the stated amount of Fas antibody plus protein G (a 4:1 ratio of Fas to protein G) for the indicated amount of time. Extracts were processed for Western blots as described above.

2-Kinase RNAi Construct Expression in 5/6-Kinase-expressing Cells—5/6-Kinase cells lines were transfected with the same 2-kinase RNAi construct used to generate stable cell lines, grown for 1 day to recover, then split into 12-well plates and grown for 24 h in the presence of tetracycline to induce 5/6-kinase expression. These cells were then treated for various times with TNFα and cycloheximide as above.

Transferrin Uptake—Cells were incubated with ^{125}I-transferrin (0.25 μg/ml) in binding medium (0.1% bovine serum albumin in Dulbecco's modified Eagle's medium) at 37 °C for 1–8 min. At the end of the incubation, the medium was aspirated, and the monolayers were rapidly washed three times with cold PBS to remove unbound ligand. The cells were then incubated for 5 min with 0.2 M acetic acid, pH 2.8, containing 0.5 M NaCl at 4 °C. The acid wash was combined with a short rinse in the same buffer and used to determine the amount of surface-bound ^{125}I-ligand. 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 min, a 100-fold molar excess of unlabeled ligand was added, and the cells were treated as before to determine the background binding.

Immunoprecipitation—For caspase 8 co-immunoprecipitations, two 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: 0.5×10^6 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.

RESULTS

HEK293 Cells Expressing a Stably Transfected RNAi Construct to the 2-Kinase Result in Altered $InsP_6$ 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 resulted in an increase in $InsP_6$ and a loss of $InsP_5$. We also generated HEK293 cells stably transfected with an RNAi construct to the 2-kinase. As a control we produced cell lines transfected with the pSUPER construct containing an RNAi insert to the luciferase gene. When these cells were labeled with [^{3}H]inositol, and their soluble inositol phosphates purified and separated by HPLC, there is a decrease of $InsP_6$ relative to the controls by about 70% (*cf.* Fig. 1, *B* to *A*) and an increase in $InsP_5$ and pyrophosphorylated $InsP_4$. This result is consistent with inositol labeling of HEK293 cells transiently transfected with siRNAs to 2-kinase as reported previously (1).

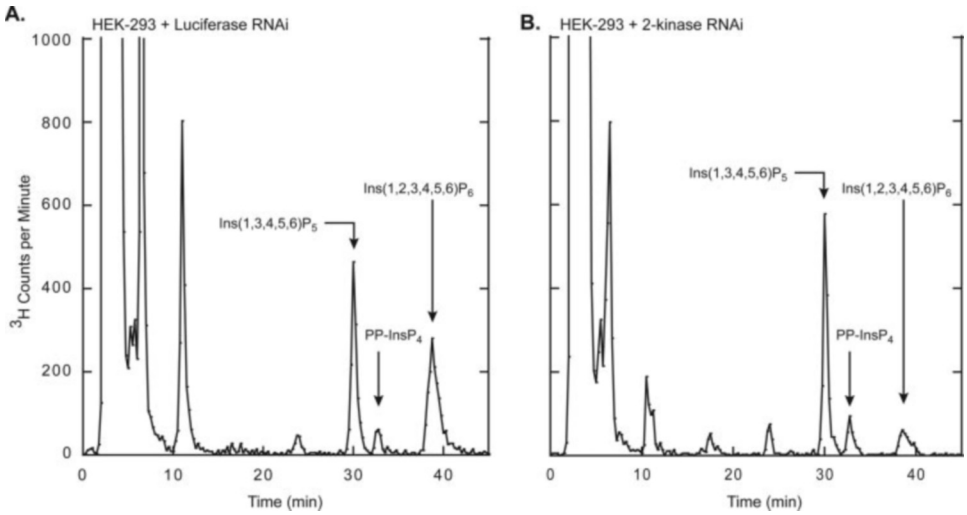

FIG. 1. **HPLC profiles of [^{3}H]inositol-labeled HEK293 cells expressing a RNAi construct to the 2-kinase: 2-kinase- or luciferase RNAi-expressing cells were grown in the presence of tetracycline and tritiated inositol for 4 days and their soluble inositols extracted and separated by HPLC.** The identity of the labeled peaks was confirmed by internal standards (data not shown).

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 $InsP_5$ and $InsP_6$ and a protection from TNFα-induced apoptosis. 2-Kinase-overexpressing cells show increased $InsP_6$ and a loss of $InsP_5$. When treated with TNFα 2-kinase-overexpressing cells (Fig. 2*B*) show a relative decrease in the number of apoptotic cells, as determined by APOPercentage staining, when compared with vector cells (Fig. 2*A*). Similarly, relative to the luciferase lines (Fig. 2*C*) the 2-kinase RNAi lines (Fig. 2*D*) 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 polyclonal 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. 3*A*). 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. 3*B*).

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 $InsP_6$ 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 $InsP_6$ or a downstream metabolite.

Increased InsP$_6$ Protects against FAS-mediated Apoptosis, while Decreased InsP$_6$ 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. 5*A*). Furthermore, the 2-kinase RNAi line showed more apoptosis as compared with the luciferase control (Fig. 5*B*). 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 InsP$_6$ 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 NFκB 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 Y*XX*W 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 $InsP_6$ 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 (κ_d), 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 $InsP_6$ and those with depleted levels of $InsP_6$. Finally, caspase 8 activity was assayed with increasing amounts of $InsP_6$ or inositol hexakissulfate to control for

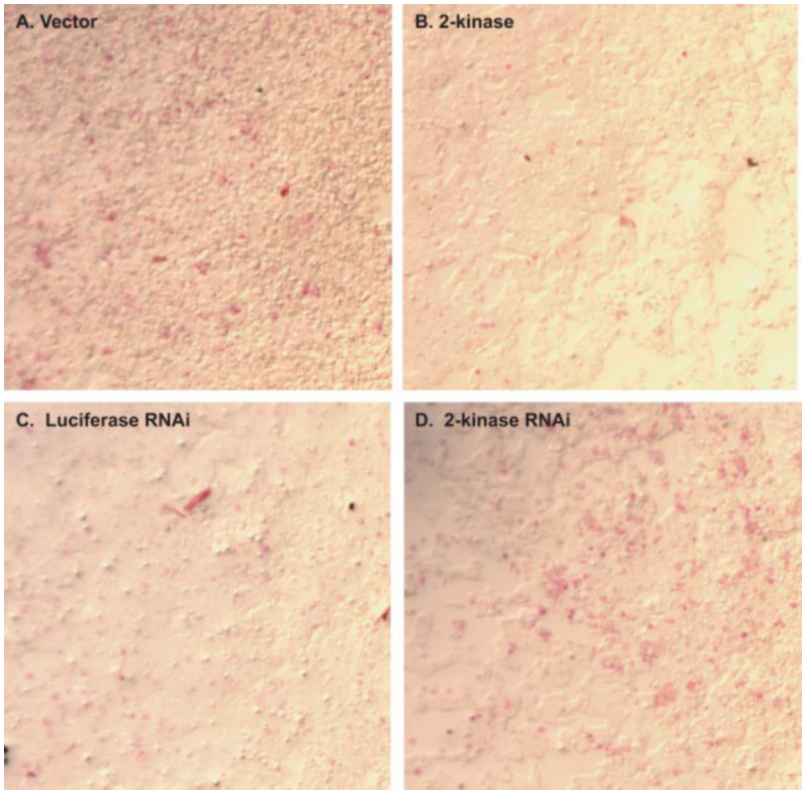

FIG. 2. **APOPercentage staining of cells expressing the 2-kinase or the 2-kinase RNAi.** TRex vector cells and 2-kinase-expressing cells were grown in the presence of tetracycline. All cell lines were treated with TNFα and cycloheximide for 16 h. 2-Kinase lines (*B*) show fewer apoptotic cells compared with the vector lines (*A*). Consistently, the 2-kinase RNAi lines (*D*) show more apoptotic cells than luciferase RNAi controls (*C*).

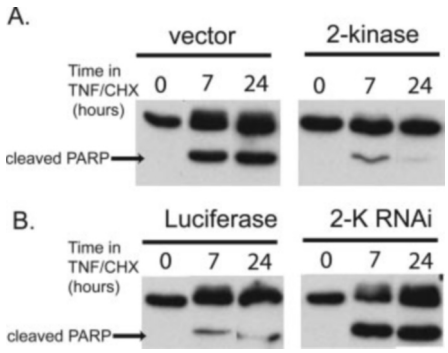

FIG. 3. **PARP Western blots of TNF-treated 2-kinase (*2-K*) lines.** 2-Kinase-expressing or vector lines (*A*), and luciferase or 2-kinase RNAi lines (*B*), were treated for 0, 7, or 24 h with TNFα/cycloheximide and their extracts blotted and probed with anti-PARP antibody.

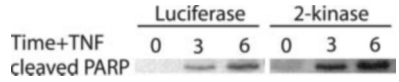

FIG. 4. **2-Kinase RNAi construct expression overcomes the protective effect of 5/6-kinase expression in HEK293 cells.** 5/6-Kinase lines were transiently transfected with a luciferase RNAi construct or a RNAi construct to the 2-kinase. These cells were then treated with tetracycline to induce 5/6-kinase expression. 24 h post-transfection, the cells were treated with TNFα/cycloheximide for 0, 3, or 6 h and their extracts blotted with PARP antibody.

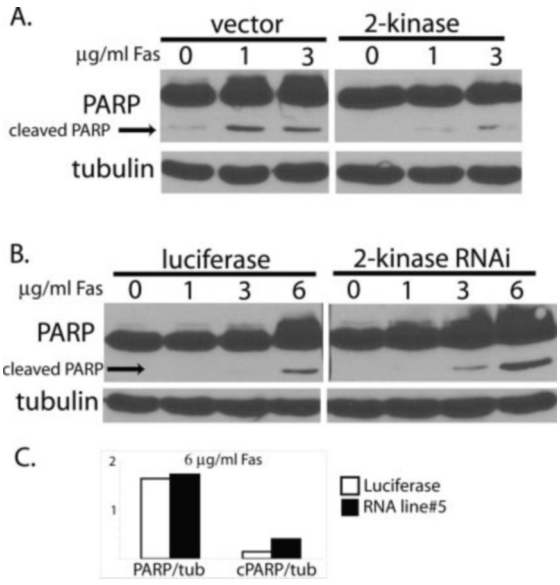

FIG. 5. **Expression of the 2-kinase affords protection from Fas-mediated apoptosis, whereas depletion of InsP$_6$ results in an increased susceptibility to Fas mediated apoptosis.** *A*, 2-kinase-expressing or vector lines were grown in tetracycline for 24 h and then treated with activating Fas antibody as indicated and blotted against anti-PARP as for TNFα treatment. *B*, luciferase or 2-kinase RNAi stable cell lines were treated with activating Fas antibody as indicated and blotted against PARP. *C*, PARP, cPARP, and tubulin bands from the 6 mg/ml Fas lane in *B* were subject to densitometry on a Eastman Kodak Co. Image Station 440 CF, and the ratio of PARP to tubulin and cPARP to tubulin was plotted for luciferase and RNAi lines.

nonspecific charge effect, and no effect was seen on caspase 8 activity up to 100 μM $InsP_6$ or inositol hexakissulfate.

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

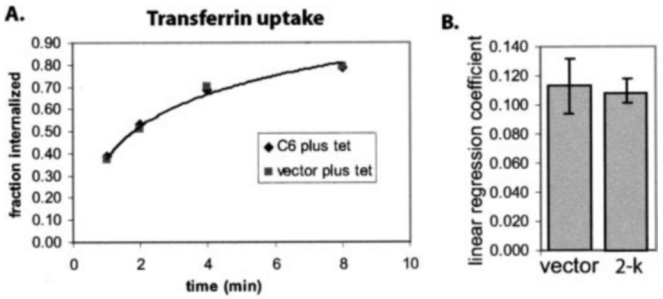

FIG. 6. *A*, transferrin uptake assays in 2-kinase-overexpressing cell lines or vector controls in the presence of tetracycline. *B*, the average linear regression coefficient for the first three time points in *A* from four different experiments representing the coefficient of internalization.

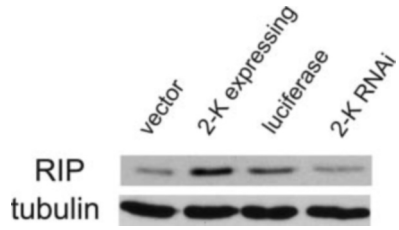

FIG. 7. **Cell lines expressing the 2-kinase contain relatively higher levels of RIP, whereas cells deficient in InsP$_6$ contain relatively lower levels of RIP.** Western blots of 2-kinase-expressing, 2-kinase RNAi, or vector control cell extracts probed with anti-RIP mAb antibody. Tubulin was used as a loading control.

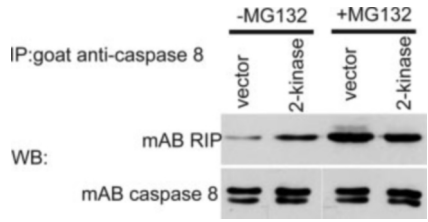

FIG. 8. **The increase in RIP levels is correlated with an increase in RIP in caspase 8 co-immunoprecipitations.** Caspase 8 was immunoprecipitated from HEK293 cells as stated with goat anti-caspase 8 antibody, and RIP was detected using a monoclonal RIP antibody. 5% of each immunoprecipitation was run on a separate gel and blotted with caspase 8 monoclonal antibody to control for the efficiency of the immunoprecipitation of caspase 8.

the complex forms to a small degree in cells unstimulated with TNFα. When HEK293 cells expressed 2-kinase, more RIP protein was precipitated with caspase 8 as compared with the vector controls (Fig. 8). Treatment of these cells with the proteosome inhibitor MG132 restored the level of RIP in the immunoprecipitations in the vector lines equal to that of the 2-kinase 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.

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 $InsP_5$ and $InsP_6$ (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 $InsP_6$, deplete $InsP_5$, and show protection from apoptosis relative to vector controls. Furthermore, HEK293 cells stably transfected with an RNAi construct to 2-kinase deplete $InsP_6$ and accumulate $InsP_5$ and show an increased susceptibility to apoptosis. In addition, expression of 5/6-kinase was not able to overcome the susceptibility to apoptosis caused by expression of the 2-kinase RNAi construct. This result confirms the data of Sun *et al.* (12) that suggested that the protective effect of the 5/6-kinase on apoptosis is not due to its protein kinase activity but rather to its inositol kinase activity. Thus, the protection from apoptosis is due to $InsP_6$ or possibly some more highly phosphorylated inositol phosphate. In addition, we have shown that overexpression of 2-kinase protects from Fas-induced apoptosis, while 2-kinase RNAi expression renders the cells more sensitive to Fas-induced apoptosis. Taken together, the two studies support each other in their implication that soluble inositol phosphates are regulators of apoptosis.

As mentioned above, our results do not conclusively implicate $InsP_6$ itself in the protection from apoptosis; we can only say that the isomer involved has to lie downstream of $InsP_6$. $InsP_6$ is metabolized to the higher inositol pyrophosphates $InsP_7$ and $InsP_8$. We, though, have seen no increase in an $InsP_7$ 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 β1-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 $InsP_6$ to the pyrophosphate $InsP_7$. 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 TNF apoptosis, *e.g.* TRAIL (TNF-related apoptosis-inducing ligand). Since $InsP_6$ kinases phosphorylate $InsP_6$, their expression may result in the depletion of cellular levels of $InsP_6$, which we show here to sensitize cells to apoptosis. Thus the balance between $InsP_6$ and the inositol pyrophosphates may act as a switch controlling apoptosis. Nonetheless, such speculation requires experiments with the $InsP_6$ 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 $InsP_6$ and saw no difference nor did we see an effect of $InsP_6$ on caspase 8 activity *in vitro*. TNF activation has recently been shown to require the internalization of the TNF receptor (19). $InsP_6$ 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 $InsP_6$ 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 affect of altered $InsP_6$ levels on endocytosis. Although it has been implicated in endocytosis by a number of studies, we did not see an effect on endocytosis when $InsP_6$ levels were elevated (Fig. 6) or depleted (data not shown).

The increase in $InsP_6$ is correlated with an increase in RIP and may provide a mechanism for the protection afforded by an increase in $InsP_6$ 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 $InsP_6$ affects RIP levels. Preliminary experiments to determine the effect of $InsP_6$ itself on the half-life of RIP did show that cells deficient in $InsP_6$ 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 $InsP_6$ 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, $InsP_6$ may affect its turnover rate by affecting the proteosome or its targeting for degradation by ubiquitination.

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