Interferon α-induced Apoptosis in Tumor Cells Is Mediated through the Phosphoinositide 3-Kinase/Mammalian Target of Rapamycin Signaling Pathway*

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Interferon (IFN) α induces a caspase-dependent apoptosis that is associated with activation of the pro-apoptotic Bak and Bax, loss of mitochondrial membrane potential, and release of cytochrome c. In addition to the onset of the classical Jak-STAT pathway, IFNα also induced phosphoinositide 3-kinase (PI3K) activity. Pharmacological inhibition of PI3K activity by Ly294002 disrupted IFN-induced apoptosis upstream of mitochondria. Inhibition of mTOR by rapamycin or by overexpression of a kinase dead mutant of mTOR, efficiently blocked IFNα-induced apoptosis. A PI3K and mTOR-dependent phosphorylation of p70S6 kinase and 4E-BP1 repressor was induced by IFNα treatment of cells and was strongly inhibited by Ly294002 or rapamycin. The activation of Jak-STAT signaling upon IFNα stimulation was not affected by abrogating PI3K/mTOR pathway. Neither was the expression of several IFNα target genes affected, nor the ability of IFNα to protect against virus-induced cell death affected by inhibition of the PI3K/mTOR pathway. These data demonstrate that an intact PI3K/mTOR pathway is necessary for the ability of IFNα to induce apoptosis, whereas activation of the Jak-STAT pathway alone appears to be insufficient for this specific IFNα-induced effect.

Interferons were the first cytokines to be identified and have since been used as a model substance in cytokine research in terms of genetic cloning, evaluation of clinical efficacy, and elucidation of signaling pathways (1). Although initially discovered through their antiviral activities, the IFNs are now known to couple PI3K to the IFNAR1 (18). PI3K activation by insulin involves the transducer and activator of transcription 3 (14–17). JAK-mediated tyrosine phosphorylation of the docking proteins involved in insulin signaling, IRS-1 and IRS-2 (14–17). Another group has shown that STAT3 can serve as an adaptor to couple PI3K to the IFNAR1 (18). PI3K activation by insulin leads to the generation of secondary messengers, phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate, that transmit signals to downstream kinases, such as PKB/Akt and PDK-1 (19). Akt kinase activity was

led to their introduction as an approved treatment for various medical conditions including tumor, viral, and neurological diseases (4). Despite the widespread use of IFNs1 as effective pharmacological agents, their mechanism of action in these diseases has remained elusive.

A direct inhibition of growth of tumor cells as well as the indirect effects of IFNs such as immunomodulation or inhibition of angiogenesis may underlie the antitumor activity of type I IFNs, demonstrated in a large number of clinical studies (5). We and others have shown that IFNα exerts potent pro-apoptotic activities in a number of established cell lines and primary tumor cells (6–10), suggesting that the induction of apoptosis is of a major importance for the anti-tumor activities of IFN.

Type I interferons transduce signals via activation of multiple downstream signaling cascades. The major IFN-activated Jak-STAT signaling pathway causes the altered transcription of several hundreds of genes with either ISRE or GAS elements, or both, in their promoter regions (11). Although the role of the Jak-STAT pathway in type I interferon signaling is well established (12), the roles that other cascades play in the induction of IFN responses remain to be defined.

Phosphoinositide 3-kinase (PI3K), which plays a critical role in cell survival, proliferation, and adhesion, is also activated by IFNα. Both the lipid and serine kinase activity of PI3K are induced by IFNα (13, 14). This stimulation occurs through JAK-mediated tyrosine phosphorylation of the docking proteins involved in insulin signaling, IRS-1 and IRS-2 (14–17). Another group has shown that STAT3 can serve as an adaptor to couple PI3K to the IFNAR1 (18). PI3K activation by insulin leads to the generation of secondary messengers, phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate, that transmit signals to downstream kinases, such as PKB/Akt and PDK-1 (19). Akt kinase activity was

1 The abbreviations used are: IFN, interferon; PI3K, phosphoinositide 3-kinase; mTOR, mammalian target of rapamycin; STAT, signal transducer and activator of transcription; cyt c, cytochrome c; KD, kinase dead; wt, wild type; PML, promyelocytic leukemia protein; IRF-9, interferon regulatory factor 9; ISRE, interferon-stimulated response element; GAS, γ interferon-activated sequence; TMRE, tetramethylrhodamine ethyl ester perchlorate; VSV, vesicular stomatitis virus; EMSA, electromobility shift assay; DAPI, 4',6-diamidino-2'-phenylindole-dihydrochloride; GFP, green fluorescent protein; PI, propidium iodide; FACS, fluorescence-activated cell sorter; HRP, horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay; SIE, sis-inducible element.

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induced by IFNα in Daudi cells, and it was required for the induction of NF-κB-specific DNA binding in EMSA (20). However, these effects were associated with cell survival rather than with inhibition of cell growth and proliferation by IFNα. In contrast, other studies demonstrated that PI3K activation induced by IFNα does not result in the activation of Akt (17) or Grb-2 (21). Another kinase, activated downstream of PI3K in response to stimulation with the mammalian target of rapamycin (mTOR) serine-threonine protein kinase (22). The best known function of mTOR is the regulation of initiation of protein translation, largely mediated by p70S6 kinase (p70S6K) and 4E-BP1 (23). A recent study demonstrated that IFNα treatment led to the phosphorylation of mTOR, as well as p70S6K and 4E-BP1, in a PI3K-dependent manner (24). In the present investigation we sought to define the role of PI3K/mTOR activation in the IFNα-mediated apoptosis, which we have characterized in previous studies (6, 7, 25).

**EXPERIMENTAL PROCEDURES**

**Cell Lines, Culture Conditions, and Treatments**—The cell lines used in this study were the multiple myeloma cell line U266 (26) and the SV40-transformed keratinocyte cell line Rhee-1 (27). The cells were cultured in high glucose DMEM (U266) or DMEM/F12 (Rhee-1) medium supplemented with 10% heat-inactivated fetal calf serum, 10 mM HEPES, 50 μM of streptomycin, 50 μg/ml of penicillin (Invitrogen) and maintained in 5% CO₂ at 37 °C.

Recombinant human IFNα (Schering-Plough, Kenilworth, NJ) had a specific activity of 2.0 × 10⁸ units/mg, and the purity was >99%. In preliminary experiments, the cellular events following IFNα treatment were found to take place in a dose-response-dependent fashion, with apoptosis occurring using doses as low as 15 units/ml and a maximum effect at 5000 units/ml, wherefore this latter dose was chosen for the remaining experiments. 10 μg of Ly294002, 100 μg of Wortmannin, or 1 μM of rapamycin (Sigma) was added 1 h prior to IFN treatment.

**Constitutes and Transient Transfections—**Transient transfections of Rhee-1 cells were performed in 6-well plates using LipofectAMINE 2000 from Invitrogen according to the manufacturer’s instructions. Eighteen hours after transfection, the cells from one well were split into three wells, allowed to attach, and either left untreated or treated with 5000 units/ml of IFNα with or without 1 μM rapamycin for 24 or 40 h. Thereafter, the cells were cytospun onto glass slides, fixed in 30 °C cold methanol:aceton (1:1), and stained using anti-FLAG antibody (Stratagene) and DAPI (included in mounting medium from Vector).

**Assessment of Apoptosis by Morphological Changes, Annexin V, and TMRE Stainings**—The cells were stained with Türk solution and counted in a light microscope, and apoptosis was assessed based on the nuclear morphology. Redistribution of plasma membrane phosphatidylserine was assessed using annexin V FLOWOS (Roche Applied Science) according to the manufacturer’s protocol. Annexin V and propidium iodide (PI) stainings were performed and analyzed by a FACScalibur flow cytometer (Becton Dickinson) and the Cell Quest software as described (7).

To detect changes in the mitochondrial membrane potential, ΔΨm, the cells were stained with TMRE (Molecular Probes Inc.) as described (25). For the double annexin/TMRE stainings, TMRE-labeled cells were then stained with annexin V FLUOS (Roche Applied Science) according to the manufacturer’s protocol. Annexin V and propidium iodide (PI) stainings were performed and analyzed by a FACScalibur flow cytometer (Becton Dickinson) and the Cell Quest software as described (25).

**Immunocytochemistry**—The cells were cytospun onto glass slides, fixed in 4% formaldehyde for 20 min and permeabilized using either digitonin (for cytochrome c) or 0.2% Triton X-100 (for STATs) in phosphate-buffered saline pH 7.4. The slides were washed twice in phosphate-buffered saline and then incubated with Vectashield with DAPI for the staining of nuclei (Vector Laboratory, Inc.). The images were acquired on a Zeiss Axioplan 2 imaging microscope with Axiovision software and processed either as gray scale or dual color TIF images in Adobe Photoshop (Adobe Systems Inc.).

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**Fluo Cytometric Analysis of Activated Bak, Bod, and Caspase 3 and of PML—**Upon induction of apoptosis, the pro-apoptotic Bak and Bod proteins undergo conformational changes and expose otherwise inaccessible N-terminal epitopes. We used monoclonal antibodies that specifically recognize these epitopes: AM03, clone TC100 against Bak (Oncogene Research Products), and clone 6A7 against Bod (Pharmin-gen-Becton Dickinson). Antibodies against active caspase 3, conjugated to fluorescein isothiocyanate, were from Becton Dickinson, and anti-PML monoclonal antibodies were from DAKO (Dokoppats AB). The stainings were performed as described (25) and analyzed using a FAC-Scalibur flow cytometer. For quantitation and comparison, the median fluorescence intensity values were calculated using Cell Quest software.

**In Vitro Caspase Assay—**Caspase activity was measured by cleavage of the following substrates; Ac-VDVAD-AMC (caspase-2), Ac-DEVAD-AMC (caspase-3 like), Ac-IETD-AMC (caspase-8), and Ac-LEHD-AMC (caspase-9) in a fluorometric assay as described (7). The experiments were performed in duplicate, and the activity was expressed as a change in fluorescence units relative to control. All of the substrates were from Peptide Institute Inc. (Osaka, Japan).

**Western Blot Analysis and Antibodies**—For Western blots, total cell extracts were prepared by direct lysis in hot Laemmli buffer. The samples corresponding to 10⁶ cells/well were separated on 10 or 12% SDS-PAGE followed by electroblotting to polyvinylidene difluoride membranes (Roche Applied Science) by semi-dry transfer. The filters were incubated with the appropriate primary antibodies overnight at 4 °C and thereafter with a secondary antibody for 1 h. The protein bands were visualized using a SuperSignal West Pico chemiluminescent substrate (Pierce) according to the manufacturer’s protocol. The images were captured using a LAS-1000 from Fujifilm. Quantification of the bands was carried out using Image Gauge (4.0) software from Fujifilm. The following antibodies were used: rabbit polyclonal IgG against phospho-STAT1 (Y701 and S727), phospho-STAT2 (Y689), phospho-STAT3 (Y705 and S727), phospho-Akt (S473 and T308), phospho-p70S6K (T389 and T421/S424), phospho-EBP1 (T574/648), and total 4E-BP1 from Cell Signaling Technology; mouse monoclonal anti-IRF-9 (ISOFG3) from Transduction Laboratories; rabbit polyclonal anti-Caspase-9, anti-STAT1, anti-STAT2, anti-STAT3, anti-MxA (myxovirus resistance protein A) chicken serum from Baclab (Pratteln); and HRP-conjugated anti-chicken from Sigma. The HRP-conjugated anti-rabbit and anti-mouse antibodies and biotinylated protein ladder that was used as a molecular weight marker and HRP-conjugated anti-biotin were from Cell Signaling.

**Analysis of PI3K Activity**—The samples of 5 × 10⁶ cells were washed with cold phosphate-buffered saline, resuspended in lysis-buffer (137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 0.5 mM Na₂VO₃, 1% Triton X-100, 10 μg/ml leupeptin, 0.2 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 10 mg/ml aprotinin) and thereafter with homogenization through a 27-gauge needle. Lyssates containing mg of protein were subjected to immunoprecipitation using either a monoclonal anti-p85 antibody (corresponding to the C-terminal SH2-domain; Transduction Laboratories) or a polyclonal rabbit serum against IRS-1 (Upstate Biotechnology Inc.). PI3K activity in the immunoprecipitates was analyzed by thin layer chromatography as described (29) using (γ³²P)ATP (PerkinElmer Life Sciences) and υ-phosphatidylinositol (Avanti Polar-Lipids, Inc.). The data were acquired by phosphorimaging using TINA software 2.09c. Quantifications were made in Photoshop.

**Preparation of Nuclear Extracts and EMSA**—The nuclear extracts were obtained as follows. Following appropriate treatment, the cells were washed twice in phosphate-buffered saline and resuspended in an extraction buffer containing 50 mM HEPES, pH 7.5, 100 mM Na₂EDTA, 0.1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 2.5 mM dithiothreitol for 20 min on ice, after which Nonidet P-40 was added to a final concentration of 0.6%. The cells were vortexed vigorously for 10 s and rapidly centrifuged to collect the nuclear pellet, which was resuspended in ice-cold buffer containing 20 mM HEPES, pH 7.9, 0.4% Triton X-100, 0.5 mM EDTA, 1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 2.5 mM dithiothreitol. The tubes were vortexed vigorously at 4 °C for 15 min and centrifuged at high speed for 10 min at 4 °C, and the supernatant containing nuclear proteins was collected and stored in aliquots at -70 °C.

The hsIE probe (5’-GGCCAGTCTTGGGTGAATATCT-3’) and the 9–27 ISRE probe (5’-GGAAGAAGAAAAGTCT-3’) were end-labeled using T4 polynucleotide kinase (New England Biolabs) according to the manufacturer’s instructions. For EMSA, between 20 and 30 μg of nuclear extracts were incubated for 5 min in binding buffer containing 25 mM

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HEPES, pH 7.9, 10% glycerol, 5 mM dithiothreitol, 150 mM KCl, and 2 μg/ml poly(dI-dC) (Amersham Biosciences). Upon the addition of labeled probe and further incubation for 30 min, the complexes were separated on a 4% nondenaturing polyacrylamide gel in Tris/glycine/EDTA buffer (40 mM Tris-base, 200 mM glycine, 0.1 mM EDTA). The gels were visualized by autoradiography.

Antiviral Assay—U266 cells were seeded in 96-well plates at a concentration of 2 × 10⁵ cell/ml in a volume of 200 μl, and corresponding treatments were carried out in triplicate 6 h before the addition of vesicular stomatitis virus (VSV). Virus was added directly to the corresponding wells in 1:400 dilution from the stock (obtained from Dr. Gunnar Alm, Uppsala, Sweden). After 18 or 24 h of incubation, 20 μl of WST-1 cell proliferation reagent (Roche Applied Science) was added to each well followed by 1.5 h of incubation at 37 °C. The absorbance at 450 nm was detected by a Bio-Rad microplate reader, and the data were further processed in Microsoft Excel. For VSV-G protein ELISA assay, U266 cells were pretreated with respective drugs for 6 h as above, infected with VSV for 1 h, washed from the unbound virus, resuspended in the new medium, and seeded in triplicate in a 96-well plate. 24 h later Nonidet P-40 (Igepal CA-630...
from Sigma) was added to a final concentration 1% to each well and cell lysis was achieved during a 15-min incubation at +4 °C. ELISA plates (Greiner Biovan) were coated with supernatants from the corresponding wells overnight at +4 °C. The plates were washed twice using wash buffer (phosphate-buffered saline with 0.1% Tween 20) and blocked for 2 h using 3% bovine serum albumin in wash buffer at room temperature. The plates were washed four times with wash buffer and incubated with HRP-conjugated anti-VSV-G antibody (ab3556; Abcam Limited).
RESULTS

IFNα-induced Apoptosis Is Blocked by Inhibition of the PI3K/mTOR Pathway—The role of PI3K in IFNα-induced apoptosis was assessed by blocking PI3K activity using the chemical inhibitor, Ly294002. The addition of 10 μM of Ly294002 1 h prior to IFN treatment nearly completely blocked IFNα-induced apoptosis as measured by annexin V/PI staining at the 24-h (data not shown) or 48-h time points (Fig. 1, A, left panels, and B). IFNα induced a loss of mitochondrial membrane potential, as demonstrated by TMRE staining, that was efficiently blocked by Ly294002 (Fig. 1, A, right panels, and B). Inhibition of IFNα-induced cell death was similarly observed when another PI3K inhibitor, wortmannin, was used (data not shown).

mTOR is one of several kinases downstream of PI3K. Inhibition of mTOR by rapamycin blocked IFNα-induced apoptosis of U266 cells in a manner similar to that of Ly294002 (Fig. 1A). The presence of either chemical inhibitor induced a slight increase in the number of apoptotic cells. However, none of the drugs induced a loss of mitochondrial membrane potential, and overall, the toxicity of the PI3K or mTOR inhibitors was minimal (Fig. 1, A and B).

We have previously shown that IFNα-induced apoptosis is largely dependent on the activation of a set of caspases (7). We therefore measured the activity of caspases-2, -3, -8, and -9 in U266 cells after 24 and 48 h of incubation with IFNα in the presence or absence of Ly294002. IFNα-induced activation of the caspases was potently inhibited in the presence of the PI3K inhibitor already after 24 h in culture (Fig. 1C; data for 48 h not shown), in line with the annexin V and TMRE data (Fig. 1, A and B).

We reported earlier that IFNα treatment induced release of cytochrome c (cyt c) from the mitochondrial intermembrane space to the cytoplasm (7). Approximately 12% of cells released cyt c at 24 h of IFNα treatment as shown in Fig. 1D. Both Ly294002 and rapamycin efficiently blocked the IFNα-induced release of cyt c from mitochondria.

Activation of the pro-apoptotic Bcl-2 family member Bak by IFNα occurs prior to cyt c release and the loss of mitochondrial membrane potential, whereas Bax is activated and translocated to mitochondria later in the apoptotic process (25). We therefore asked whether Ly294002 or rapamycin could block this activation. Treatment of U266 cells with IFNα for 48 h induced activation of both Bak (Fig. 1E) and Bax (data not shown). This was abrogated in the presence of either Ly294002 or rapamycin (Fig. 1E, upper panel, left and right columns, respectively). In line with these data, activation of caspase-3 by IFNα was efficiently blocked by either drug (Fig. 1, E lower panel; and chart for data quantification). Thus, inhibition of IFNα-mediated apoptosis by blocking either PI3K or mTOR activity occurred upstream of Bak activation.

To test whether the block of IFNα-induced apoptosis by PI3K or mTOR inhibitors is not a phenomenon specific to U266 cells, we used another cell line, Rhek-1 keratinocytes, in similar experiments. 32% of Rhek-1 cells underwent apoptosis after 48 h of IFNα treatment as measured by annexin V/TMRE double stainings in a representative experiment (Fig. 1F). Both Ly294002 and rapamycin efficiently protected from IFNα-induced apoptosis of this cell line as shown in a representative experiment in Fig. 1F. This demonstrated that the effect of Ly294002 or rapamycin on IFNα-induced apoptosis is not specific to the U266 cell line.

To substantiate the data obtained using a pharmacological inhibitor against mTOR, we transfected Rhek-1 cells with constructs encoding either wt mTOR or a kinase dead (KD) mutant of mTOR or, with G-FPP as control, treated the cells with IFNα, prepared slides, and stained for transfected cells using an anti-FLAG antibody while the nuclei were stained with DAPI. The F-GFP-transfected cells were stained only with DAPI. The transfection efficiency of F-GFP and mTOR constructs varied between 12 and 6%, respectively. Therefore the effect of IFNα was evaluated at the single cell level using fluorescent microscopy. F-GFP and FLAG-positive cells were counted manually, and the amount of apoptotic cells among them was assessed according to nuclear morphology (Fig. 2, A and B). The results of three independent experiments are shown in Fig. 2C. While cells transfected with either F-GFP or wt mTOR died during IFNα treatment, the cells expressing KD mTOR were protected from IFNα-induced death. Also, rapamycin protected F-GFP as well as wt mTOR-expressing cells from death but did not have any additional effect on the cells that expressed KD mTOR (Fig. 2C).

Activation of PI3K by IFNα Is Inhibited by LY294002—To confirm that PI3K activity was indeed induced by IFNα in our system and that Ly294002 inhibited this activation, U266 cells were either grown under normal growth conditions (Fig. 3A, lanes 5–9) or serum-starved for 24 h prior to the treatment (Fig. 3A, lanes 1–4). The cells were stimulated for 20 min with either IFNα or insulin, which was used as a positive control. Under low serum conditions, the endogenous PI3K activity was decreased as compared with the cells cultured in the presence of serum (compare lanes 1 and 5), and Ly294002 inhibited the endogenous PI3K activity (lane 7). Insulin treatment induced PI3K lipid kinase activity as expected (Fig. 3A, lane 2). IFNα treatment induced PI3K activity to a similar extent as insulin under low serum conditions (compare lanes 2 and 3) and in the presence of serum (lane 6). The addition of Ly294002 abrogated PI3K activity induced by IFNα under either culture condition (Fig. 3A, lanes 4 and 8). A summary of three experiments where induction of PI3K activity by insulin or IFNα was studied is also presented as a bar graph in Fig. 3A.

Inhibition of PI3K or mTOR Leads to Reduced Activity of p70S6K and 4E-BP1—Next, we sought to establish which of the downstream targets of PI3K that are activated in response to IFNα. Akt/PKB is a well established target of the PI3K, for its full activation, phosphorylation of both Ser473 and Thr384 is required (19). Western blot analysis using phosphorylation state-specific antibodies against these sites showed no induction of phosphorylation of Akt by IFNα on either Ser473 or Thr384 (data not shown), which is in line with previously published data (17).

The PI3K-signaling pathway leads to phosphorylation of both the p70S6 kinase and the 4E-BP1 repressor of translation, resulting in induction of translation of ribosomal and other proteins. This regulation has been shown to involve the activity of mTOR. Akt/PKB is a well established target of the PI3K, for its full activation, phosphorylation of both Ser473 and Thr384 is required (19). Western blot analysis using phosphorylation state-specific antibodies against these sites showed no induction of phosphorylation of Akt by IFNα on either Ser473 or Thr384 (data not shown), which is in line with previously published data (17).

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IFNα treatment also induced phosphorylation of p70S6K on both the Thr421/Ser423 and Thr389 sites (Fig. 3C). Ly294002 and
rapamycin efficiently blocked this phosphorylation. These data are in agreement with a recently published study (24).

STAT Phosphorylation and Activation Are Not Affected by Inhibition of the PI3K or mTOR Activity—Activation of the STAT proteins and induction of the downstream target genes upon IFN\(\alpha\)/H9251 stimulation is well studied (12). To determine whether STAT activation was affected by the inhibition of PI3K or mTOR activity, we assessed STAT1, STAT2, and STAT3 phosphorylation, nuclear translocation, and the STAT homo- and heterodimer binding to the SIE or ISRE sequences in the presence or absence of Ly294002 or rapamycin (Fig. 4). STAT3 phosphorylation on Tyr705 was induced by IFN\(\alpha\)/H9251, and this was not affected by the presence of Ly294002 or rapamycin (Fig. 4A, upper panel). The phosphorylation on Ser\(^{727}\) of STAT3 and Ser\(^{277}\) of STAT1 and tyrosine phosphorylation of STAT1 and STAT2 were not affected by the inhibition of either PI3K or mTOR activity (Fig. 4).

Thirty minutes of IFN\(\alpha\) treatment of U266 cells induced nuclear translocation of STAT1, STAT2, and STAT3 as detected by immunostaining using antibodies against these proteins (data not shown). Phosphorylation site-specific antibodies against STAT1-ph-Y701, STAT2-ph-Y689, and STAT3-ph-Y705 detected accumulation of the phosphorylated proteins in the nuclei (not shown and Fig. 4B). Neither Ly294002 nor rapamycin affected this nuclear translocation as exemplified by staining for STAT3-ph-Y705 in Fig. 4B.

Stimulation with type I IFNs leads to the formation of the ISGF3 complex that predominantly mediates the activation of specific gene targets through binding to ISRE promoter elements (12). We monitored the kinetics of ISGF3 binding to the consensus ISRE sequence upon IFN\(\alpha\) treatment in the presence or absence of Ly294002 by EMSA. Thirty minutes of IFN\(\alpha\) treatment induced DNA binding of ISGF3, which was not inhibited when PI3K was blocked at this or later time.
FIG. 3. IFNα-induced activation of the PI3K/mTOR pathway. A, U266 cells were grown in absence (lanes 1–4) or presence of serum (lanes 5–9) for 24 h and thereafter treated for 20 min with either IFNα alone (lane 3 and 6) or in the presence of Ly294002 (lanes 4 and 8). Ly294002 alone was used in one of the samples (lane 7). The cells were immunoprecipitated with anti-p85 antibodies, and protein complexes were assayed for PI3K activity by measuring the amount of radioactive labeled phosphatidylinositol 3,4,5-trisphosphate (PIP3, lanes 1–8). Normal rabbit serum was used as negative control (lane 9). Twenty minutes of insulin treatment was used as positive control (lane 2). A representative of two experiments is shown. A summary of three experiments is presented as a chart. Quantitations were made in Photoshop. B, immunoblot analysis of total cell lysates from U266 cells that were either left untreated (control) or were treated with either IFNα alone or in the presence of Ly294002 or rapamycin as indicated. The membranes were probed with antibodies against total or Thr(P)37/46 4E-BP1 protein. Protein loading was monitored by probing...
points (Fig. 4C, upper panel). Furthermore, the amounts of IFNα-induced STAT3, STAT3, and STAT1 dimers binding to a GAS/SIE element were not inhibited either by Ly294002 at this or later time points (Fig. 4C, lower panel). Some amounts of active STAT3, STAT3, and STAT1 complexes were constitutively present in control cells because of the autocrine interleukin-6 stimulation in U266 cells (30). In conclusion, IFNα-induced STAT activation was not impaired by inhibition of the PI3K/mTOR pathway.

Expression of IFNα Target Genes Is Not Affected by Inhibition of the PI3K/mTOR Pathway—The PI3K/mTOR pathway regulates cell proliferation and survival in part through the activation of transcription of ribosomal and other proteins. Ly294002 and rapamycin treatment leads to inhibition of p70S6K and also prevents phosphorylation of the 4E-BP1 repressor (Fig. 3). This may likely lead to inhibition of translation of the IFN downstream target genes. However, neither Ly294002 nor rapamycin prevented the induction of four different well established IFNα-target proteins (Fig. 5). The MxA gene has an ISRE element in its promoter and is regulated predominantly by type I IFNs (31); IRF-1 and IRF-9 are the two examples of responsive genes that are common to IFNα and IFNγ and have a GAS/SIE element in their promoter regions (32); the PML gene contains both ISRE and GAS elements (33). Expression of MxA, IRF-9, and IRF-1 was studied by immunoblotting (Fig. 5A and not shown), whereas the induction of PML was tested by immunostaining and flow cytometry (Fig. 5B).

These data demonstrate that inhibition of PI3K/mTOR signaling does not prevent the induction of these genes by IFNα. This also substantiated the finding that the activity of Jak-STAT signaling resulting in the activation of these genes was not impaired by the inhibition of either PI3K or mTOR. Specifically, IFNα induces MxA, IRF-9, and IRF-1 expression that is dependent on PI3K activity (35). This implies that STAT activation and its downstream signaling may be affected by the inhibition of PI3K or mTOR. However, although thoroughly investigated, we obtained no data to support such a notion, because neither serine phosphorylation of STAT1 or STAT3 nor the binding of the ISGF3 or STAT homo- or heterodimers to the specific DNA sequences was affected by the inhibition of either PI3K or mTOR. Neither did Ly294002 nor rapamycin affect the activation of four different IFNα-induced genes containing either ISRE or GAS elements, or both, in their promoters. It has been reported, however, that inhibition of PI3K activity selectively abrogated IFNβ-mediated induction of the β-R1 (SCYB11) gene (36), suggesting that there could exist specific targets of type I IFNs that require PI3K activity for their transcriptional up-regulation. We therefore tested the consequence of Ly294002 and rapamycin treatment on the biological effects of Jak-STAT signaling. The ability of IFNα to promote an anti-viral state, known to be largely dependent on the Jak-STAT signaling, was not impaired by either drug. Thus, although the cells were almost fully protected from IFNα-induced apoptosis by these drugs, the Jak-STAT pathway activation as well as a major IFN-mediated biological effect were kept intact. Our results also confirm previously published data that the Jak-STAT and the PI3K pathways, activated by IFNα, act separately from each other (21, 37). Furthermore, on the basis of the data presented, we suggest that Jak-STAT signaling is not sufficient to induce apoptosis.

Castedo et al. (38) demonstrated that mTOR, although generally considered as a growth promoting enzyme, participates in the induction of apoptosis. mTOR was shown to phosphorylate the p53 tumor suppressor protein on Ser15, thus mediating human immunodeficiency virus, type 1-encoded envelope glycoprotein-induced syncytial apoptosis of lymphoblasts. The cells used in our study either have mutant p53 (U266) or express the SV40LT antigen that inactivates endogenous wt p53 (Rhek-1). Therefore IFNα-induced apoptosis in these systems is not dependent on p53 activity, and the block of apoptosis by mTOR inhibition cannot be accounted for by the inhibition of p53 phosphorylation. However, one cannot exclude other substrates of mTOR that might be involved in the induction of apoptosis by IFNα.

There is no consensus in the literature about activation of the kinases downstream of PI3K by type I IFN. In Daudi and 2F-TGH fibrosarcoma cells, Akt was activated, whereas in U266 cells this was not the case (17, 20, 36). We found that IFNα did not induce Akt phosphorylation in U266 cells (data not shown). This brings up the question of whether activation of PI3K by IFNα in these cells may play a role other than promoting

of the same membrane with anti-tubulin antibodies. In the bar graph, the bands from the blot were quantified using Image Gauge software, and the ratio with tubulin is presented. C, immunoblot of the same lysates as in B was performed using antibodies against the phosphorylation sites Thr492/Ser494 or Thr627 of p70S6K. The membranes were probed with anti-tubulin and bands were quantified as in B. A representative of two experiments is shown.

DISCUSSION

In this study we have shown that activation of the PI3K/mTOR signaling pathway by IFNα is critical for the induction of apoptosis. This is, to our knowledge, the first report that links one specific branch of the IFNα signaling cascade to a specific cellular effect.

One question that arises from this finding is how is a survival pathway, such as the PI3K pathway, may be converted into a mediator of cell death. A possible explanation could be that both mTOR and PI3K, as serine kinases, may be required for the phosphorylation of STATs on serine. There is at least one publication demonstrating that IFNγ-induced serine phosphorylation of STAT1 and subsequent activation of gene expression are dependent on PI3K activity (35). This implies that STAT activation and its downstream signaling may be affected by the inhibition of PI3K or mTOR. However, although thoroughly investigated, we obtained no data to support such a notion, because neither serine phosphorylation of STAT1 or STAT3 nor the binding of the ISGF3 or STAT homo- or heterodimers to the specific DNA sequences was affected by the inhibition of either PI3K or mTOR. Neither did Ly294002 nor rapamycin affect the activation of four different IFNα-induced genes containing either ISRE or GAS elements, or both, in their promoters. It has been reported, however, that inhibition of PI3K activity selectively abrogated IFNβ-mediated induction of the β-R1 (SCYB11) gene (36), suggesting that there could exist specific targets of type I IFNs that require PI3K activity for their transcriptional up-regulation. We therefore tested the consequence of Ly294002 and rapamycin treatment on the biological effects of Jak-STAT signaling. The ability of IFNα to promote an anti-viral state, known to be largely dependent on the Jak-STAT signaling, was not impaired by either drug. Thus, although the cells were almost fully protected from IFNα-induced apoptosis by these drugs, the Jak-STAT pathway activation as well as a major IFN-mediated biological effect were kept intact. Our results also confirm previously published data that the Jak-STAT and the PI3K pathways, activated by IFNα, act separately from each other (21, 37). Furthermore, on the basis of the data presented, we suggest that Jak-STAT signaling is not sufficient to induce apoptosis.

Castedo et al. (38) demonstrated that mTOR, although generally considered as a growth promoting enzyme, participates in the induction of apoptosis. mTOR was shown to phosphorylate the p53 tumor suppressor protein on Ser15, thus mediating human immunodeficiency virus, type 1-encoded envelope glycoprotein-induced syncytial apoptosis of lymphoblasts. The cells used in our study either have mutant p53 (U266) or express the SV40LT antigen that inactivates endogenous wt p53 (Rhek-1). Therefore IFNα-induced apoptosis in these systems is not dependent on p53 activity, and the block of apoptosis by mTOR inhibition cannot be accounted for by the inhibition of p53 phosphorylation. However, one cannot exclude other substrates of mTOR that might be involved in the induction of apoptosis by IFNα.

There is no consensus in the literature about activation of the kinases downstream of PI3K by type I IFN. In Daudi and 2F-TGH fibrosarcoma cells, Akt was activated, whereas in U266 cells this was not the case (17, 20, 36). We found that IFNα did not induce Akt phosphorylation in U266 cells (data not shown). This brings up the question of whether activation of PI3K by IFNα in these cells may play a role other than promoting...
Fig. 4. STAT phosphorylation and activation are not affected by the inhibition of the PI3K or mTOR. U266 cells were treated with 5000 units/ml of IFNα alone or together with Ly294002 or rapamycin for the indicated times. A, immunobots for the phosphorylation specific sites of STAT3, STAT1, and STAT2 were performed. The same membranes were reprobed with anti-tubulin antibodies as a control for loading. B, cells were cytospun onto glass slides, fixed, and stained with rabbit serum against phosphotyrosine-STAT3, and detected by using fluorescein isothiocyanate-conjugated secondary antibody. The nuclei were stained with DAPI. DMSO, dimethyl sulfoxide. C, nuclear extracts from the indicated samples were incubated with 32P-labeled ISRE (top panel) or SIE (bottom panel) double-stranded oligonucleotides followed by EMSA.
survival signals. It is conceivable, for example, that IFN signaling to PI3K may specifically result in the stimulation of the negative feedback loop, normally activated by insulin in parallel to Akt stimulation (39). Thus, IFN would interfere with surviving signals from insulin or other growth factor receptors leading to cell death.

There is no absolute consensus in the literature about the specificity of the pharmacological inhibitors of PI3K or mTOR. Some investigators show that Ly294002 is specific to PI3K, whereas wortmannin may also inhibit other PIKK family members (38, 40). On the other hand, there is evidence that Ly294002 can inhibit other kinases from the PIKK family, including DNA-PK and mTOR (19). In contrast, rapamycin is quoted in numerous reports as being a very specific mTOR inhibitor (41). To minimize the side effect of the drugs on other possible targets, we used low concentrations of Ly294002 (10 μM) and rapamycin (1 μM), which were comparatively tested in a cell-based assay and shown to be specific to PI3K and mTOR, respectively (41). We then showed that these amounts of Ly294002 were sufficient to inhibit PI3K activity and the phosphorylation of the downstream target proteins, p70S6K and 4E-BP1. To substantiate these data we have also used a dominant negative kinase dead mutant of mTOR that is unable to phosphorylate target proteins (28). In a single cell-based assay, we have found that overexpression of this mutant blocked IFNα-induced apoptosis, confirming the data obtained using rapamycin.

The block of IFNα-induced apoptosis by Ly294002 or rapamycin was more efficient than that achieved using a pan-caspase inhibitor (7). Furthermore, all of the hallmarks of IFNα-induced apoptosis, including Bak activation, which precedes cytochrome c release and loss of mitochondrial membrane potential (25), were blocked through inhibition of the PI3K/mTOR pathway. Data from other groups have suggested that IFNα-induced apoptosis was dependent on activation of TRAIL/Apo2L and, consequently, on a TRAIL-induced caspase-8-mediated apoptotic pathway (42, 43). Ly294002 efficiently blocked caspase-8 activation in our experiments, demonstrating that the PI3K pathway lies upstream of caspase-8 in IFNα-induced apoptosis. All of these facts have led us to propose a model where PI3K and mTOR activation are upstream events in IFNα-induced apoptosis.

The fact that IFNα acts as a potent inducer of apoptosis in a number of malignant cell lines and in primary tumor cells (6–10, 44) allows the suggestion that the induction of programmed cell death may be one of the actual mechanisms for the anti-tumor effects of IFN. One could draw a parallel with the cytotoxic drugs used for chemotherapy, the major mechanism

Fig. 5. Expression of the IFNα target genes is not affected by inhibition of the PI3K/mTOR pathway. A, U266 cells were treated with 5000 units/ml of IFNα for 24 and 48 h either alone or in the presence of Ly294002 or rapamycin and were directly lysed in the SDS buffer followed by SDS-PAGE, immunoblotting and incubation with antibodies against IRF-9, and subsequently against MxA proteins. The same membrane was reprobed with anti-CDK4 antibodies as a control for loading. B, U266 cells were treated with 5000 units/ml of IFNα for 48 h either alone or in the presence of Ly294002 or rapamycin, fixed, stained with anti-PML antibodies and secondary fluorescein isothiocyanate-conjugated antibodies, and analyzed by FACS. Because all cells uniformly expressed certain levels of PML protein, the representative analysis included cells with strong induction of PML protein, presented as a bar graph. A representative of two experiments is shown.

Fig. 6. IFNα-mediated protection from virus production and virus-induced cell death is not impaired by the PI3K or mTOR inhibitors. A, U266 cells were seeded in a 96-well plate, pretreated with IFNα alone or in the presence of Ly294002 or rapamycin (Rapa) for 6 h, and infected with VSV for another 24 h followed by WST proliferation assay. A summary of three experiments is shown. B, cells were treated as in A, infected with VSV for 1 h, washed, and seeded in a fresh medium in a 96-well plate. 24 h later, the cells were lysed using 1% Nonidet P-40, and the resultant lysate plus conditioned medium were used to coat ELISA plates. The envelope G protein of VSV was detected using HRP-conjugated anti-VSV-G antibody.
of action of which is induction of apoptosis of tumor cells (45). As with cytotoxic drugs, many tumor types are resistant to IFN therapy for unknown reasons. Therefore the knowledge of the signaling pathways responsible for the apoptosis induction is extremely important for understanding of the variability in cellular responses to IFN treatment.

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Interferon α-induced Apoptosis in Tumor Cells Is Mediated through the Phosphoinositide 3-Kinase/Mammalian Target of Rapamycin Signaling Pathway

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