Activation of the STAT Signaling Pathway Can Cause Expression of Caspase 1 and Apoptosis

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Protein tyrosine kinases activate the STAT (signal transducer and activator of transcription) signaling pathway, which can play essential roles in cell differentiation, cell cycle control, and development. However, the potential role of the STAT signaling pathway in the induction of apoptosis remains unexplored. Here we show that gamma interferon (IFN- γ) activated STAT1 and induced apoptosis in both A431 and HeLa cells, whereas epidermal growth factor (EGF) activated STAT proteins and induced apoptosis in A431 but not in HeLa cells. EGF receptor autophosphorylation and mitogen-activated protein kinase activation in response to EGF were similar in both cell lines. The breast cancer cell line MDA-MB-468 exhibited a similar response to A431 cells, i.e., STAT activation and apoptosis correlatively resulted from EGF or IFN- γ treatment. In addition, in a mutant A431 cell line in which STAT activation was abolished, no apoptosis was induced by either EGF or IFN- γ . We further demonstrated that both EGF and IFN- γ induced caspase 1 (interleukin-1 β converting enzyme [ICE]) gene expression in a STAT-dependent manner. IFN- γ was unable to induce ICE gene expression and apoptosis in either JAK1-deficient HeLa cells (E2A4) or STAT1-deficient cells (U3A). However, ICE gene expression and apoptosis were induced by IFN- γ in U3A cells into which STAT1 had been reintroduced. Moreover, both EGF-induced apoptosis and IFN- γ -induced apoptosis were effectively blocked by Z-Val-Ala-Asp-fluoromethylketone (ZVAD) in all the cells tested, and studies from ICE-deficient cells indicated that ICE gene expression was necessary for IFN- γ -induced apoptosis. We conclude that activation of the STAT signaling pathway can induce apoptosis through the induction of ICE gene expression.

Apoptosis, or programmed cell death, is a process essential for normal development and homeostasis in multicellular organisms and provides a defense against oncogenesis or viral invasion (18, 43, 53, 58, 67, 68). It is known that a number of factors and pathways can lead to apoptosis. For example, p53 is involved in apoptosis in response to DNA damage and other cellular damage (11, 38). Certain growth-inhibitory cytokines are capable of inducing apoptosis independent of p53. Tumor necrosis factor alpha (TNF- α) and Fas ligand (FasL) can both trigger cell death through a proteolytic signaling cascade in the absence of de novo RNA or protein synthesis (5, 12, 22, 28, 48, 61). The key players in TNF- α /FasL-induced apoptosis are the members of the interleukin-1 β converting enzyme (ICE) family of proteases, recently renamed caspase (1). The mammalian ICE protease family has at least 10 members, and they are homologs of the *Caenorhabditis elegans* programmed cell death gene *ced-3* (70). It is possible that these different ICE family members may function in different apoptosis pathways.

Many growth factors and cytokines activate protein tyrosine kinase (PTK) signaling pathways leading ultimately to gene expression (29, 55, 65). The PTK pathways are believed to play crucial roles in cell proliferation and survival. For many cells, either cell growth arrest or cell apoptosis will occur with deprivation of growth factors or cytokines (12, 63). Thus, growth factors, such as insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF), which usually induce mitogenic responses, act as survival factors (4, 20, 27, 31, 63). However, some PTK-activating cytokines, such as interferons, can trigger cell cycle arrest and

cell death in many cells (16, 37). EGF and PDGF stimulate cell growth and protect many cells from apoptosis but also inhibit cell growth and induce apoptosis in some mammalian cells (2, 7, 8). Compared with the fast-accumulating knowledge on the mechanisms of TNF/FasL-induced apoptosis, little is known about how apoptosis is induced by activation of tyrosine kinase pathways and what mediators of signal transduction are involved.

In these PTK pathways, binding of a growth factor or cytokine to its cell surface receptor results in the activation of the receptor tyrosine kinase or receptor-associated tyrosine kinase. The ultimate activation of mitogen-activated protein (MAP) kinases (e.g., ERK, extracellular signal-regulated kinase) involves recruitment of signaling molecules containing SH2 (Src homology region 2) domains, and activation of Ras, Raf, and MAP kinases (51, 55). MAP kinases translocate to the nucleus upon activation and phosphorylate transcription factors (e.g., c-Jun and TCF) (see reference 33). MAP kinase activation therefore provides a link between cytoplasmic and nuclear signaling events. However, it is not known how cell survival and apoptosis are regulated through this signaling cascade. A parallel direct signaling pathway has been discovered over the past few years. In this pathway, PTKs directly activate STAT (signal transducer and activator of transcription) proteins, which are a class of transcription factors with SH2 domains (13, 23, 24, 29, 40, 47, 54, 56a, 66). Although JAK kinases were initially found as the tyrosine kinases activating STATs, a variety of other protein tyrosine kinases, such as EGF receptor kinase, can also activate STAT proteins independent of JAK kinases (25, 50, 52). Thus, we use the term “the PTK-STAT signaling pathway” in this paper.

Recent work demonstrated that the Ras-MAP kinase pathway and the PTK-STAT pathway are both activated at the

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same time by many growth factors or cytokines, including interferons and EGF. Previous work from this laboratory and others indicated that STAT activation by interferons, EGF, or interleukin 6 (IL-6) inhibits cell proliferation and leads to cell differentiation (10, 45). In this study, we examined the potential role of STAT1 in cell apoptosis. We present evidence that the PTK-STAT pathway activation is critical for either gamma interferon (IFN- γ)- or EGF-induced apoptosis. The induction of ICE gene expression, which plays an essential role in apoptosis in response to these two cytokines, is dependent on the activation of STAT1 protein.

MATERIALS AND METHODS

Cell culture, extracts, EMSA, immunoprecipitation, and Western blot analysis. A431, MDA-MB-468, and HeLa cells (American Type Culture Collection) were grown in monolayers at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) or calf serum. 2FTGH and U3A cells, obtained from G. Stark, were grown in DMEM supplemented with 10% FBS and 400 μ g of hygromycin/ml. U3A-S1-2 cells were grown in DMEM supplemented with 10% FBS and 400 μ g of G418/ml.

Whole-cell extracts were prepared as described previously (10). Briefly, cells were starved overnight and treated with 50 to 200 ng of EGF (Gibco) ml⁻¹ or 10 ng of IFN- γ (Genzyme) ml⁻¹ for 30 min. Phosphate-buffered saline (PBS)-rinsed cells were lysed in 20 mM HEPES (pH 7.9) buffer containing 0.2% Nonidet P-40, 400 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 1 mM sodium vanadate, 0.5 mM phenylmethylsulfonyl fluoride, and 1 μ g (each) of aprotinin, leupeptin, and pepstatin/ml. After 30 min of gentle agitation at 4°C, the supernatants were collected by centrifugation.

For all electrophoretic mobility shift assays (EMSA), M67-SIE was used as the probe (10). DNA-protein binding reactions (15 μ l) were performed by incubation of the whole-cell extracts in a solution containing 10 mM HEPES (pH 7.9), 50 mM NaCl, 0.1 mM EDTA, 5% glycerol, 50 μ g of poly(dI-dC) (Pharmacia)/ml, 0.5 mM dithiothreitol, and 0.01% Nonidet P-40 for 10 min at room temperature, followed by an additional 30-min incubation with ³²P-end-labeled M67-SIE probe (0.1 ng) at room temperature. DNA-protein complexes were separated on 6% nondenaturing acrylamide gels in 0.5 $\times$ Tris-borate-EDTA and detected by autoradiography.

Anti-EGF receptor antibody was purchased from Gibco, and a purified antiphosphotyrosine polyclonal antibody was a generous gift from Jun-Lin Guan (Cornell University). The EGF receptor immunoprecipitation and phosphotyrosine antibody blotting were performed as previously described (25). Anti-MAP kinase (ERK-2) and anti-ICE (p10) antibodies were from Santa Cruz Biotech, Inc., and the Western blot assays using these two antibodies were performed according to the manufacturer's protocol.

Analysis of apoptotic cells. Morphological changes in the nuclear chromatin of cells undergoing apoptosis were detected by staining with the DNA-binding fluorochrome bis-benzimide (Hoechst 33258; Sigma) as described in reference (49). Briefly, monolayer cells (3×10^5 to 6×10^5) were grown in 6-well plates and were treated with or without EGF (100 to 200 ng/ml) or IFN- γ (80 to 160 ng/ml) in the presence of 1% calf serum or FBS for different times. After treatment, cells were collected and pelleted at $300 \times g$ for 5 min and washed once with PBS. Cells were resuspended in 100 μ l of 3% paraformaldehyde in PBS and incubated for 15 min at room temperature. After fixation, the cells were washed once with PBS and were stained with 15 μ l of bis-benzimide (16 μ g/ml) in PBS. Following 15 min of incubation at room temperature, a 5- μ l aliquot of cells was placed on a glass slide, and the average number of nuclei per field was scored for the incidence of apoptotic chromatin changes under a fluorescence microscope. Cells with three or more condensed chromatin fragments were considered apoptotic.

ICE inhibitor Z-Val-Ala-Asp-(O-methyl)-fluoromethylketone (ZVAD) was purchased from Enzyme Systems Products and dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 10 mM. A431, MDA-MB-468, and HeLa cells (5×10^5) were grown in 6-well plates and treated with EGF (100 ng/ml) or IFN- γ (100 ng/ml) for 24 h, followed by treatment with 20 μ M ZVAD or DMSO alone for an additional 12, 24, or 48 h. After harvest, the cells were washed with PBS and mixed with 0.2% trypan blue, and the concentrations of live and dead cells were determined.

Northern blot. Total RNA was prepared with an RNA isolation kit from Gibco-Life Science. RNA (40 μ g) was analyzed by electrophoresis in a 1.2% agarose-formaldehyde gel and transferred to a nylon membrane (Zeta-Probe; Bio-Rad). Hybridization was performed at 65°C overnight in 0.25 M Na₂PO₄ (pH 7.2)-7% sodium dodecyl sulfate (SDS)-1 mM EDTA. The wash was performed at 65°C in 0.04 M Na₂PO₄ (pH 7.2)-1% SDS. The probes (ICE cDNA and CPP32 cDNA) were labeled with a random-primed DNA labeling kit (Boehringer Mannheim).

Primary spleen cell preparation, cell viability determination, and DNA fragmentation assay. Mouse (ICE^{+/+} or ICE^{-/-}) spleens were washed with PBS twice and chopped up with a sterilized blade. The chopped spleen cells were then

treated with trypsin at 37°C for 10 min. The trypsinized spleen cells were then suspended in RPMI medium supplemented with 10% FBS, 100 U of penicillin per ml, and 100 ng of streptomycin sulfate per ml. After setting in a 15-ml tube for 1 to 2 min, the suspended single cells near the top were removed and cultured at a concentration of 5×10^6 cells/ml in 6-well plates. Cells were treated with IFN- γ for 48 h, and cell viability was determined by trypan blue exclusion. To examine DNA fragmentation, approximately 1×10^7 to 2×10^7 cells were seeded in a 100-mm dish and treated with IFN- γ (50 to 100 U/ml) or left untreated. After 48 h, cells were harvested and washed with cold PBS twice, and the DNA was isolated as follows. Lysis buffer (10 mM Tris-HCl [pH 7.5], 10 mM EDTA, 0.2% Triton X-100) (0.6 ml) was added to the cells, the lysis was allowed to proceed at room temperature for 15 min, and then the lysate was centrifuged for 10 min at 12,000 rpm in an Eppendorf microfuge. The supernatant was collected and mixed with an equal volume of phenol and centrifuged for 10 min at 12,000 rpm. The aqueous layer was adjusted to 300 mM NaCl and mixed with 2 volumes of ethanol to precipitate the DNA. After centrifugation for 10 min at 12,000 rpm, the DNA pellet was resuspended in 20 μ l of Tris-EDTA buffer and digested with 0.2 μ g of RNase at 37°C for 30 min. The fragmented DNA was analyzed by running a 2% agarose gel and staining with ethidium bromide.

RESULTS

Apoptosis induction by EGF and IFN- γ correlated with STAT activation. We have shown previously that unlike IFN- γ , which activates STAT in most mammalian cells, EGF activates STAT proteins only in a few types of cells (10), such as A431 cells, which express a large amount of EGF receptors and have been widely used in studies of EGF receptor activation and function (26). Here we compared STAT activation in HeLa cells with that in A431 cells in response to EGF and IFN- γ . Cells were treated with either EGF or IFN- γ for 30 min, and protein extracts were prepared as described in Materials and Methods. EMSAs using M67-SIE as the probe showed that in A431 cells treated with EGF, DNA-bound STAT dimers were formed (Fig. 1A, lanes 7 and 8) (for detailed characterization of these complexes, see references 10 and 71). In contrast, no obvious STAT activities were detected in HeLa cells treated with EGF under the same conditions, except that STAT3 might be weakly activated (Fig. 1A, lanes 3 and 4). IFN- γ induced strong STAT1 activation in both cell types (Fig. 1A, lanes 2 and 6).

We then examined the morphological changes of these two cell lines in response to EGF and IFN- γ treatments. In A431 cells, EGF treatment not only inhibited cell growth, as we and others have reported previously (10, 26), but also induced cell apoptosis (Fig. 1B). In contrast, EGF exhibited a strong mitogenic effect on HeLa cells (data not shown), and no apoptosis was observed (Fig. 1B). As expected, IFN- γ induced apoptosis in both A431 and HeLa cells (Fig. 1B). The apoptotic cells were clearly identified by altered nuclear structure, with condensed chromatin fragments seen under fluorescence microscopy after staining with the fluorochrome bis-benzimide (49, 64). Either EGF- or IFN- γ -induced apoptosis was further confirmed by DNA fragmentation assays (data not shown).

Since EGF elicited very different responses in these two cell lines, we examined EGF receptor autophosphorylation and MAP kinase activity in A431 and HeLa cells. Cell extracts were prepared from each cell line with or without EGF treatment and were immunoprecipitated with anti-EGF receptor antibody. The immunoprecipitated proteins were resolved by SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and probed with antiphosphotyrosine antibody. Fig. 1C (top) shows that EGF treatment led to EGF receptor autophosphorylation in both A431 and HeLa cells. The same protein extracts were also probed with anti-MAP kinase antibody in Western blot assays. As shown in Fig. 1C (bottom), MAP kinase (ERK-2) was phosphorylated (slowed mobility) and therefore activated after EGF treatment in both A431 and HeLa cells. These data indicate that the failure of EGF to activate STAT proteins in HeLa cells was not due to an

FIG. 1. EGF and IFN- γ induced STAT activation and apoptosis, and EGF induced EGF receptor autophosphorylation and MAP kinase activation, in HeLa and A431 cells. (A) STAT activation. EMSAs were conducted by using M67-SIE as the probe to examine STAT activation in whole-cell extracts, prepared from HeLa and A431 cells, that were left untreated (mock, lanes 1 and 5) or treated with either IFN- γ (lanes 2 and 6) or EGF (lanes 3, 4, 7, and 8) for 30 min. SIF-A, STAT3 homodimer; SIF-C, STAT1 homodimer; SIF-B, STAT1-STAT3 heterodimer. (B) Alterations of nuclear morphology. HeLa cells and A431 cells were left untreated (top) or treated with EGF (100 ng/ml) or IFN- γ (100 ng/ml) for 72 h. Cells were fixed with 2% paraformaldehyde and stained with bis-benzimide (magnification, $\times 480$). (C) EGF receptor and MAP kinase phosphorylation. (Top) Cells were starved overnight and then treated without (–) or with 100 ng of EGF (E) ml^{-1} for 30 min before lysis. EGF receptor was immunoprecipitated, electrophoresed, transferred to a nitrocellulose membrane, and immunoblotted with antiphosphotyrosine antibodies. (Bottom) Western blot analysis of the cell lysates with anti-MAP kinase antibody.

EGF receptor defect. Currently, it is not clear why different cells respond to EGF in STAT activation so differently. The above data also indicate that EGF's inhibitory effect on cell growth or survival in A431 cells was not due to inactivation of the Ras-MAP kinase pathway.

Additional evidence for the correlation between STAT activation and apoptosis was obtained from the studies of MDA-MB-468 cells, a breast cancer cell line that expresses high levels of EGF receptors (2, 8). It was reported that EGF inhibits cell growth and induces apoptosis in this cell line (2, 21). As expected, STAT proteins were activated (as shown by EMSA) and apoptosis was induced by both EGF and IFN- γ treatments in MDA-MB-468 cells (Fig. 2A and B). With A431-R cells, an A431 variant isolated by G. Gill's group based on its resistance to the growth-inhibitory effects of high concentrations of EGF (26, 35), we found that STAT was activated neither by EGF nor by IFN- γ (Fig. 2C). Consequently, no apoptosis was induced by EGF or IFN- γ in A431-R cells (Fig. 2D). We also selected A431 and MDA-MB-468 variants in which EGF was unable to activate STAT or induce apoptosis (data not shown).

In A431, A431-R, and MDA-MB-468 cells, apoptotic cells were enumerated by trypan blue exclusion at various time points following the addition of EGF or IFN- γ (Fig. 2E). The data were consistent with the results obtained with bis-benzimide staining and DNA fragmentation assays (data not

shown). As determined by EMSAs (STAT activation), bis-benzimide staining, and trypan blue exclusion with all these independent cell lines, STAT activation indeed was closely correlated with apoptosis.

ICE expression correlated with EGF- and IFN- γ -induced apoptosis. We next sought to explore the cellular target(s) of the activated STAT for apoptosis induction. Since the ICE protease family and the *bcl-2* family play important roles in apoptosis (22, 43, 62, 70), we examined the gene expression patterns of most members of these two apoptosis gene families by Northern blot analysis. Among the genes we tested, ICE (caspase 1) expression was upregulated in a STAT-dependent

FIG. 2. Effects of EGF and IFN- γ on STAT activation and apoptosis in MDA-MB-468 and A431-R cells. (A) STAT activation in MDA-MB-468 cells. EMSA was performed as described in the Fig. 1 legend and Materials and Methods. Whole-cell extracts, prepared from untreated cells or from cells stimulated with EGF (E) or IFN- γ (γ), were examined for DNA binding activity with M67-SIE as the probe. The SIF complexes are indicated. (B) Alterations of nuclear morphology in MDA-MB-468 cells. Bis-benzimide staining (magnification, $\times 600$) after EGF and IFN- γ treatments of the cells for 48 h shows apoptotic nuclei (appearance of condensed or fragmented chromatin structures). (C) EMSA was conducted with whole-cell extracts obtained from EGF- or IFN- γ -treated A431-R or A431 (parental cell line) cells. (D) A431-R cells were treated with EGF and IFN- γ for 72 h and stained with bis-benzimide. (E) Quantitation of apoptotic cells induced by EGF or IFN- γ . A431, A431-R, and MDA-MB-468 cells were cultured in 6-well plates at a concentration of 5×10^5 cells/well and treated with EGF or IFN- γ for the time intervals indicated. After bis-benzimide staining, apoptotic cell numbers were counted under the fluorescent microscope. Percent apoptotic cells represents the mean value $\pm$ standard deviation from three independent experiments.

manner. As shown in Fig. 3B and C, EGF and IFN- γ induced ICE gene expression in both A431 and MDA-MB-468 cells. In HeLa cells, a time-dependent induction of ICE expression occurred only with IFN- γ treatment (Fig. 3A). A small increase of ICE expression was observed with EGF treatment of these cells only after 24 h, probably as a result of weakly activated STAT3 (Fig. 1A, lanes 3 and 4). It is worthwhile to point out that starving HeLa cells of FBS for a period from 12 to 24 h might also induce ICE expression (Fig. 3A). In addition, in A431-R cells, which are defective in STAT activation, ICE mRNA expression was uninducible (data not shown).

To confirm that ICE induction occurred at the protein level, Western blot analysis of whole-cell protein extracts from A431, HeLa, and MDA-MB-468 cells treated with or without EGF or IFN- γ was performed and revealed that ICE protein levels increased following EGF and IFN- γ treatments in A431 and MDA-MB-468 cells and following IFN- γ treatment in HeLa cells (Fig. 3D). Interestingly, a proteolytically cleaved form of ICE, p10, was clearly observed in A431 and HeLa cells after either IFN- γ or EGF treatment. However, p10 seemed very unstable and difficult to detect in some experiments we have done with MDA-MB-468 cells, whereas the level of p45 was clearly increased in response to either IFN- γ or EGF (Fig. 3D, lanes 7 to 9). With repeated experiments, this p10 form of ICE could be observed as faint bands in Western blots of MDA-MB-468 cells treated with either IFN- γ or EGF (Fig. 3D, lanes 10 and 11). Taking these data together, we conclude that induction of ICE mRNA and protein may be parallel to STAT activation in these cells, suggesting that the ICE protease may be involved in IFN- γ - and EGF-induced apoptosis.

To obtain further evidence for the involvement of ICE in EGF- and IFN- γ -induced apoptosis, we tested whether ZVAD, an irreversible inhibitor of ICE family proteases (17, 32), can block EGF- and IFN- γ -induced apoptosis. All the cells were treated with EGF and IFN- γ for 24 h, and ZVAD (20 μ M) was then added for an additional period of time (e.g., 12, 24, or 48 h). As shown in Fig. 4, ZVAD effectively blocked either EGF- or IFN- γ -induced apoptosis in all three types of cells tested. A small fraction of cells became apoptotic before ZVAD addition, which may explain why the protective action of ZVAD was not complete. ZVAD also rescued HeLa cells from apoptosis induced by FBS withdrawal (data not shown). These findings further suggest a possible role of ICE in EGF- and IFN- γ -induced apoptosis.

The JAK-STAT pathway is essential for ICE expression and apoptosis induction in response to IFN- γ . Like many other cytokines, interferons may activate the Ras-MAP kinase pathway in addition to the JAK-STAT pathway (14, 69). To confirm that ICE expression and apoptosis occurrence in the presence of IFN- γ is indeed mediated by the JAK-STAT pathway, we investigated induction of both ICE mRNA expression and apoptosis in JAK- and STAT-deficient cell lines in response to IFN- γ treatment. E2A4 is a JAK1 kinase-deficient cell line derived from HeLa cells (42). DNA binding activity of STAT activation was absent as determined by EMSAs in E2A4 cells (Fig. 5A). The strong induction of ICE mRNA normally seen upon IFN- γ treatment in the parental HeLa cells was completely abolished in this JAK1-deficient cell line, as shown in Fig. 5B. Moreover, bis-benzimide staining showed that E2A4 cells did not undergo apoptosis in the presence of IFN- γ (Fig. 5C). These results suggest that JAK1 kinase is essential for the induction of both ICE expression and apoptosis by IFN- γ .

We then analyzed the effects of IFN- γ on U3A cells, a STAT1-defective cell line (44). As can be seen in Fig. 6, IFN- γ failed to activate STAT, induce ICE mRNA expression, or lead to apoptosis in U3A cells. In contrast, IFN- γ was able to

FIG. 3. Induction of ICE mRNA and protein expression. (A) Total RNA was prepared from HeLa cells that were treated with EGF or IFN- γ for various intervals of time (as indicated) and subjected to Northern blot analysis with the 1.0-kb ICE cDNA as the probe. The right panel shows ICE mRNA expression in HeLa cells that were deprived of FBS for different time intervals as indicated. 28S RNA was used to show that an equal amount of RNA was loaded in each sample. (B and C) Under the same conditions, ICE mRNA induction was examined in A431 cells (B) and MDA-MB-468 cells (C) in response to EGF and IFN- γ treatments. (D) ICE protein levels were examined in A431, HeLa, and MDA-MB-468 cells. All the cells were cultured in 100-mm plates and left untreated or treated with EGF or IFN- γ for 48 h. Twenty micrograms of denatured whole-cell extracts were loaded for Western blot analysis. The polyclonal anti-ICE antibody recognizes both the precursor form (p45) and the enzymatic form (p10) of the ICE protein.

induce STAT-DNA binding activity, ICE mRNA, and apoptosis in both the U3A parental cell line, 2fTGH cells, and U3A-S1-2 cells, in which STAT1 had been reintroduced. U3A cells that were stably transfected with the vector alone as a control were unable to respond to IFN- γ (data not shown). The expression of CPP32, an ICE family member which is believed to play a crucial role in many apoptotic processes (19, 22), was not affected by IFN- γ treatment in all the cells tested (Fig. 6C and data not shown). Taken together, our results strongly indicate that activation of the JAK-STAT pathway is essential for the induction of ICE expression and apoptosis by IFN- γ .

FIG. 4. Inhibitory effect of ZVAD on EGF- and IFN- γ -induced apoptosis. A431, MDA-MB-468, and HeLa cells cultured in 6-well plates at 5×10^5 cells/well received either no treatment (-) or treatment with EGF or IFN- γ for 24 h, followed by treatment with 20 μ M ZVAD or DMSO alone for an additional 48 h. Apoptotic cells were counted by trypan blue exclusion.

FIG. 5. JAK1 is required for IFN- γ to induce ICE mRNA expression and apoptosis. (A) EMSA was performed as described for Fig. 1. In E2A4 cells, a JAK1 kinase-deficient cell line, STAT activity was not induced with either EGF or IFN- γ treatment. However, STAT activity was induced by IFN- γ in the parental cell line, HeLa cells. (B) ICE mRNA was undetectable in the E2A4 cell line treated by IFN- γ but was induced in its parental HeLa cells. 28S RNA was used as an indicator of loading RNA with an equal amount in each sample. Numbers at the top of the gel represent hours of treatment. (C) Nuclear morphology was examined as described for Fig. 1. All nuclei of E2A4 cells (untreated or treated with EGF or IFN- γ for 72 h) were stained uniformly with bis-benzimidazole, indicating intact nuclei.

The ICE gene is essential for IFN- γ -induced apoptosis. To further confirm that ICE expression induced by IFN- γ is critical for provoking apoptosis, we isolated the primary spleen cells from ICE $^{-/-}$ and ICE $^{+/+}$ mice (39) and compared their responses to IFN- γ treatment. Although STAT1 can be activated by IFN- γ in both ICE $^{-/-}$ and ICE $^{+/+}$ cells (Fig. 7A), IFN- γ -induced DNA fragmentation was significantly reduced in ICE $^{-/-}$ cells compared with that in ICE $^{+/+}$ cells (Fig. 7B). The cell viability assays (trypan blue exclusion) showed that IFN- γ triggered much more apoptosis in ICE $^{+/+}$ cells than in ICE $^{-/-}$ cells, in a dose-dependent manner (Fig. 7C). Thus,

FIG. 6. STAT1 is required for IFN- γ to induce ICE mRNA expression and apoptosis. (A) EMSA analysis of STAT-DNA complex formation in the whole-cell extracts of 2fTGH, U3A, and U3A-S1-2 cells as described in Materials and Methods. The STAT activity in SIE binding (SIF-C) was induced by IFN- γ in 2fTGH and U3A-S1-2 cells but not in U3A cells. U3A cells are derived from the 2fTGH cell line and are STAT1 deficient. U3A-S1-2 cells are derived from the U3A cell line with a stable transfectant of STAT1 as described previously (10). (B) Northern blot analysis, performed as described in Materials and Methods, showing a time-dependent induction of ICE mRNA expression by IFN- γ treatment in both 2fTGH and U3A-S1-2 cells but not in U3A cells. (C) A similar Northern blot analysis showing the effect of IFN- γ on CPP32 mRNA expression in 2fTGH, U3A, and U3A-S1-2 cells. (D) Bis-benzimide staining of 2fTGH, U3A, and U3A-S1-2 cells. After receiving no treatment or IFN- γ treatment for 72 h, all the cells were stained with bis-benzimide and examined under a fluorescence microscope. The condensed and/or fragmented nuclei were observed in 2fTGH and U3A-S1-2 cells treated with IFN- γ .

FIG. 7. ICE is involved in IFN- γ induced apoptosis. Primary spleen cell cultures were prepared as described in Materials and Methods. (A) Gel shift assay illustrating altered DNA mobility due to STAT1 binding (SIF-C) following IFN- γ treatment for 30 min in both ICE wild-type (+/+) (WT) and ICE-deficient (-/-) cells. (B) DNA fragmentation assay (see Materials and Methods) shows that after treatment with IFN- γ for 48 h, DNA fragmentation occurred in ICE^{+/+} cells (lane 2) but not in ICE^{-/-} cells (lane 4). (C) Cell viability analysis of ICE^{+/+} and ICE^{-/-} cells. ICE^{+/+} and ICE^{-/-} cells were plated at a density of 5×10^6 in 6-well plates and incubated for 48 h with increasing amounts of IFN- γ , as indicated. Cell numbers (live and dead) were determined by trypan blue exclusion. Data are means $\pm$ standard deviations of three experiments.

ICE expression plays an important role in IFN- γ -induced apoptosis.

DISCUSSION

It has been known for some time that IFN- γ inhibits cell growth and induces apoptosis in many types of cells, and EGF can also inhibit cell growth and induce apoptosis in some cells, such as A431 and MDA-MB-468 cells (2, 8, 10). We and others recently demonstrated that PTK-STAT activation and cell cycle inhibitor p21^{WAF1/CIP1} expression through STAT activation may be responsible for cell growth arrest induced by interferons and EGF (10). The present studies demonstrated that the PTK-STAT pathway mediates not only cell growth arrest but also a cytokine-regulated type of programmed cell death. Moreover, ICE expression is necessary for this process and is turned on by activated STAT proteins.

Many growth factors and cytokines have dual effects on cell

FIG. 8. A yin (negative) and yang (positive) signaling model of PTK-STAT induced apoptosis. Ligand-bound receptors induce receptor dimerization, receptor autophosphorylation, and phosphorylation of receptor-associated tyrosine kinases such as JAK kinases (55). The phosphorylated tyrosine residues on the receptors serve as the docking sites for SH2-containing proteins: STAT proteins, adapter proteins (51), and other substrates (9), which then become tyrosine phosphorylated. The tyrosine-phosphorylated STAT proteins translocate to the nucleus and regulate genes, such as the ICE gene, which negatively control cell growth and survival. Simultaneously, a mitogenic pathway, such as the Ras-MAP kinase pathway, which regulates the expression of genes, such as oncogenes (34), that positively control cell growth and survival is also activated. Whether an occupied receptor will produce a positive or a negative effect is determined by the relative strengths of these positive and negative signals and their effectors.

growth (10, 57). IL-6 stimulates hepatocyte proliferation and prevents apoptosis from occurring, but IL-6 inhibits cell growth and induces apoptosis in some myeloma cell lines (45). Like EGF, PDGF is required for proliferation and survival in many types of cells but also inhibits cell growth and accelerates apoptosis in some other cells (36). Based on our findings, we propose a yin-yang signaling model to explain this fickleness. As summarized in Fig. 8, a cytokine, by binding to its receptor, can turn on at least two separate signaling pathways: activation of the Ras-MAP kinase pathway (or other pathways such as PI3 kinase pathway) for cell growth/survival (yang) and activation of the STAT pathway for cell arrest/death (yin). The intracellular homeostasis requires a balance between growth/survival and arrest/death signaling events. Different cells may have different dynamic states and hence different phenotypic outputs. In HeLa cells, the STAT pathway is not active and EGF mainly triggers the MAP kinase pathway, so cells proliferate and survive. In A431 or MDA-MB-468 cells, the STAT pathway is more sensitive in response to EGF, so cells arrest and die.

Recently, TNF- α signaling was found to initiate at least two

opposing events: induction of apoptosis through the activation of a protease signaling pathway and activation of transcription factor NF- κ B, which suppresses TNF- α -induced apoptosis (3, 41). The present experiments showed that unlike TNF- α -induced apoptosis, in which gene expression is not required, both EGF- and IFN- γ -induced apoptosis processes require coordinate expression of a specific gene(s), such as the ICE gene. In MDA-MB-468 cells, ICE mRNA was quickly induced (in less than 1 h), indicating that STAT may directly regulate ICE gene expression. Since ICE gene induction was much slower in A431 and HeLa cells, an indirect regulation mechanism of ICE gene expression by STAT1 (e.g., via interferon regulatory factor-1, IRF-1) is not excluded (60). We identified several SIE- or ISRE-like sequences within 1 kb of the 5'-flanking region of the ICE gene. However, STAT-DNA binding activity was not detected in EMSAs by using any of these SIE- or ISRE-like sequences as a probe (data not shown). In addition, we subcloned the ICE gene promoter (1 kb) into a luciferase reporter gene vector. After transfection of COS or 293T cells with this construct and treatment of cells with IFN- γ or EGF, no significant increase of luciferase activity was detected. Therefore,

the STAT regulatory element(s) may be located outside this 1-kb promoter region.

The greatly reduced apoptotic response in ICE^{-/-} cells constitutes definitive evidence for a requirement for ICE in IFN- γ -induced cell death. While ICE expression is necessary for EGF- and IFN- γ -induced apoptosis, as demonstrated here, our data do not indicate whether ICE alone is sufficient. Cathepsin D was recently claimed as a protease responsible for IFN- γ -induced apoptosis in HeLa cells (15). However, it is not clear whether there is any link between ICE and cathepsin D; alternatively, the ICE family inhibitor ZVAD may cross-interact with cathepsin D. In our hands, ZVAD effectively blocked both IFN- γ - and EGF-induced apoptosis in different cell types. Although ICE is not essential for development as shown in ICE^{-/-} mice, ICE is involved in apoptosis resulting from several pathways. For instance, ICE is involved in DNA damage-induced and IRF-1-mediated apoptosis (60), FasL-induced apoptosis (39), granzyme B-induced apoptosis (56), degradation of basement membrane extracellular matrix (ECM)-induced apoptosis in mammary epithelial cells (6), and spontaneous cell death of thymocytes (70a). Thus, the elevated expression of ICE in cells, either by ICE gene transfection (46) or by cytokine induction (as presented in this paper), will lead to cell death.

Some diseases resulting from inappropriate cell arrest and apoptosis may be due to an overactive PTK-STAT pathway. Like JAK and EGF receptor kinases, focal adhesion kinase (FAK) and fibroblast growth factor (FGF) receptor tyrosine kinase can also activate STAT1 and induce apoptosis (69a). Data from this laboratory showed that constitutive activation of STAT1 by mutant FGF receptor kinase may be responsible for the improper growth arrest or apoptosis in bone development disorders (59). Thus, these additional data provide further support for STAT activation as a broad molecular signal mediating apoptosis.

The finding that activation of STAT by EGF receptor kinase leads to apoptosis in the breast cancer cell line MDA-MB-468 may have significant implications for the role of the EGF receptor family in cancer development, since aberrant expression of EGF receptor or its closely related receptor erbB2/neu has been implicated in the malignant behavior of a number of cancers, including breast cancer. MDA-MB-231, another breast cancer cell line that did not induce STAT activation by EGF treatment, is more transforming and invasive than MDA-MB-468 cells (30), and the less malignant phenotype of MDA-MB-468 cells may correlate with differential STAT activity in these cells (10a).

In light of the role of STAT1 in apoptosis as uncovered here, it would be interesting to investigate the susceptibility of STAT1^{-/-} mice to cytokine-induced apoptosis. STAT activation may be used by organisms as a means of killing cytokine-targeted cells. Interferons have been used in the treatment of cancers and other disorders. Our discovery that STAT/ICE mediates apoptosis contributes to an understanding of the mechanism of how interferons work and may lead to additional therapeutic approaches against both cancer and viral infectious diseases.

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