Defective TNF-α–Induced Apoptosis in STAT1-Null Cells Due to Low Constitutive Levels of Caspases

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Signal transducers and activators of transcription (STATs) enhance transcription of specific genes in response to cytokines and growth factors. STAT1 is also required for efficient constitutive expression of the caspases Ice, Cpp32, and Ich-1 in human fibroblasts. As a consequence, STAT1-null cells are resistant to apoptosis by tumor necrosis factor α (TNF-α). Reintroduction of STAT1α restored both TNF-α–induced apoptosis and the expression of Ice, Cpp32, and Ich-1. Variant STAT1 proteins carrying point mutations that inactivate domains required for STAT dimer formation nevertheless restored protease expression and sensitivity to apoptosis, indicating that the functions of STAT1 required for these activities are different from those that mediate induced gene expression.

The binding of TNF-α to its high-affinity receptor results in new gene expression and apoptosis in various cell types (*1*). We investigated whether Janus protein kinases (JAKs) or STATs, which were discovered through their roles in interferon (IFN) signaling pathways (*2*), participate in TNF-α–induced apoptosis. Treatment of cells with IFN-γ causes activation of JAK1 and JAK2, leading to activation of STAT1 by phosphorylation of Tyr^{701}. STAT1 dimerizes through reciprocal interactions of phosphotyrosine 701 and an Src homology 2 (SH2) domain and activates genes containing GAS (gamma-activated sequence) elements. Interferon α or β (IFN-α or -β) activates the tyrosine kinases JAK1 and TYK2, leading to the phosphorylation on tyrosine of STAT1 and STAT2, which allows them to heterodimerize. The heterodimer, together with another protein, p48, forms the complex transcription factor ISGF3, which regulates genes containing interferon-stimulated response elements (ISREs) (*3*, *4*).

Mutant human fibroblast cell lines have been isolated that lack a single JAK, STAT, or other component of IFN signaling pathways (*2*). We treated cell lines representing seven complementation groups with TNF-α and actinomycin D (Table 1). Actinomycin D greatly enhances the induction of apoptosis by TNF-α in many cell types, including fibroblasts (*5*). To quantify the response, we measured exclusion of the dye trypan blue by living cells (*6*). After 18 hours of treatment with TNF-α and actinomycin D, U3A cells were resistant to apoptosis, whereas the 2fTGH parental cells and all the other mutant cell lines examined were sensitive (Table 1). Similar results were obtained with U3X (Table 1), an independent STAT1-deficient clone (*7*). Differential splicing leads to two different forms of STAT1, α (p91) and β (p84). The latter lacks 38 COOH-terminal residues (*8*). In U3A-R cells, the expression of STAT1α has been restored by stable transfection with an expression vector (*9*). Protein immunoblot analysis indicates that U3A-R and parental 2fTGH cells express similar amounts of STAT1α (Fig. 1B). The U3A-R and 2fTGH cells respond similarly to the TNF-α and actinomycin D. This induced cell death depends on STAT1α (Table 2). Sensitive cells killed by TNF-α and actinomycin D had the hallmarks of apoptosis (*10*), including membrane blebbing and cellular fragmentation (*7*). 2fTGH cells treated with TNF-α and actinomycin D yielded a distinctive ladder of genomic DNA fragments (*11*), which was not seen in U3A cells and was restored in U3A-R cells (Fig. 2).

Table 1. Cell viability after treatment with TNF-α and actinomycin D. Cell lines lacking specific IFN signaling components were treated (*6*) with Human TNF-α and actinomycin D for 18 hours and assayed by exclusion of trypan blue.

Cell line	Missing protein	Viable cells (%)
2fTGH	None	8
U1A	TYK2	9
U2A	p48	15
U3A	STAT1	82
U3X	STAT1	84
U4A	JAK1	8
U5A	IFNAR2	2
U6A	STAT2	1
2C4	None	12
γ2A	JAK2	5

To understand why apoptosis depends on STAT1, we examined the expression of Ice family (caspase) and Fas genes, known to mediate apoptosis (*12*). Engagement of the receptor Fas with Fas ligand or anti-Fas initiates apoptosis, which can be inhibited by antisense RNA to Ice family proteases or Ice protease inhibitors such as CrmA (*12*). We examined Ice family mRNA levels (Fig. 3) using the reverse transcriptase–polymerase chain reaction (RT-PCR) in 2fTGH, U3A, U3A–IRF-1, and U3A-R cells, using specific primers (*13*). The amount of Ice mRNA was low in U3A cells (Fig. 3A), as were the amounts of Cpp32 and Ich-1 mRNAs (Fig. 3B), but Ice/rel2, Mch2α, and Mch3 mRNAs were expressed in comparable amounts in 2fTGH and U3A cells (Fig. 3B). Reintroduction of STAT1α into U3A cells restored Ice mRNA expression (Fig. 3C). The amount of Fas mRNA was the same in parental 2fTGH and U3A cells (Fig. 3, A and C). Amounts of glyceralde-

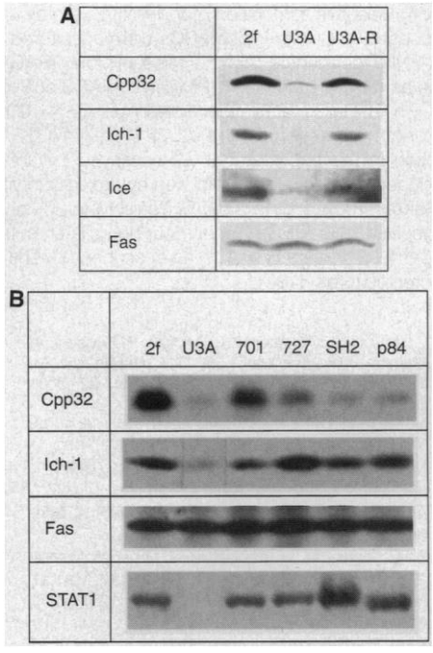

Fig. 1. Expression of Ice family proteases in 2fTGH, U3A, U3A-R, and U3A cells expressing STAT1 variant proteins. (**A**) Protein immunoblot analysis of Ich-1, Cpp32, Ice, and Fas in whole-cell extracts (100 μg) from 2fTGH (2f), U3A, and U3A-R cells. (**B**) Protein immunoblot analysis of Ich-1, Cpp32, Fas, and STAT1 in whole-cell extracts (100 μg) from 2fTGH (2f), U3A, U3A-701 (701), U3A-727 (727), U3A-SH2 (SH2), and U3A-p84 (p84) cells.

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hyde-3-phosphate dehydrogenase mRNA were similar in all the cells examined (Fig. 3, A and C). Because the transcription factor IRF-1 can mediate the apoptosis induced by DNA damage in mouse lymphocytes and can induce expression of the Ice gene (*14*) and because expression of IRF-1 is deficient in U3A cells (9), we studied U3A–IRF-1 cells, a stable transfectant of U3A in which IRF-1 expression is comparable to that of 2fTGH cells (*15*). The resistance to apoptosis of U3A–IRF-1 cells and U3A cells was comparable after treatment with TNF-α and actinomycin D (Tables 1 and 2), and the expression of Ice mRNA was not restored in U3A–IRF-1 cells (Fig. 3A).

We analyzed the levels of Ice family proteins in 2fTGH, U3A, and U3A-R cells (*16*). The expression of Ice, Cpp32, and Ich-1 in U3A cells was one-tenth to one-fifteenth that of 2fTGH cells (Fig. 1). In U3A-R cells, the expression of these three proteins was restored (Fig. 1A). Fas protein expression was comparable in all the cells tested (Fig. 1). Thus, STAT1α is apparently required to mediate the expression of these Ice family members. We observed no increase in expression of the proteins Cpp32, Ich-1, Bcl2, or Bcl-x (*17*) in 2fTGH cells treated with TNF-α alone, IFN-α, or IFN-γ (*15*).

A crucial question is whether STAT1 homodimer is the transcription factor required for efficient constitutive expression of Ice family genes or whether STAT1 monomers can perform this function, either acting alone or in concert with other as yet unknown proteins. Formation of STAT1 dimers requires the phosphorylation of Tyr^{701}, leading to reciprocal phosphotyrosine-SH2 domain interactions between the monomeric units (*18–20*). STAT1 can be activated in response to some growth factors (*2*), and it is possible that a small amount of phosphorylated STAT1, because of exposure to growth factors, might be sufficient to drive efficient expression of Ice family genes. Therefore, we investigated STAT1 variants in which either Tyr^{701} or the SH2 domain were mutated: $Tyr^{701} \rightarrow Phe^{701}$ (*21*) and $Arg^{602} \rightarrow Leu^{602}$ (*18*). Like 2fTGH parental cells, both U3A-701 and U3A-SH2 cells were sensitive to apoptosis induced by TNF-α and actinomycin D (Table 2 and Fig. 2). The expression of Ich-1 and Cpp32 proteins was reduced only slightly in U3A-701 cells compared with 2fTGH cells (Fig. 1B). Thus, in every assay, U3A-701 cells and 2fTGH cells were similar (*22*). U3A-SH2 and 2fTGH cells expressed similar amounts of Ich-1, but the expression of Cpp32 was much lower in U3A-SH2 cells (Fig. 1B). Therefore, the SH2 domain is required for STAT1-mediated expression of at least one gene, but dimerization of STAT1 apparently is not required for constitutive expression of Ice family proteases in these cells.

Ser^{727} in STAT1α is required for full activation of STAT1α by IFN-γ. The $Ser^{727} \rightarrow Ala^{727}$ (S727A) mutant protein is phosphorylated normally on tyrosine, and dimerizes and binds to GAS elements, but IFN-γ–dependent transcription is reduced by as much as 80% (*23*). U3A-727 cells were resistant to apoptosis induced by TNF-α and actinomycin D (Table 2) and displayed only weak DNA laddering (Fig. 2). In these cells, the amount of Ich-1 was similar to that in 2fTGH cells, but the amount of Cpp32 was one-third to one-fourth that in 2fTGH cells (Fig. 1B). Although the expression of Ich-1 and Cpp32 was similar in U3A-727 and U3A-SH2 cells (Fig. 1B), the phenotypes were different (Table 2 and Fig. 2), suggesting that one or more genes that were not investigated may be poorly expressed in U3A-727 cells. Thus, the Ser^{727} residue has an important role in the STAT1-dependent constitutive expression of some genes. STAT1α can restore IFN-γ–dependent signaling to STAT1-null cells, but the truncated STAT1β [p84, lacking the COOH-terminal 38 residues, including Ser^{727} (*8*)] cannot (9). U3A-p84 cells were sensitive to apoptosis induced by TNF-α and actinomycin

Table 2. Same as Table 1, except that U3A cells expressing the indicated STAT variant were used. STAT1-WT indicates the wild-type STAT1.

Cell line	Missing protein	Viable cells (%)
U3A–IRF-1	STAT1	80
U3A-R	None	6
U3A-701	STAT1-WT	18
U3A-727	STAT1-WT	68
U3A-SH2	STAT1-WT	15
U3A-p84	STAT1α	21

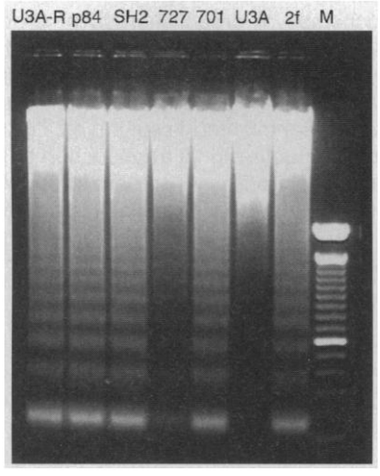

Fig. 2. Analysis of genomic DNA after treatment of cells with TNF-α and actinomycin D. 2fTGH (2f), U3A, and complemented U3A cells were treated with human TNF-α and actinomycin D (6) for 12 hours. Genomic DNA was analyzed in an agarose gel (1.0%) (*11*). M is a 100-bp DNA marker.

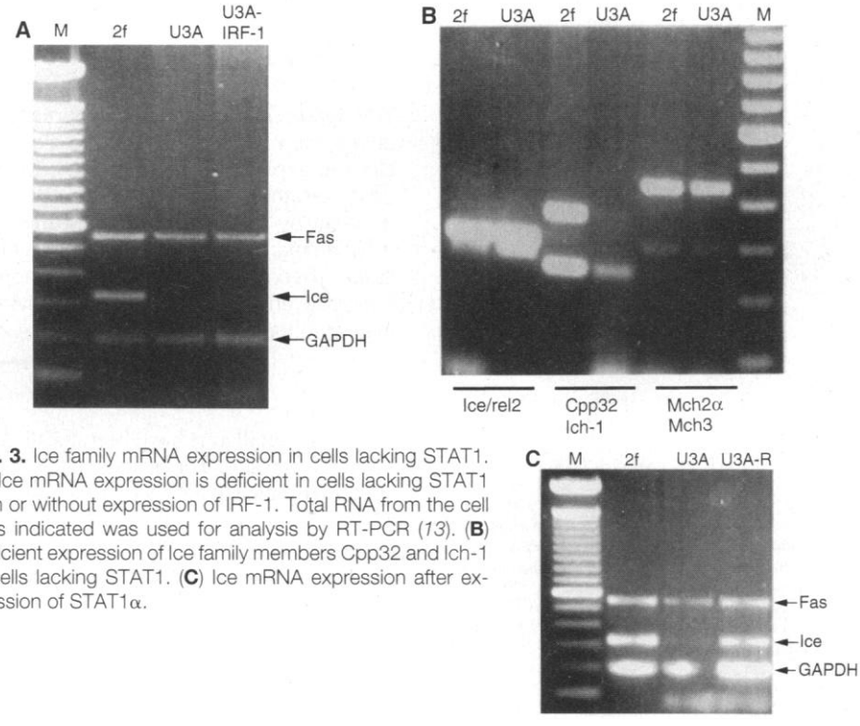

Fig. 3. Ice family mRNA expression in cells lacking STAT1. (**A**) Ice mRNA expression is deficient in cells lacking STAT1 with or without expression of IRF-1. Total RNA from the cell lines indicated was used for analysis by RT-PCR (*13*). (**B**) Deficient expression of Ice family members Cpp32 and Ich-1 in cells lacking STAT1. (**C**) Ice mRNA expression after expression of STAT1α.

D (Table 2 and Fig. 2). The expression of Ich-1 in those cells was comparable to that in 2fTGH cells, but the amount of Cpp32 was reduced (Fig. 1B). Therefore, p84 and S727A have different apoptotic profiles. We conclude that the constitutive expression of Ich-1 and Cpp32 does not require formation of STAT1 homodimers. However, STAT1 might form complex transcription factors by interacting with other proteins (*24*).

STAT1-null mice are defective in all responses to IFN-α or IFN-γ (*25*, *26*), as expected from the properties of STAT1-null human cells (*27*). However, in contrast to Cpp32-null mice (*28*), STAT1-null mice show no gross developmental abnormalities (*25*, *26*). If, like human cells, mouse cells require STAT1 for efficient expression of Cpp32, the reduced levels of this protease in STAT1-null mouse cells must still be sufficient to support the apoptosis required for nearly normal development. Apoptosis of underlying keratocytes that follows the ablation of corneal epithelial cells is grossly defective in STAT1-null mice (*29*), revealing that at least one form of stress-induced apoptosis is defective in the absence of STAT1.

STAT1 is also required for constitutive expression of LMP2 and LMP7 (low molecular mass polypeptides 2 and 7) (*30*), in addition to Ice, Ich-1, Cpp32, and IRF-1. The finding that STAT1 is required for efficient constitutive expression of several genes reveals a more general role for this ubiquitous transcription factor.

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13. Total RNA was extracted from 4×10^6 cells with TRIzol (Gibco-BRL) according to the manufacturer's directions. First-strand cDNA synthesis was performed, using 2 μg of each RNA sample primed with oligo(dT) in a 30-μl reaction volume with 400 U of Moloney murine leukemia virus reverse transcriptase (Promega). All PCRs were performed on a portion (2 μl) of each cDNA mixture in a 50-μl reaction volume containing 25 pmol of each upstream and downstream primer, 2.5 U of Taq DNA polymerase (Boehringer Mannheim), 0.125 mM of each deoxynucleoside triphosphate, 2.0 mM $MgCl_2$, and 1× reaction buffer (Boehringer Mannheim). The Fas primer pair, 5′-CCCAAATAGGAGTGTATGCAGAGG-3′ and 5′-GCCATTAAGATGAGCACCAAGG-3′, generated a 547–base pair (bp) product. The Ice primer pair, 5′-TTGCTCCCTAGAAGAAGCTCAAAG-3′ and 5′-GCCTTCCCGAATACCATGAGAC-3′, generated a 321-bp product. The Cpp32 primer pair, 5′-TGGAACAAATGGACCTGTTGACC-3′ and 5′-AGGACTCAAATTCTGTTGCCACC-3′, generated a 365-bp product. The Ice/rel2 primer pair, 5′-CGCTGAGGGCATTTGCTACC-3′ and 5′-CACTTCCAAGGATGCTGGAGAG-3′, generated a 272-bp product. The Ich-1 primer pair, 5′-TCCAGCTCCAAGAGGTTTTTCAG-3′ and 5′-GTCCCTTTGAGGCAGGCATAG-3′, generated a 250-bp product. The Mch2α primer pair, 5′-CTGTTAGCCACGCAGATGCC-3′ and 5′-TGTTCACCAGTGTGAGGAGTTCTG-3′, generated a 447-bp product. The Mch3 primer pair, 5′-GCTGAGAAGCAATGGGTCACTC-3′ and 5′-TGCAGTGGACACAGCCATGAG-3′, generated a 300-bp product. The glyceraldehyde phosphate dehydrogenase (GAPDH) primer pair, 5′-CCATGGAGAAGGCTGGGGC-3′ and 5′-CCAAAGTTGTCATGGATGTCC-3′, generated a 195-bp product. All products were analyzed by 1.0% agarose gel electrophoresis.
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Independent Photoreceptive Circadian Clocks Throughout *Drosophila*

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Transgenic *Drosophila* that expressed either luciferase or green fluorescent protein driven from the promoter of the clock gene *period* were used to monitor the circadian clock in explanted head, thorax, and abdominal tissues. The tissues (including sensory bristles in the leg and wing) showed rhythmic bioluminescence, and the rhythms could be reset by light. The photoreceptive properties of the explanted tissues indicate that unidentified photoreceptors are likely to contribute to photic signal transduction to the clock. These results show that autonomous circadian oscillators are present throughout the body, and they suggest that individual cells in *Drosophila* are capable of supporting their own independent clocks.

Circadian oscillators have been localized in several organisms. For example, the suprachiasmatic nucleus (SCN) is important for mammalian rhythms (*1*), whereas *Iguana iguana* has at least three independent oscillators: the retina, parietal eye, and pineal gland (*2*). Sparrows show activity rhythms that can be altered by lesioning the pineal gland (*3*); this operation reveals the influence of other oscillators on the bird's behavior. The brain controls behavioral rhythms in moth (*4*) and *Drosophila* (*5*, *6*), whereas sperm release in the moth is controlled by an independent oscillator (*7*). Recently, free-

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