The C-terminal activation domain of the STAT-1 transcription factor is necessary and sufficient for stress-induced apoptosis

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Abstract

It has previously been demonstrated that the STAT-1 transcription factor plays a key role in apoptosis induced by the cellular regulatory factors interferon γ and TNF- α . Here we demonstrate that cells lacking STAT-1 show reduced cell death/apoptosis in response to stressful stimuli such as heat or ischaemia. Expression of STAT-1 in these cells does not enhance basal cell death but restores sensitivity to stress-induced death whereas this effect is not observed upon over-expression of STAT-3. Enhanced sensitivity to stress-induced cell death requires the C-terminal activation domain of STAT-1 and the phosphorylation sites at tyrosine 701 and serine 727. Moreover, we show for the first time in any system that the isolated C-terminal domain of STAT-1 is able to enhance stress-induced cell death in the absence of the DNA binding domain or any other region of STAT-1. Hence, STAT-1 plays a key role in stress-induced cell death, potentially acting via a novel co-activator-type mechanism and represents a possible therapeutic target for strategies aimed at minimising cell death, for example, following ischaemic injury.

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Abbreviations: DMEM, Dulbecco's modified eagle medium; DMSO, dimethyl sulphoxide; FBS, foetal bovine serum; JAK, janus activated kinase; MAPK, mitogen activated protein kinase; PBS, phosphate buffered saline; STAT, signal transducers and activators of transcription; TNF- α , tumour necrosis factor- α

Introduction

The STAT (Signal Transducer and Activator of Transcription) family of transcription factors contains six members which are known as STAT-1 to STAT-6 (for reviews see^{1–4}). All have a high degree of conservation of their structural domains which

include, a DNA binding domain, a dimerisation domain and a C-terminal transcriptional activation domain which contains conserved tyrosine and serine residues allowing phosphorylation by respectively JAKs and MAPKs.⁵

The different STAT factors play key roles in mediating the transcriptional response to specific interferons and cytokines. Thus, for example, treatment with interferon γ results in the phosphorylation of STAT-1 on tyrosine residue 701 and serine residue 727 leading to homodimerisation, DNA binding and transcriptional activation of its target genes.^{1–4} In contrast, STAT-3 is activated by specific cytokines of the IL-6 family which use the gp130 receptor subunit. Interestingly, activation of STAT-3 has been linked to enhanced cell proliferation⁶ and over-expressed constitutively active STAT-3 has been shown to act as an oncogene able to transform cells either alone or in co-operation with other oncogenes.^{6,7}

In contrast to this role for STAT-3 in cellular proliferation, STAT-1 appears to have a role in the induction of programmed cell death (apoptosis). Thus, interferon γ treatment induces apoptosis and activation of the proteolytic enzyme caspase-1 in several different cell lines.⁸ Moreover, both apoptosis and caspase activation in response to interferon γ are abolished in cells lacking either functional STAT-1 or the Jak1 kinase necessary for its activation.⁸ Similarly, STAT-1 negative cells show reduced caspase expression and TNF- α -induced apoptosis compared to closely matched cells expressing functional STAT-1.⁹

These findings indicate therefore that activation of STAT-1 plays a key role in inducing apoptosis in response to regulatory factors such as interferon γ or TNF- α . To investigate the role of STAT-1 in stress-induced apoptosis we have recently investigated its role in ischaemia/reoxygenation injury in cardiac cells. We have shown that STAT-1 is expressed at enhanced levels following ischaemia/reoxygenation and is phosphorylated on tyrosine 701 and serine 727.^{10,11} Moreover, inhibition of STAT-1 expression using an anti-sense approach protects the cardiac cells from apoptosis induced by ischaemia/reoxygenation.¹⁰

To probe further the features of STAT-1 required for stress-induced apoptosis we have used the U3A cell line which lacks functional STAT-1.¹² We show that these cells show a lower rate of cell death than the parental 2FTGH cells. Moreover, by transfecting the U3A cells with expression vectors encoding different forms of STAT-1 we show that the isolated C-terminal activation domain of STAT-1 is necessary and sufficient for stress-induced apoptosis and can produce this effect in the absence of the DNA binding domain.

Results and Discussion

To compare the effect of stress on U3A cells lacking STAT-1 and the parental 2fTGH cells expressing STAT-1, the cells were exposed to ischaemia for various periods and cell death measured on the basis of the ability of live cells to exclude trypan blue. As shown in Figure 1a, 2fTGH cells consistently showed greater cell death than the U3A cells at each time point and the difference between the two cell types became greater, the greater the period of the stressful stimulus.

In order to confirm that this difference between the two cell lines was not specific to ischaemia, we also exposed the cells to heat shock. As shown in Figure 1b, heat stress also produced more cell death in the parental 2fTGH cells compared to the U3A cells, with this effect again being more marked with severe heat stress at 44°C.

As trypan blue exclusion measures total cell death, we next investigated whether the cells expressing STAT-1 exhibited enhanced apoptosis (programmed cell death) when exposed to ischaemia or elevated temperature. In these experiments, apoptosis was assayed by measuring two distinct features of this process, using TUNEL staining

to measure DNA cleavage and annexin V surface staining to measure translocation of phosphatidyl serine to the external surface of the plasma membrane. In these experiments (Figure 2), the 2fTGH cells expressing STAT-1 clearly showed enhanced apoptosis in response to ischaemia or heat shock compared to the U3A cells as assayed by both measures of apoptosis.

These findings therefore demonstrate that U3A cells lacking STAT-1 do show enhanced resistance to apoptosis induced by stressful stimuli compared to the parental 2fTGH cells expressing STAT-1. Although the U3A cells are derived from 2fTGH¹² it is possible that other differences between the cell lines may have occurred during the course of their derivation and/or subsequent culture. To confirm that the effects we observe were due to STAT-1, the U3A cells were therefore transfected with either a STAT-1 expression vector or the corresponding vector lacking any insert and exposed to heat shock or ischaemia as before. In all cases, cells were co-transfected with an expression vector encoding β -galactosidase to allow successfully transfected cells to be identified. Apoptotic cell death was quantitated by TUNEL staining since other apoptosis assays such as annexin V staining or the trypan blue assay cannot be used together with β -galactosidase staining. Values are therefore expressed as the percentage of total β -galactosidase positive cells which are also positive for TUNEL. We have previously used this method to successfully quantify the effect of specific transfected plasmids on apoptosis in a variety of cell types.^{10,11,13,14}

In these experiments (Figure 3), transfection of STAT-1 into U3A cells had only a minimal effect on the extent of apoptosis observed in the absence of a stressful stimulus. However, a clear enhancement of apoptosis was observed in the U3A cells transfected with STAT-1 and then exposed to heat or ischaemia with approximately fourfold more apoptotic transfected cells being observed in each case

Figure 1 Cell death in parental 2fTGH cells (expressing STAT-1) or their U3A derivative (lacking STAT-1) following exposure to either simulated ischaemia for the period indicated (a) or to heat shock for 2 h at 43°C (b). Values indicate the percentage of dead cells unable to exclude trypan blue and are the mean of six determinations whose standard error is shown by the bars. * $P < 0.05$ U3A versus 2fTGH

Figure 2 Apoptotic cell death in 2fTGH cells or their U3A derivative following exposure to heat shock or ischaemia as assayed by TUNEL or annexin V staining. Values are the mean of six determinations whose standard error is shown by the bars. * $P < 0.05$ U3A versus 2fTGH

compared to the cells transfected with expression vector alone.

In the next series of experiments, we tested whether this effect of STAT-1 could be reproduced with the related protein STAT-3 or with mutant or truncated derivatives of STAT-1. In our previous experiments, these factors have been shown to be expressed at similar levels in the transfected cells,^{10,11} and this was confirmed in our current experiments (see for example, Figure 4). Initially, we tested the effect of transfecting a STAT-3 expression vector. In these experiments (Figure 5) STAT-3 produced a small enhancement of apoptotic cell death following exposure to heat or ischaemia which was much smaller than that observed with STAT-1 and was not statistically significant. Moreover, co-transfection of STAT-1 and STAT-3 abolished the enhanced apoptosis induced by STAT-1. This suggests that STAT-3 interferes with the action of STAT-1, possibly

Figure 3 Apoptotic cell death in U3A cells transfected with expression vector lacking any insert or with STAT-1 expression vector and then either left untreated or exposed to simulated ischaemia for 4 h or heat shock at 43°C for 2 h. Values indicate the percentage of successfully transfected (β -galactosidase positive) cells which are positive in the TUNEL assay of apoptosis and are the mean of six determinations whose standard error is shown by the bars. * $P < 0.05$ versus control U3A cells

Figure 4 Western blot with an antibody to STAT-1 using extracts of U3A cells transfected with wild-type STAT-1 (track 1), the tyrosine 701 to phenylalanine mutant (track 2) and the 390–750 C-terminal construct (track 3)

Figure 5 Apoptotic cell death in U3A cells transfected with empty expression or expression vectors encoding STAT-1 or STAT-3, singly or in combination and then either left untreated or exposed to heat shock at 43°C for 2 h or simulated ischaemia for 4 h. Values indicate the percentage of successfully transfected cells which are TUNEL positive and are the mean of six determinations whose standard error is shown by the bars. * $P < 0.05$ versus empty expression vector transfected cells

by forming a STAT-1/STAT-3 heterodimer which is unable to induce enhanced cell death or by competing with STAT-1 for binding to a cellular target required for death induction.

The difference in the effect of STAT-1 and STAT-3 allowed us to use chimaeric constructs containing different regions from STAT-1 or STAT-3 (see Figure 6 and reference¹⁵) in order to probe the region(s) of STAT-1 required for its effect on apoptosis. In these experiments (Figure 7) exchange of the DNA binding domain between STAT-1 and STAT-3 had no effect, with a STAT-1 protein containing the central DNA binding domain still inducing enhanced apoptosis (construct ^{1,3}297–514³) whereas only a small increase in apoptosis which was not statistically significant was observed with the reciprocal STAT-3 construct in which the DNA binding domain has been replaced by that of STAT-1 (construct ^{3,1}293–508³). Moreover, a construct containing amino acids 1–293 of STAT-3 linked to amino acids 293–750 (C-terminus) of STAT-1 (construct ³296¹) induced enhanced cell death indicating that the N-terminus of STAT-1 is not essential for this effect (Figure 7).

This was confirmed by demonstrating that a construct encoding only the DNA binding domain and C-terminus of STAT-1 (amino acids 390–750) (see Figure 6) was able to induce enhanced cell death, although to a lesser extent than wild-type STAT-1. Hence, the effect observed with the chimaeric construct is not due to the N-terminal domain of STAT-3 substituting for that of STAT-1. Rather, enhanced apoptosis can still be observed in the absence of the N-terminal domain. In contrast, a construct containing a stop codon at position 695 of STAT-1 and therefore encoding a

Figure 6 Structure of the STAT-1/STAT-3 chimaeric constructs and the other STAT-1 constructs used in this study

truncated protein was unable to induce enhanced apoptosis (Figure 8). Hence, the C-terminal domain of STAT-1 downstream of amino acid 695 is essential for the effects we observe.

To determine whether the isolated C-terminal domain without the DNA binding domain is not only necessary but also essential for enhanced stress-induced apoptosis, we prepared a construct expressing only amino acids 691–750 of STAT-1 (Figure 6). When this construct was used in our experiments (Figure 7), it was able to induce similar (ischaemia) or enhanced (heat shock) cell death compared to that observed with full-length STAT-1. Hence, the C-terminal domain of STAT-1 is not only necessary for enhanced stress-induced apoptosis but is also sufficient for this effect, producing it in the absence of the adjacent

Figure 7 Apoptotic cell death in U3A cells transfected with expression vectors encoding STAT-1, STAT-3, STAT-1/STAT-3 chimaeras or constructs encoding amino acids 390–750 or 691–750 of STAT-1 (see Figure 6) and then exposed to heat shock at 43°C for 2 h or to simulated ischaemia for 4 h. Values indicate the percentage of successfully transfected cells which are TUNEL positive and are the mean of six determinations whose standard error is shown by the bars. **P* < 0.05 versus empty expression vector transfected cells

Figure 8 Apoptotic cell death in U3A cells transfected with expression vectors encoding full-length STAT-1, a construct encoding amino acids 1-694 of STAT-1 with a stop codon at position 695 or two constructs (D694A or D694E) in which amino acid 694 has been mutated to a residue which prevents caspase-mediated cleavage (see Figure 6). Transfected cells were either left untreated or exposed to heat shock at 43°C for 2 h or to simulated ischaemia for 4 h. Values indicate the percentage of successfully transfected cells which are TUNEL positive and are the mean of six determinations whose standard error is shown by the bars. **P* < 0.05 versus empty expression vector transfected cells

DNA binding domain or other regions of STAT-1. Although the C-terminal domain has previously been shown to be involved in STAT-1 induced cell death,^{9,11} this is to our knowledge the first demonstration that the isolated domain can produce this effect in any system.

Interestingly, it has been shown that STAT-1 can be cleaved by caspases such as caspase-3 at position 694.¹⁶ To determine whether such cleavage to release an active C-terminal fragment was essential for stress-induced apoptosis, we used two constructs in which the aspartic acid residue at position 694 has been replaced by either alanine or glutamic acid residues rendering the protein non-cleavable. As illustrated in Figure 8, both these constructs were still able to induce enhanced cell death, although to a lesser extent than the wild-type. Cleavage of STAT-1 to release a free C-terminal fragment is therefore not essential for the effects we observe. Hence, the C-terminal domain can produce its effects either within the intact molecule or when present as an isolated domain produced artificially or naturally by caspase cleavage.

Interestingly, this C-terminal region has been shown to act as a transcriptional activation domain which can interact with transcriptional regulators such as CBP,¹⁷ BRCA-1¹⁸ and the chromatin remodelling factor MCM5.¹⁹ It is probable therefore that the C-terminal domain interacts with DNA-bound transcription factors and is therefore recruited to the DNA independent of the STAT-1 DNA binding domain. This co-activator role for STAT-1 would allow the C-terminal domain to enhance cell death by activating specific target genes when present either alone or linked to the remainder of the STAT-1 molecule. Interestingly, the ability of STAT-3 to prevent death induction by STAT-1 (Figure 5) suggests that STAT-3 may also bind to the same DNA-bound factor as STAT-1 but that such binding does not induce enhanced cell death, as observed with STAT-1.

The C-terminal region contains two phosphorylation sites, tyrosine 701 which is phosphorylated by JAK family kinases and serine 727 which is phosphorylated by MAP kinases. Phosphorylation on tyrosine 701 has been shown to be essential for specific aspects of the interferon γ response which also involves serine 727 phosphorylation. Recent data have indicated however, that some STAT-1 dependent genes are still activated when tyrosine 701 is mutated to a non-phosphorylatable phenylalanine residue.²⁰ In contrast, phosphorylation on serine 727 is essential for the interaction of STAT-1 and BRCA-1 or MCM5.^{18,19} Moreover, phosphorylation on serine 727 is not coupled to prior phosphorylation on tyrosine 701 following stress, although this is the case following interferon- γ treatment.²¹

We therefore tested the effect of STAT-1 mutants in which either tyrosine 701 or serine 727 have been altered to non-phosphorylatable residues (phenylalanine and alanine respectively). In these experiments (Figure 9), both of the mutants were impaired in their ability to induce apoptosis following exposure of U3A cells to thermal or ischaemic stress.

This suggests that in our system, phosphorylation on both tyrosine 701 and serine 727 is necessary for enhanced apoptotic cell death following stress. This is in contrast to the situation in U3A cells exposed to TNF- α where the tyrosine 701 mutant is able to induce enhanced apoptosis whereas the serine 727 mutant cannot.⁹ Similarly, we observed enhanced apoptosis in normal cardiac cells exposed to ischaemia/reoxygenation following

Figure 9 Apoptotic cell death in U3A cells transfected with expression vectors encoding wild-type STAT-1 or STAT-1 701 and 727 mutants, and then exposed to heat shock at 43°C or simulated ischaemia for 4 h. Values indicate the percentage of successfully transfected cells which are TUNEL positive and are the mean of six determinations whose standard error is shown by the bars. * $P < 0.05$ versus empty expression vector transfected cells

transfection of wild type STAT-1 or the tyrosine 701 mutant but not following transfection of the serine 727 mutant.¹¹ This difference may reflect the use of TNF- α rather than thermal or ischaemic stress in the experiments of Kumar *et al.*⁹ and the use of a different cell type expressing endogenous STAT-1 in our experiments.¹¹ It will evidently be of particular interest to identify the transcription factor(s) with which the C-terminal domain of STAT-1 interacts to achieve its potential co-activator effect in enhancing apoptosis and to determine the dependence of this interaction on phosphorylation at tyrosine 701 and serine 727.

Whatever the case, our data show that the presence of STAT-1 results in enhanced sensitivity upon exposure to thermal or ischaemic stress compared to cells where it is absent. Moreover, we demonstrate for the first time that the C-terminal activation domain of STAT-1 is both necessary and sufficient for this effect.

Materials and Methods

DNA constructs

The STAT-1 expression vector was kindly provided by Dr. C Horvath. The STAT-3 expression vector was kindly provided by Dr. S Akira (Hyogo College of Medicine, Japan). STAT-1/STAT-3 chimeric constructs were a kind gift of Dr. C Horvath.¹⁵ The STAT-1 tyrosine-phenylalanine 701 and serine-alanine 727 mutants were kind gifts of Dr. C Horvath (Mount Sinai Medical Centre, New York, USA). Mutants at position 694/695 (D/A and D/E) of STAT-1 were a kind gift of Dr. S Goodbourn.¹⁶ Primers 5'-GTCATCCGACATGGAAGAATCCAC-CAACGG-3' and 5'-GTCACCTCGAGGACCGTGTGTATCTGCGCT-3' were used to construct an expression vector for amino acids 390–750 of STAT-1. A C-terminal STAT-1 construct consisting of sequence 691–750 was constructed using the following primers: 5'-GGATC-CATGGAACCTTGATGGCCC-3' and 5'-GCGGCCGCTACTGTGTT-CATCATACTGTC-3'.

Cell culture

2FTGH and U3A human fibrosarcoma cell lines were obtained from the Imperial Cancer Research Fund (ICRF, London, UK). They were maintained in DMEM supplemented with 10% FBS and 100 units/ml penicillin and streptomycin (all Life Technologies, UK) in an incubator at 37°C, 5% CO₂, 95% humidity until 70–80% confluent and then plated in 6-well or 24-well (Falcon, USA) to a density of 10⁶ cells per ml.

Transient transfection

All transfections were performed by the calcium phosphate method. 2.5 µg of STAT expression vector was co-transfected into cells with pCMVβ-galactosidase reporter vector (1.5 µg/well). DNA precipitates were left on cells for 16 h.

Stress treatment

Media were replaced with either 1 ml DMEM supplemented with 1% FBS for heat shock treatment or ischaemic buffer (137 mM NaCl, 12 mM KCl, 0.49 mM MgCl₂, 0.9 mM CaCl₂·2H₂O, 4 mM HEPES, 10 mM deoxyglucose, and 20 mM sodium lactate pH 6.7).

For a heat shock treatment, cells were placed in a water bath for 2 h at 43°C. For ischaemic treatments, cells were placed in an ischaemic chamber for 4 h in an atmosphere of 0% oxygen, 5% CO₂, 95% argon (BOC).

Trypan blue exclusion assay

Cells were washed in PBS and trypsinised for 1 min in 0.25 µ/ml trypsin in versene (Gibco, Life Technologies, UK). The cell suspension was centrifuged for 5 min at 1000 r.p.m., and the supernatant aspirated. The cell pellet was resuspended in 100 µl PBS and stored on ice. Cell counts were made, using a haemocytometer, by addition of 0.4% trypan blue (Sigma) to equal volume of cell suspension. The percentage of blue cells/total cells was counted by scoring 100 cells per well three times.

X-gal staining

After stress treatments, cells were washed with PBS and fixed with 1 ml 0.5% glutaraldehyde in PBS for 10 min. The fixative was removed and cells were washed twice with PBS. X-gal stain (12 mM MgCl₂, 200 mM potassium ferrocyanide, 200 mM potassium ferricyanide, and 0.4 mg/ml 5-Bromo-4-Chloro-3-tridoyl-β-D-galactopyranoside in dimethyl sulfoxide (DMSO, Sigma)) was added to each well (1 ml) and incubated for 16 h at 37°C. After washing twice in PBS, cells were ready to be assessed for apoptosis.

Assessment of apoptosis by TUNEL

Cells were stained with Terminal deoxynucleotidyl transferase (3'-OH labelling of DNA) and 2 mM Fluorescein-12-2'-deoxy-uridine-5'-triphosphate (Roche, Germany) and incubated for 1 h 30 min in a humidified incubator at 37°C in the dark. Cells were washed with PBS and subsequently viewed under a phase contrast microscope (Zeiss) for the total number β-galactosidase (β-gal) positive cells and fluorescent microscopy for β-gal and TUNEL positive cells. The percentage of apoptotic cells was expressed as the percentage of total β-gal positive cells which were TUNEL positive.

Assessment of apoptosis by Annexin V

To assess early apoptosis and the changes that occur on the cell surface, Annexin-V-Fluos (Roche, Germany) staining was applied

after heat shock or ischaemic stress. Incubation buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 5 mM CaCl₂) was prepared. Annexin-V-Fluos-labelling solution was prepared by pre-diluting 20 µl of Annexin-V-Fluos labelling reagent in 1000 µl of incubation buffer and 20 µl of propidium iodide (PI) (50 µg/ml stock). PI was included to differentiate necrotic cells from apoptotic cells. A labelling solution of 200 µl was added per well and incubated for 15 min in dark at room temperature. Annexin-V binding cells were observed under fluorescent light (Zeiss). Cells from three separate fields of view from the same well were counted and apoptotic cells were expressed as a percentage of total cells.

Western blotting

Transfected cells were harvested, and protein concentrations were determined by the Bradford protein assay.²² Extracts were run on a 10% acrylamide gel and transferred to Hybond C nitrocellulose membrane (Amersham Biosciences, UK). The membrane was probed with an antibody to STAT-1 (epitope mapped at the carboxy terminus of STAT-1α p91) (Santa Cruz Biotechnologies). Bands were visualised by chemiluminescence (Amersham Biosciences, UK) and exposure to photographic film.

Statistics

Values were expressed as the mean±the standard error of *n* experiments. Statistical analysis was performed by the two-tailed Student's *t*-test for unpaired data, with *P* values <0.05 considered statistically significant.

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