

Role of the JNK/c-Jun/AP-1 signaling pathway in galectin-1-induced T-cell death

B Brandt¹, EF Abou-Eladab², M Tiedge¹ and H Walzel^{1*}

Galectin-1 (gal-1), an endogenous β -galactoside-binding protein, triggers T-cell death through several mechanisms including the death receptor and the mitochondrial apoptotic pathway. In this study we first show that gal-1 initiates the activation of c-Jun N-terminal kinase (JNK), mitogen-activated protein kinase kinase 4 (MKK4), and MKK7 as upstream JNK activators in Jurkat T cells. Inhibition of JNK activation with sphingomyelinase inhibitors (20 μ M desipramine, 20 μ M imipramine), with the protein kinase C- δ (PKC δ) inhibitor rottlerin (10 μ M), and with the specific PKC θ pseudosubstrate inhibitor (30 μ M) indicates that ceramide and phosphorylation by PKC δ and PKC θ mediate gal-1-induced JNK activation. Downstream of JNK, we observed increased phosphorylation of c-Jun, enhanced activating protein-1 (AP-1) luciferase reporter, and AP-1/DNA-binding in response to gal-1. The pivotal role of the JNK/c-Jun/AP-1 pathway for gal-1-induced apoptosis was documented by reduction of DNA fragmentation after inhibition JNK by SP600125 (20 μ M) or inhibition of AP-1 activation by curcumin (2 μ M). Gal-1 failed to induce AP-1 activation and DNA fragmentation in CD3-deficient Jurkat 31-13 cells. In Jurkat E6.1 cells gal-1 induced a proapoptotic signal pattern as indicated by decreased antiapoptotic Bcl-2 expression, induction of proapoptotic Bad, and increased Bcl-2 phosphorylation. The results provide evidence that the JNK/c-Jun/AP-1 pathway plays a key role for T-cell death regulation in response to gal-1 stimulation.

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Apoptosis in the immune system is a process that ensures the proper removal of autoreactive T cells during thymic development and T-cell homeostasis as well as the downregulation of peripheral immune responses against antigens. Thus T-cell apoptosis is essential for induction of central and peripheral tolerance and for prevention of autoimmunity.¹ Peripheral autoreactive lymphocytes can be deleted by developmental arrest (anergy) or by apoptosis through receptor-mediated activation-induced cell death (AICD). Following an immune response most of the activated T cells need to be deleted by apoptosis, which requires a switch from an apoptosis-resistant to an apoptosis-sensitive state. This can be mediated by cytokines, death receptors, and proapoptotic proteins.² Apoptosis can be initiated by two different pathways: the receptor-mediated AICD and the intrinsic mitochondria-dependent pathway.^{3,4} Both pathways ultimately activate

a cascade of cysteine proteases (caspases) and cells are committed to death thereby terminating immune responses.

Galectin-1 (gal-1), a prototype member of the family of endogenous β -galactoside-binding proteins, is widely expressed in lymphoid and nonlymphoid tissues and confers a variety of immunoregulatory functions. Functional gal-1 is a homodimer of noncovalently associated 14 kDa subunits with two carbohydrate recognition domains. This enables cell adhesion and cross-linking of several glycoproteins preferentially with branched or repeating Gal β 1-4GlcNAc sequences on T cells including the signaling proteins CD2, CD3, CD7, CD43, and CD45.⁵⁻⁹ Gal-1 induces apoptosis of immature cortical thymocytes, activated T cells, and T-cell lines,^{7,9,10} but also sensitizes resting human T lymphocytes to Fas Apo-1, CD95)-mediated cell death.¹¹ Gal-1 is exported by an endoplasmic reticulum/Golgi-independent pathway and is

¹Medical Faculty, Department of Medical Biochemistry and Molecular Biology, University of Rostock, Rostock, Germany and ²Department of Chemistry, Faculty of Science, Mansoura University, New Damietta City, Egypt

*Corresponding author: H Walzel, Medical Faculty, Department of Medical Biochemistry and Molecular Biology, University of Rostock, Schillingallee 70, Rostock 18057, Germany.

Tel: +49 381 494 5759; Fax: +49 381 494 5752; E-mail: hermann.walzel@med.uni-rostock.de

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Abbreviations: AICD, activation-induced cell death; AP-1, activating protein-1; bp, base pair; BSA, bovine serum albumin; CD, cluster of differentiation; DTT, dithiothreitol; ECL, enhanced chemiluminescence; EDTA, ethylene-diaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; FCS, fetal calf serum; gal-1, galectin-1; GST, glutathion-S-transferase; HEPEs, *N*-2-hydroxyethyl-piperazine-*N*-2-ethanesulfonic acid; HRP, horseradish peroxidase; IgG, immunoglobulin G; JNK, c-Jun N-terminal kinase; kDa, kilo Dalton; mAb, monoclonal antibody; MKK, mitogen-activated protein kinase; NP-40, Nonidet P-40; pAb, polyclonal antibody; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PKC, protein kinase C; RNase, ribonuclease; SD, standard deviation; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline; TCR, T-cell receptor; TPA, 12-O-tetradecanoylphorbol-13-acetate; TRE, TPA-response element; T_{reg}, regulatory T cells; Tris, tris(hydroxymethyl) aminomethane; T, Tween 20

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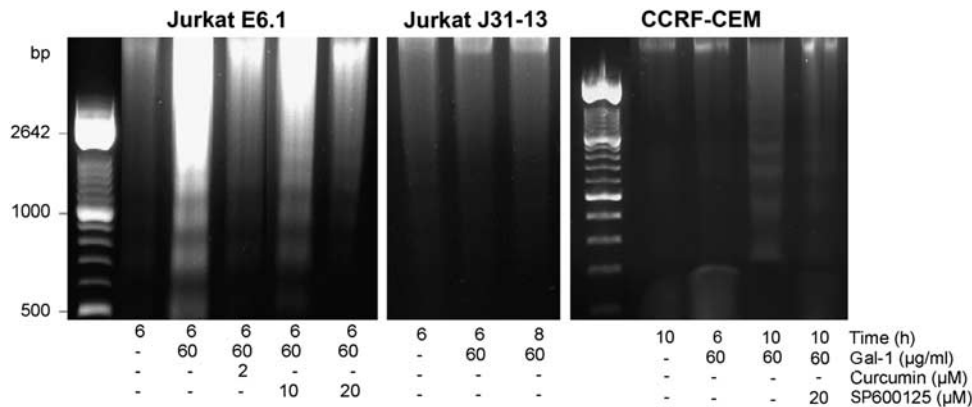


Figure 1 Inhibition of Galectin-1 (gal-1)-induced apoptosis of Jurkat E6.1 and CCRF-CEM cells by the inhibitor of activating protein-1 (AP-1) activation curcumin and by the ATP-competitive c-Jun N-terminal kinase (JNK) inhibitor SP600125. The cells were cultured with curcumin (2 μM) for 14 h and with SP600125 (10 and 20 μM) for 30 min. Cells were incubated at 37°C in 24-well plates at a density of 2×10^6 cells per well in RPMI 1640 medium in the presence or absence of gal-1 for the periods of time as indicated. Extracted DNA was separated in 1.5% agarose gels. Molecular weight standards (100 bp DNA ladder) are indicated on the left. Shown is a representative image of three independent experiments

presented by cell-surface glycoconjugates.¹² It has been shown that gal-1 presented by extracellular matrix more effectively induces apoptosis of susceptible T cells than the soluble form.¹³ Gal-1 synthesis is strongly upregulated after peptide antigen-induced activation of murine T cells and inhibits antigen-induced proliferation of activated T cells.¹⁴ These data strongly suggest a potential autocrine suicide mechanism to achieve homeostasis during termination of an immune response. CD4⁺CD25⁺ regulatory T (T_{reg}) cells express gal-1 at high level and this expression is upregulated upon T-cell receptor (TCR) activation.¹⁵ Surface-bound gal-1 was found to be a key effector of regulation mediated by T_{reg} cells, which is consistent with the non-antigen-specific nature of suppression. The secretion of gal-1 by decidual natural killer cells, its abundance in various cell types of human placenta, and the involvement in apoptotic depletion of alloreactive T cells contribute to the generation of an immune-privileged environment at the maternal–fetal interphase.¹⁶ Although cancer patients develop adaptive immune responses, tumors evade an effective immunosurveillance by induction of T-cell apoptosis.¹⁷ Increased gal-1 expression by activated tumor endothelial cells may locally contribute to the apoptotic elimination of infiltrating effector T cells and favor tumor progression.¹⁸ Blockage of gal-1 expression stimulates the generation of a tumor-specific T-cell-mediated response and tumor rejection in a syngeneic model of murine melanoma.¹⁹

It has been suggested that the gal-1 death pathway in T cells is entirely different from that mediated by Fas/FasL or glucocorticoids.⁷ However, recent data have shown that gal-1 cooperates with Fas-induced apoptosis in peripheral T cells¹¹ and stimulates the death receptor pathway in human Jurkat T lymphocytes.²⁰ Caspase activation has been shown by several authors as death effectors,^{21,22} although it has been reported that gal-1 induces T-cell death in a caspase- and cytochrome *c*-independent manner by translocation of endonuclease G from mitochondria to the nucleus.²³ Activation of the activating protein-1 (AP-1) transcription factor and downregulation of Bcl-2 have also been shown after exposure to gal-1.²⁴ Gal-1-induced activation of the

TCR ζ /Lck/ZAP70 pathway was proved to be essential to stimulate ceramide release and to trigger the mitochondrial pathway of apoptosis.²⁵

The stimulation of the mitochondrial apoptotic pathway by gal-1 initiated ceramide release²¹ and the requirement of AP-1 for ceramide-induced apoptosis²⁶ prompted us to investigate the role of the JNK/c-Jun/AP-1 pathway for cell death.

In this study we could show that gal-1 induces the activation of c-Jun N-terminal kinase (JNK), the phosphorylation of c-Jun, enhanced AP-1 luciferase reporter, and AP-1/DNA-binding activities as measured by immunoblot analyses, kinase assays, reporter and gel shift assays, and by enzyme-linked immunosorbent assay (ELISA). The results provide evidence for a pivotal role of the JNK/c-Jun/AP-1 pathway for T-cell death regulation.

Results

Evidence for a role of the JNK/c-Jun/AP-1 pathway in gal-1-induced cell death. Jurkat T lymphocytes exposed to gal-1 for 6 h underwent apoptosis as indicated by DNA fragmentation (Figure 1). To elucidate the role of JNK and AP-1 for gal-1 induced death, we preincubated the cells with the reversible ATP-competitive JNK inhibitor SP600125 (10 μM, 20 μM) and with curcumin (2 μM), an inhibitor of AP-1 activation. Both inhibitors effectively blocked gal-1-stimulated DNA fragmentation in Jurkat E6.1 cells (Figure 1). DNA fragmentations and inhibition by SP600125 were also recorded in gal-1-treated CCRF-CEM cells indicating that this effect was not specific for Jurkat T cells (Figure 1). Interestingly, gal-1 failed to generate DNA fragmentation in CD3-deficient Jurkat J31-13T cells, which shows defects in gal-1-induced TCR/CD3 signaling.⁸

Gal-1 induces JNK activation. Next we studied whether gal-1 is able to activate JNK and its downstream pathways to T-cell death. The inhibition of gal-1-induced DNA fragmentation with the specific JNK inhibitor SP600125 indicated that JNK is involved in the apoptotic pathway. Western blot analysis

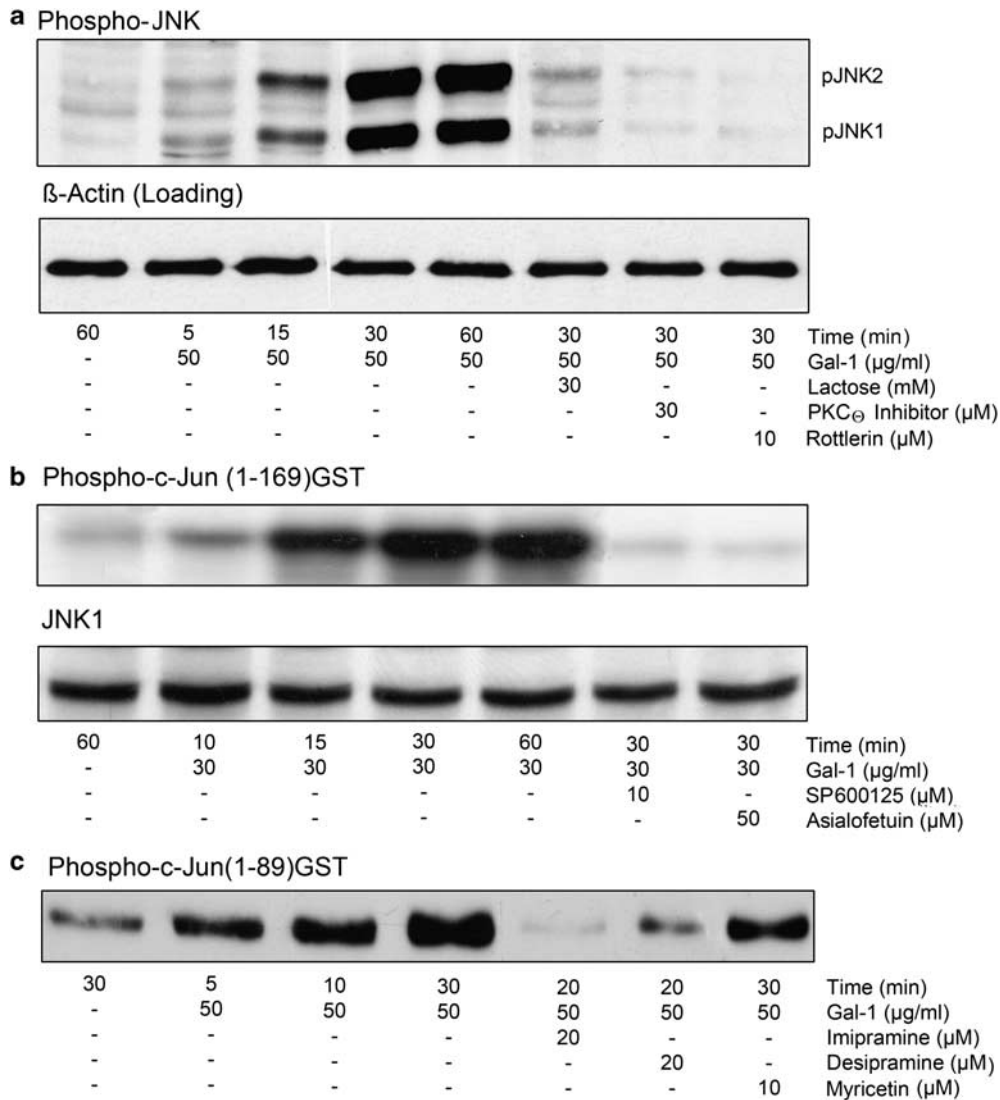


Figure 2 Galectin-1 (Gal-1)-induced phosphorylation of c-Jun N-terminal kinase 1 (JNK1) and JNK2 (a) and JNK activation with c-Jun(1-169)-GST (b), and c-Jun(1-89)-GST (c) as kinase substrates. Jurkat E6.1 cells (2×10^6 per ml RPMI 1640 medium) were incubated with protein kinase C- θ (PKC θ) inhibitor and PKC δ inhibitor rottlerin for 1 h, with the sphingomyelinase inhibitors desipramine and imipramine for 2 h, as well as with the ATP-competitive inhibitor for JNK SP600125 and for mitogen-activated protein kinase kinase 4 (MKK4) myricetin for 30 min as indicated. Control cells were incubated in medium alone. Cells were then stimulated with gal-1 without and in the presence of lactose or asialofetuin as indicated in panels a, b, and c. (a) For immunoblot analysis cell extract proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Blots were analyzed with a phospho-JNK (Thr183/Tyr185) monoclonal antibody (mAb). The bands were luminographically visualized on X-ray films using ECL Plus reagents. Equal loading of gel lanes was verified by reprobing the blots for expression of β -actin. (b) After termination of the kinase reactions with $3 \times$ SDS sample buffer, samples were electrophoretically separated and blotted on PVDF membranes. The [^{32}P]-labeled substrate c-Jun(1-169)-GST was recorded by autoradiography. To control loading, we separated $50 \mu\text{g}$ cell extract protein/lane and blotted it on PVDF membranes. Membranes were probed with a JNK1 polyclonal antibody (pAb). (c) After termination of the kinase reactions, samples were separated and blotted on Hybond ECL membranes. Blots were analyzed for substrate phosphorylation with a phospho-c-Jun (Ser63) pAb. The bands were luminographically visualized on X-ray films using ECL Plus reagents. Shown are representative blots from three independent experiments

revealed that the stimulation of Jurkat T cells with gal-1 effectively increased the dual phosphorylation of JNK1 and JNK2 (Figure 2a). JNK phosphorylation in Jurkat T cells by gal-1 ($50 \mu\text{g/ml}$) could be blocked by the disaccharidic competitor lactose (30 mM), the protein kinase C- δ (PKC δ) inhibitor rottlerin ($10 \mu\text{M}$), and the specific PKC θ pseudosubstrate inhibitor ($30 \mu\text{M}$). JNK activation was additionally verified by kinase assays as indicated by increased phosphorylation of JNK substrates in a time-dependent manner (Figures 2b and c). Exposure of the cells with the specific JNK inhibitor

SP600125 ($10 \mu\text{M}$) or stimulation of the cells with gal-1 ($50 \mu\text{g/ml}$) in the presence of asialofetuin ($50 \mu\text{M}$) decreased the phosphorylation of the JNK substrate to the level of control cells (Figure 2b). Furthermore, treatment of the cells with the sphingomyelinase inhibitors imipramine and desipramine ($20 \mu\text{M}$) before stimulation with gal-1 strongly reduced the activation of JNK, whereas myricetin ($10 \mu\text{M}$), an ATP-competitive inhibitor of mitogen-activated protein kinase kinase 4 (MKK4), showed only marginal inhibitory effects (Figure 2c). The data suggest that PKC δ , PKC θ , and MKK4

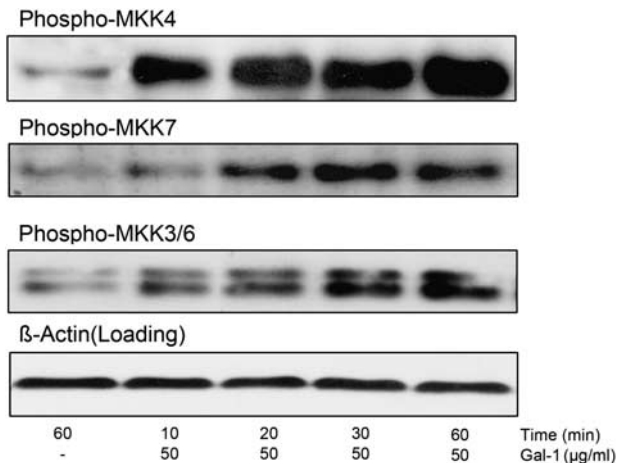


Figure 3 Kinetics of galectin-1 (gal-1)-induced phosphorylation of mitogen-activated protein kinase kinase (MKK4), MKK7, and MKK3/6 in Jurkat E6.1 cells. Cells (2×10^6 per ml) were incubated in medium alone or stimulated with gal-1 as indicated. Separated cell extract proteins were analyzed by immunoblotting using phospho-MKK4 (Ser257/Thr261) polyclonal antibody (pAb), phospho-MKK7 (Ser271/Thr275) pAb, and a phospho-MKK3/6 (Ser189/Thr207) monoclonal antibody (mAb). The bands were luminographically visualized on X-ray films using ECL Advance reagents. Equal loading of gel lanes was verified by reprobing the blots for expression of β -actin. Shown are blots from three independent experiments

as upstream kinases as well as ceramide contribute to JNK phosphorylation and activation.

Gal-1 activated MKKs in Jurkat E6.1 cells. The stimulating effects of gal-1 on JNK phosphorylation and inhibition of JNK substrate phosphorylation by myricetin prompted us to investigate whether cell stimulation also induces the phosphorylation and activation of MKK4, MKK7, and MKK3/6. MKK7 is a specific upstream JNK activator, MKK4 phosphorylates JNK and p38 MAP kinase groups whereas MKK3/6 are specific for p38 MAP kinase.²⁸ Full activation of JNK requires the synergistic action of MKK4 and MKK7 phosphorylating different sites of JNK. Stimulation of Jurkat E6.1 cells with gal-1 resulted in a strong phosphorylation of MKK4 in comparison to nonstimulated controls (Figure 3). The kinetics clearly showed an increased phosphorylation after 10 min with slight progression up to 1 h (Figure 3). Gal-1 also effectively induced the phosphorylation of MKK7 (Figure 3). MKK7 phosphorylation was detectable 10 min after stimulation and then gradually increased with exposure time. Weaker phosphorylation reactions were recorded for MKK3 and MKK6 (Figure 3). The activation of MKK4 and MKK7 as well as inhibition of gal-1-induced JNK substrate phosphorylation with the MKK4 inhibitor myricetin (Figure 2c) provides evidence that both MKKs function as upstream JNK kinases.

Gal-1 stimulated the phosphorylation of c-Jun. JNK activates the transcription factor c-Jun by phosphorylation of serine 63 and 73. JNK also activates other Jun-family proteins that are involved in the AP-1 transcription factor complex.²⁹ Western blot analysis showed that gal-1 (50 µg/ml) strongly increased the phosphorylation of c-Jun in a

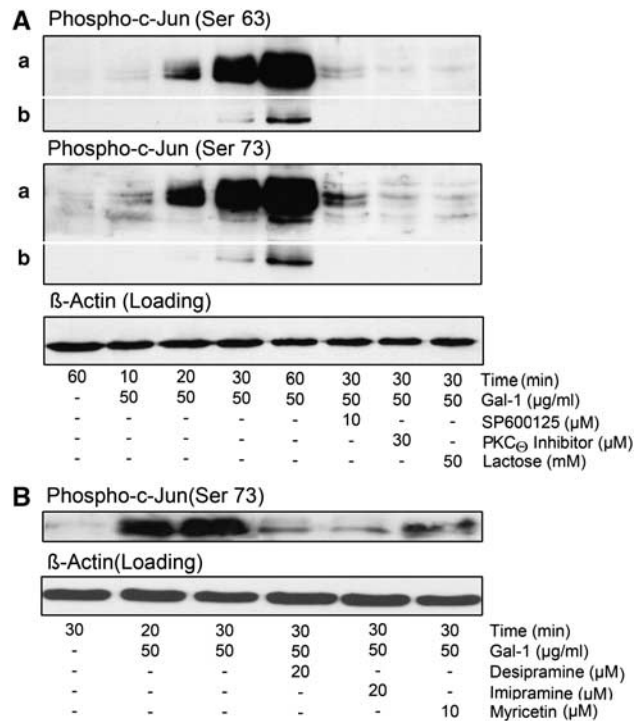


Figure 4 Galectin-1 (Gal-1)-induced phosphorylation kinetics of c-Jun and inhibition of c-Jun Ser63/73 phosphorylation with SP600125, protein kinase C- θ (PKC θ) inhibitor, and lactose (A) and with desipramine, imipramine, and myricetin (B). Jurkat E6.1 cells (2×10^6 per ml RPMI 1640 medium) were incubated with PKC θ inhibitor for 1 h, with the sphingomyelinase inhibitors desipramine and imipramine for 2 h, and with the ATP-competitive inhibitor for c-Jun N-terminal kinase (JNK) SP600125 and for mitogen-activated protein kinase kinase 4 (MKK4) with myricetin for 30 min as indicated. Control cells were incubated in medium alone. Non- and inhibitor-treated cells were then stimulated with gal-1 without and in the presence of lactose. Cell extract proteins were analyzed on blots with a phospho-c-Jun (Ser63) polyclonal antibody (pAb) and a phospho-c-Jun (Ser73) pAb without (A, panel a; B) and in the presence of c-Jun (Ser63) and c-Jun (Ser73) blocking peptides at 4 µg/ml (A, panel b). The bands were luminographically visualized on X-ray films using ECL Plus reagents. Equal loading of gel lanes was verified by reprobing the blots for expression of β -actin. Shown are representative blots from three independent experiments

time-dependent manner (Figure 4A, panel a). Exposure of the cells with the JNK inhibitor SP600125 (10 µM) or the PKC θ pseudosubstrate inhibitor (30 µM) before stimulation and stimulation of the cells with gal-1 in the presence of 50 mM lactose blocked the phosphorylation of c-Jun. Specificity of c-Jun phosphorylation was verified by inhibition in the presence of phospho-c-Jun (Ser63) and phospho-c-Jun (Ser73) blocking peptides (Figure 4A, panel b). Gal-1-induced phosphorylation of c-Jun was also effectively inhibited when Jurkat E6.1 cells were preincubated with the sphingomyelinase inhibitors desipramine and imipramine at 20 µM as well as 10 µM of the MKK4 inhibitor myricetin (Figure 4B).

Gal-1 induces AP-1 reporter activity and increased AP-1 DNA-binding activity. The construct pAP1(PMA)-TA-Luc comprises six tandem copies of the AP-1 enhancer. Stimulation of transiently transfected E6.1 and CCRF-CEM cells with 5, 10, 15, and 20 µg/ml gal-1 increased the

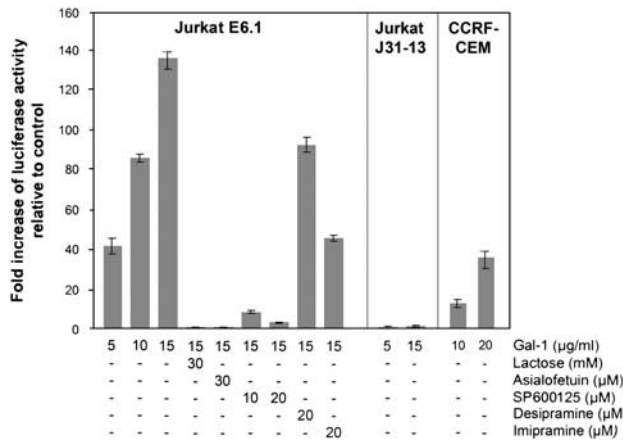


Figure 5 Activating protein-1 (AP-1) reporter gene assay. Jurkat E6.1, CCRF-CEM, and J31-13 cells were transiently transfected with pAP1(PMA)-TA-Luc. The cells were incubated at 5×10^6 per 4 ml RPMI 1640 medium with the ATP-competitive inhibitor for c-Jun N-terminal kinase (JNK) SP600125 and for mitogen-activated protein kinase kinase 4 (MKK4) with myricetin for 30 min and with the sphingomyelinase inhibitors imipramine and desipramine for 2 h as indicated. Then the cells were stimulated with galectin-1 (gal-1) without and in the presence of lactose and asialofetuin for 3 h. A representative of three independent experiments carried out in triplicates is shown. Error bars indicate the S.D. of three determinations

expression of the reporter in a concentration-dependent manner (Figure 5). The luciferase reporter activity increased 135.4-fold when transfected E6.1 cells were stimulated with 15 μ g/ml gal-1 for 3 h relative to transfected but non-stimulated cells (control). The basal luciferase activity was about 10-fold lower than the control in cell lysates from E6.1 cells transfected with the pTA-Luc construct lacking the AP-1 enhancer element (negative control). Gal-1 did not induce the reporter activity of this construct (data not shown). The gal-1-mediated induction of the AP-1 reporter was completely inhibited in the presence of 30 mM lactose, as well as by 30 μ M asialofetuin (Figure 5). Cellobiose at 30 mM did not show any inhibitory effects (data not shown). The JNK inhibitor SP600125 at 10 and 20 μ M resulted in a 93.7 and 97.6% decrease of gal-1-induced reporter activity. Furthermore, inhibition of ceramide synthesis by desipramine and imipramine (20 μ M) reduced the gal-1-stimulated luciferase reporter activity by 32.1 and 66.4%. Stimulation of transiently transfected CCRF-CEM cells with 20 μ g/ml gal-1 resulted in a 38.0-fold increase of the luciferase reporter activity (Figure 5). However, gal-1 failed to induce the AP-1 reporter construct in CD3-deficient Jurkat J31-13 cells (Figure 5).

In addition to AP-1 reporter gene assays, electrophoretic mobility shift assays (EMSA) were performed to verify if treatment with gal-1 has stimulating effects on the binding of nuclear extracts to an AP-1 consensus oligonucleotide. Gal-1 increased the binding of nuclear extracts to AP-1 consensus oligonucleotides when compared with nonstimulated cell cultures (Figure 6a). Specificity of DNA-protein complex formation was confirmed by competition experiments with 100-fold molar excess of unlabeled AP-1 consensus and mutant oligonucleotide (Figure 6a).

We next analyzed the assembly of AP-1 protein complexes with an immobilized oligonucleotide containing a 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-response element (TRE) using ELISA (Figure 6b). Stimulation of Jurkat E6.1 cells with gal-1 increased the binding of phospho-c-Jun (Ser73), phospho-JunD (Ser100), c-Fos, Jun B, and Fos B to the immobilized AP-1 consensus oligonucleotide relative to nonstimulated control cells (lane c). Specificity of DNA-protein complex formation was verified by competition experiments with unlabeled mutant and consensus oligonucleotides at 20 pM per well. DNA-protein complex formation was blocked by excess of the AP-1 consensus oligonucleotide, whereas complex formation in the presence of the mutant form was comparable to that in its absence.

Effects of gal-1 on pro- and antiapoptotic proteins. Different AP-1 dimer combinations may recognize different sequence elements in the promoter and enhancer regions of target genes.³⁰ Therefore, it is conceivable that AP-1 differentially regulates pro- and antiapoptotic genes in response to extracellular stimuli. Gal-1 moderately decreased Bcl-2 protein expression in agreement with previous studies^{21,24} (Figure 7). However, we recorded a significant upregulation of proapoptotic Bad expression by gal-1 before the onset of DNA fragmentation. Inhibition of JNK with SP600125 and of AP-1 activation with curcumin²⁶ blocked the upregulation of Bad (Figure 7). Western blot analysis and densitometric quantification of the immunoreactive protein bands revealed that gal-1 triggered the dual phosphorylation of Bcl-2 on serine 70 and threonine 56 (Figure 7) that suppresses its antiapoptotic function.^{31,32} Inhibition of JNK with SP600125 blocked the phosphorylation at both sites indicating that JNK functions as a Bcl-2 kinase (Figure 7).

Gal-1-induced processing of procaspase-9 and procaspase-3 required the JNK/c-Jun/AP-1 pathway. Downregulation and phosphorylation of Bcl-2 as well as upregulation of Bad may induce depolarization of mitochondria and subsequent translocation of cytochrome *c* into the cytosol. Because cytochrome *c* is essentially required for procaspase-9 activation, we investigated the effects of gal-1 on initiator procaspase-9 and downstream effector procaspase-3 fragmentation. Processing of procaspase-9 and procaspase-3 was detectable 4 h after stimulation of Jurkat E6.1 cells with gal-1 and cleavage products further increased with exposure time (Figure 8). Blocking of the JNK/c-Jun/AP-1 pathway by SP600125 impeded the subsequent activation of both procaspases (Figure 8).

Discussion

There is cumulative evidence that JNK has an essential role in apoptosis induced by UV radiation, growth factor withdrawal, chemotherapeutic drugs, and ceramide.^{33,34} In this study we could show that JNK activation is also required for apoptosis of human lymphoblastoid Jurkat T cells induced by gal-1. JNK activation occurred rapidly within 10 min after gal-1 exposure, as shown by kinase assays and increasing levels of phospho-JNK1 and phospho-JNK2 isoforms. Apoptotic cell death is significantly promoted in cells expressing JNK, but effectively

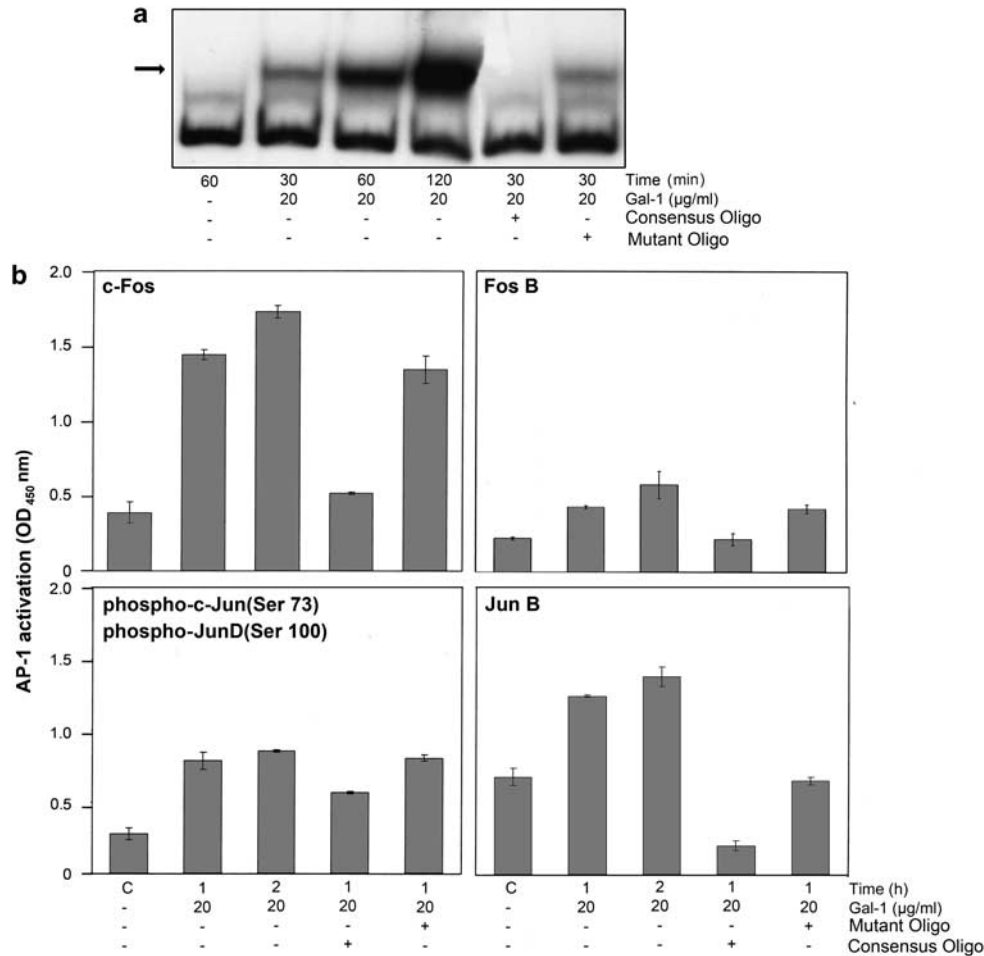


Figure 6 Kinetics of galactin-1 (gal-1)-induced activating protein-1 (AP-1)/DNA binding activity as detected by electrophoretic mobility shift assay (EMSA) (a) and analysis of immobilized AP-1-consensus oligonucleotide complexes for phospho-c-Jun (Ser 73), phospho-JunD (Ser100), c-Fos, Jun B, and Fos B by enzyme-linked immunosorbent assay (ELISA) (b). Jurkat E6.1 cells (2×10^6 per ml RPMI 1640 medium) were cultured with gal-1 as indicated. (a) The preparation of nuclear extracts and the binding to the 32 P-labelled consensus oligonucleotide were performed in the absence of a competitor or in the presence of 100-fold excess of the unlabeled AP-1 consensus or the mutant AP-1 oligonucleotide. Bands were visualized by autoradiography. (a) Nuclear extracts ($5 \mu\text{g}$ per well) were incubated in 96-well plates coated with an immobilized oligonucleotide containing the TRE sequence for AP-1 binding. For assay specificity, binding reactions were also performed in the presence of consensus and mutant oligonucleotide at 20 pM per well. Complexes were analyzed with specific antibodies for the AP-1 proteins by ELISA as indicated. A representative of three independent experiments carried out in triplicates is shown. Error bars indicate the S.D. of three determinations

suppressed in cells expressing a dominant-negative JNK1 mutant or JBD, a JNK inhibitor protein.³⁴ In agreement with these data we also found that JNK activation is efficiently prevented by the reversible ATP-competitive inhibitor of JNK SP600125 and this perturbation of JNK activation resulted in prevention of DNA fragmentation.

In a recent report we verified that gal-1-induced DNA laddering corresponds to phosphatidylserine exposure and DNA-strand breaks as analyzed by TUNEL assay.²⁰ However, in some T-cell lines gal-1-induced phosphatidylserine translocation was not associated with apoptotic progression.³⁵ Therefore, we studied the inhibitory effects of SP600125 and curcumin on gal-1-induced apoptosis in Jurkat E6.1 and CCRF-CEM cells by DNA fragmentation as a reliable apoptotic marker.

JNK activity is differentially regulated by various different upstream kinases including MKK4, MKK7, PKC δ , ASK1, and mixed lineage kinases.^{27,28,34,36} Thus, the blockade of JNK

activation by inhibitors of PKC θ , PKC δ , and MKK4 is consistent with these data. Interestingly, JNK, MKK4, and MKK7 activities increased in parallel after gal-1 stimulation indicating that these kinases are linked. Lactose and asialofetuin completely inhibited JNK activation providing evidence that gal-1 prefers glycoproteins with biantennary and triantennary N-linked glycan chains presenting terminal Gal β 1-4GlcNAc sequences for recognition available on asialofetuin.³⁶ Therefore, the differential glycosylation state of cell-surface glycoproteins during immune cell activation and differentiation can selectively regulate cellular signaling. This may explain the unequal susceptibility of effector T-cell subsets to gal-1-induced cell death.³⁷

There is experimental evidence that gal-1 induces partial TCR ζ -chain phosphorylation generating inhibitory pp21 ζ , limits receptor clustering at the TCR contact site,³⁸ and promotes apoptosis. Deficiency in p56^{lck} and ZAP70 tyrosine kinases abolishes gal-1-induced T-cell death, and reexpress-

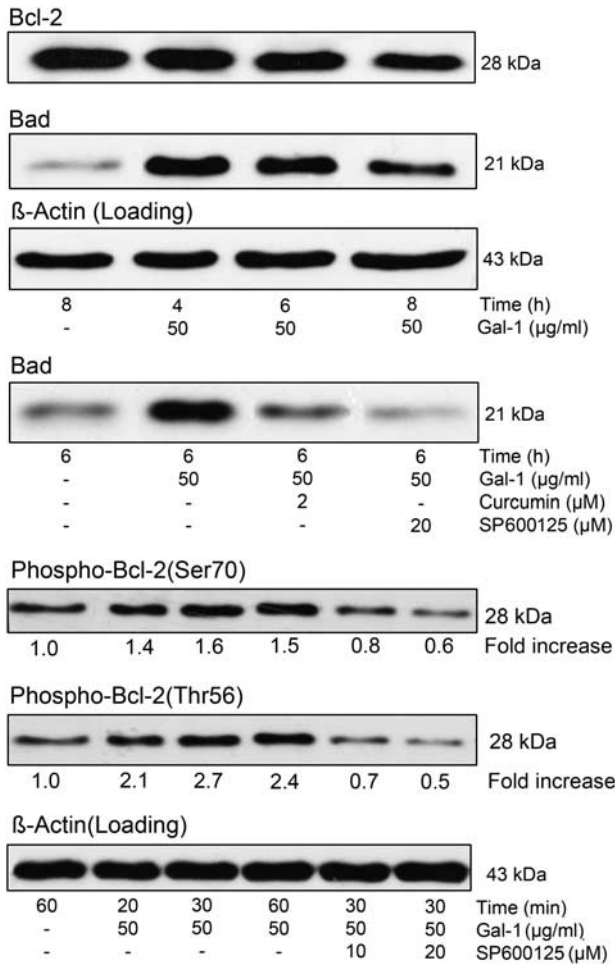


Figure 7 Effects of galectin-1 (gal-1) on Bcl-2 and Bad protein levels and on Bcl-2 phosphorylation. Jurkat E6.1 cells were cultured with curcumin for 14 h and with SP600125 for 30 min. Non- and inhibitor-treated cells (2×10^6 cells per well) were stimulated with gal-1 for the periods of time as indicated. Control cells were incubated in medium alone. Cell extract proteins were analyzed on the blots with a Bcl-2 polyclonal antibody (pAb), Bad pAb, phospho-Bcl-2 (Ser70) monoclonal antibody (mAb), and with a phospho-Bcl-2 (Thr56) pAb. The bands were luminographically visualized on X-ray films by enhanced chemiluminescence (ECL). Equal loading of gel lanes was verified by the expression of β -actin. Phospho-Bcl-2 bands were analyzed by densitometry using the Odyssey application software (version 3.0.16) from LICOR (Bad Homburg, Germany). Data are expressed as x -fold relative to the control after normalization to the corresponding β -actin bands (loading). Shown are representative blots from three independent experiments

sion restores apoptosis.²⁵ The results of our study support these data, as gal-1 failed to induce DNA fragmentation of CD3-deficient J31-13 cells. Gal-1 also initiates the sphingomyelinase-mediated release of ceramide of activated peripheral and leukemic T cells. Further downstream events such as the decrease of antiapoptotic Bcl-2, depolarization of mitochondria, and activation of caspase-9 and caspase-3 depend on increased ceramide levels. Ceramide production and all the downstream events require the presence of the protein tyrosine kinases p56^{lck} and ZAP70.²¹ We show here that blocking of ceramide production by desipramine and imipramine abrogated the induction of JNK activity by gal-1. To exert its proapoptotic effects, it seems that ceramide transduces the intracellular signals by activating JNK.³³

Furthermore, our study result shows that Bcl-2 undergoes increased phosphorylation by JNK in Jurkat E6.1 cells as JNK inhibition by SP600125 effectively decreased the phosphorylation of Bcl-2. The inhibition of Bcl-2 phosphorylation by SP600125 below the basal level in absence of gal-1 emphasizes the role of JNK as Bcl-2 kinase (Figure 7). The phosphorylation of Bcl-2 decreases its ability to heterodimerize with proapoptotic Bax.^{31,32} Thus, JNK-mediated phosphorylation of Bcl-2 suppresses its antiapoptotic function in mitochondria-related cell death mechanisms.

Our study results further show a gal-1-induced signaling pathway that connects the activation of JNK, the selective phosphorylation of c-Jun, and the activation of the AP-1 transcription factor complex.^{30,39} Blocking of JNK activation by SP600125 or inhibition of ceramide generation with imipramine and desipramine effectively decreased gal-1-induced phosphorylation of c-Jun and the AP-1 luciferase reporter activity of Jurkat E6.1 cells. Gal-1 induced the AP-1 reporter construct to a lesser extent in CCRF-CEM cells, however, failed to activate the construct in CD3-deficient J31-13 cells. This indicates that TCR/CD3 signaling is required for initiation of apoptosis.⁴⁰ Gel shift assays and analysis of immobilized AP-1/oligonucleotide complexes by ELISA revealed that gal-1 rapidly enhanced the binding of the AP-1 complex to the TRE and induced differential increases of the AP-1 proteins phospho-c-Jun, phospho-JunD, c-Fos, Jun B, and Fos B. This suggests that gal-1 promotes the assembly of various dimer combinations with cell- and stimulus-specific transcriptional activities.^{30,41} Inhibition of c-Jun/AP-1 binding to its TRE site by curcumin may be responsible for the subsequent inhibitory effects on c-Jun/AP-1-mediated gene expression.⁴² Gal-1-stimulated phosphorylation of c-Jun and the increased levels of phosphorylated c-Jun and JunD of AP-1 oligonucleotide complexes may also contribute to AP-1 activation.³⁰ Blocking of DNA fragmentation by exposure of Jurkat E6.1 cells to curcumin and to the JNK inhibitor SP600125 before gal-1 stimulation suggests a pivotal role of the JNK/c-Jun/AP-1 pathway for apoptosis. In gal-1-induced cells, the upregulation of Bad in conjunction with low levels of antiapoptotic Bcl-2 and its increased phosphorylation generate a proapoptotic cascade leading to cytochrome *c*-mediated initiator procaspase-9 and downstream effector procaspase-3 activation.²² In line with our previous study that showed a gal-1-induced coincidence of cytochrome *c* release and caspase-9 activation, the present data can be interpreted as a clear sign for involvement of the mitochondrial compartment in gal-1-induced apoptosis.²²

The data presented in this study provide the first experimental evidence indicating the pivotal role of JNK as well as of c-Jun/AP-1, Bcl-2, and Bad as targets of the signal transduction pathway triggered in gal-1-induced apoptosis. A profound knowledge about the immunoregulatory mechanisms of gal-1 on T cells opens the perspective to use this endogenous lectin for immunomodulatory strategies in autoimmune diseases, infection, and cancer.

Materials and Methods

Materials. Asialofetuin, curcumin, desipramine, dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), β -lactose, anti-rabbit IgG-HRP, imipramine, myricetin, NP-40, *N*-2-hydroxyethyl-piperazine-*N*-2-ethanesulfonic acid (HEPES),

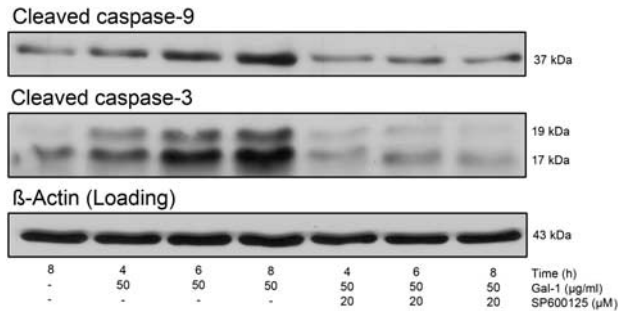


Figure 8 Effects of galactin-1 (gal-1) on initiator procaspase-9 and effector procaspase-3 processing and inhibition by the ATP-competitive inhibitor of c-Jun N-terminal kinase (JNK) SP600125. Jurkat E6.1 cells (2×10^6 per ml RPMI 1640 medium) were incubated with SP600125 for 30 min at 37°C. Non- and inhibitor-treated cells were stimulated with gal-1 as indicated. Control cells were incubated in medium alone. Cell extract proteins were analyzed on the blots with a cleaved caspase-9 (Asp315) polyclonal antibody (pAb) and with a cleaved caspase-3 (Asp175) rabbit monoclonal antibody (mAb). The bands were luminographically visualized on X-ray films by enhanced chemiluminescence (ECL). Equal loading of gel lanes was verified by reprobing the blots for expression of β -actin. Shown are representative blots from three independent experiments

ribonuclease A, SDS, SP600125 (anthra(1,9-*cd*)pyrazol-6(2*H*)-one), Tris, and Triton X-100 were from Sigma (Deisenhofen, Germany). Aprotinin, DNA molecular weight marker 100 bp ladder, leupeptin, pepstatin, pefabloc, poly[dI-dC], proteinase K, and T4 polynucleotide kinase were purchased from Roche Molecular Biochemicals (Mannheim, Germany) and JNK1 polyclonal antibody (pAb), c-Jun(1-169)-GST were from Biomol (Hamburg, Germany). Fetal calf serum (FCS), kanamycin, RPMI 1640 medium were from Gibco BRL (Eggenstein, Germany), enhanced chemiluminescence (ECL) detection reagents, Hybond ECL nitrocellulose membranes, protein G agarose, PVDF membranes, and [γ - 32 P]ATP were from GE Healthcare Europe (Freiburg, Germany). Rottlerin and the PKC θ pseudosubstrate inhibitor (Myr-LHQRRAIKQAKVHHVKC-NH $_2$) were from Merck-Biosciences (Schwalbach, Germany). The reporter gene constructs, pAP1(PMA)-TA-Luc and pTA-Luc, were from Clontech (Heidelberg, Germany) and actin (1-19) pAb, double-stranded AP-1 consensus (sc-2501), and the mutant (sc-2514) oligonucleotide were from Santa Cruz Biotechnology (Heidelberg, Germany). Bad pAb, Bcl-2 pAb, phospho-Bcl-2 (Ser70) monoclonal antibody (mAb), phospho-Bcl-2 (Thr56) pAb, cleaved caspase-9 (Asp315) pAb, cleaved caspase-3 (Asp175) rabbit mAb, phospho-c-Jun (Ser63) pAb, phospho-c-Jun (Ser63) blocking peptide, phospho-c-Jun (Ser73) pAb, phospho-c-Jun (Ser73) blocking peptide, phospho-MKK3/6 (Ser189/Thr207) mAb, phospho-MKK7 (Ser271/Thr275) pAb, phospho-JNK (Thr183/Tyr185) mAb, phospho-MKK4 (Ser257/Thr261) pAb, and the JNK assay kit were from New England Biolabs (Frankfurt, Germany). The Trans-AM AP-1 transcription factor assay kit was from Active Motif North America (Carlsbad, CA, USA).

Cell lines. The human leukemic T-cell line Jurkat (clone E6.1; European Collection of Cell Cultures, Salisbury, UK) and the CD3-deficient Jurkat 31-13 cell clone, kindly provided by A. Alcover (Institut Pasteur, Paris, France), were maintained at 37°C and 5% CO $_2$ in RPMI 1640 medium supplemented with 10% FCS and 10 μ g/ml kanamycin. Human CCRF-CEM lymphoblastic leukemia T cells (DSMZ – Search for Human and Animal Cell Lines, Braunschweig, Germany) were cultured in RPMI 1640 medium containing 10% FCS, penicillin (100 mU/ml), and streptomycin (50 μ g/ml).

Preparation of recombinant human gal-1. The preparation of gal-1 cDNA, ligation into the isopropyl- β -D-thiogalactopyranoside-inducible expression vector pET22b(+), and expression in *E. coli* were performed as previously described.²⁰ After cell lysis in EDTA-MEPBS (20 mM Na $_2$ HPO $_4$ (pH 7.2), 150 mM NaCl, 4 mM 2-mercaptoethanol, 2 mM EDTA) by sonication on ice, gal-1 was purified by affinity chromatography on lactosyl agarose.⁴³ The gal-1 protein was verified as a 14 kDa band in silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels.

AP-1 reporter gene assay. The pAP1(PMA)-TA-Luc *cis*-reporter vector and the pTA-Luc vector as a negative control are designed for monitoring the induction of AP-1-mediated signaling events by assaying the luciferase activity. The cells (1×10^7 per 0.8 ml RPMI 1640 medium) mixed with 25 μ g of the reporter constructs were electroporated in 0.4 cm cuvettes with a gene pulser from Bio-Rad (Munich, Germany) at 350 V and 900 μ F. Then the cells were cultured at 5×10^6 per 4 ml RPMI 1640 medium for 30 min at 37°C without or with SP600125 followed by stimulation with gal-1 without or in the presence of lactose or asialofetuin for 3 h as indicated in the figure legend. Cell lysis and the measurement of reporter luciferase activity were performed by applying the luciferase assay system (Promega, Mannheim, Germany).

Preparation of nuclear protein extracts. Jurkat E6.1 cells (2×10^6 per ml RPMI 1640 medium) were stimulated with gal-1 (20 μ g/ml) at 37°C and washed once with ice-cold phosphate-buffered saline (PBS). The cells were pelleted and solubilized with 0.1 ml 20 mM HEPES (pH 7.9; 10 mM KCl, 1 mM EDTA, 1 mM pefabloc, 0.1 mM Na $_3$ VO $_4$, 0.2% Triton X-100, 10% glycerol, and 1 μ g/ml each of aprotinin, pepstatin, and leupeptin) for 15 min on ice.⁴⁴ After centrifugation at $16\,000 \times g$ for 2 min, the pellets were extracted for 20 min on ice with 50 μ l 10 mM HEPES (pH 7.9; 350 mM NaCl, 10 mM KCl, 1 mM EDTA, 1 mM DTT, 1 mM pefabloc, 0.1 mM Na $_3$ VO $_4$, 20% glycerol, and 1 μ g/ml each of aprotinin, pepstatin, and leupeptin). The extracts were centrifuged at $16\,000 \times g$ for 5 min and stored at -80°C .

Electrophoretic mobility shift assay. The double-stranded AP-1 consensus oligonucleotide (sc-2501) was 32 P-labelled with [γ - 32 P]ATP using T4 polynucleotide kinase. Nuclear extract (4 μ l) was mixed with 20 μ l of binding buffer (10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM DDT, 0.1% Triton X-100, 5% glycerol, 1 mg/ml bovine serum albumin (BSA), and 2 mg/ml poly[dI-dC] containing 65 000 c.p.m. of end-labeled probe. For competition studies, the binding reactions were performed in the presence of 100-fold excess of unlabeled AP-1 consensus or mutant oligonucleotides. After 30 min on ice, the complexes were separated on a native 6% polyacrylamide gel. Dried gels were exposed to X-ray films (HT1.000G Plus; Agfa-Gevaert, Mortsel, Belgium) at -70°C with intensifying screens.

ELISA-based detection and quantification of AP-1 transcription factor activation. The DNA-binding activity of AP-1 was quantified by ELISA using the Trans-AM AP-1 transcription factor assay. Nuclear extracts (5 μ g per well) were incubated in 96-well plates coated with an immobilized oligonucleotide containing a TRE with the 5'-TGA(C/G)TCA-3' sequence that primarily binds AP-1 dimers. For assay specificity, binding reactions were performed in the presence of consensus and mutated oligonucleotides at 20 pM per well. AP-1 binding to the immobilized probe was detected by incubation with primary antibodies specific for c-Fos, c-Jun, Fos B, and Jun B. The c-Jun antibody detects phosphorylated c-Jun (Ser73) and JunD (Ser100). The addition of a secondary horseradish peroxidase (HRP)-conjugated antibody and developing solution provided a colorimetric readout that was spectrophotometrically quantified at 450 nm. Background binding was subtracted from the value obtained for binding to the consensus DNA sequence.

DNA extraction and analysis by agarose gel electrophoresis. Degraded low-molecular-weight DNA from apoptotic cells was selectively extracted with phosphate-citrate (PC) buffer.⁴⁵ The cells were collected by centrifugation, fixed in 70% ethanol, and were stored at -25°C for 16 h. Then the cells were pelleted at $800 \times g$ for 5 min. Cell pellets (2×10^6 cells) were suspended in 40 μ l of PC buffer (0.2 M Na $_2$ HPO $_4$ adjusted with 0.1 M citric acid to pH 7.8) at room temperature for 30 min. After centrifugation at $1000 \times g$ for 5 min, supernatants were evaporated in a vacuum concentrator 5301 (Eppendorf, Hamburg, Germany) for 15 min. Then 3 μ l 0.25% NP-40 was added to the cell extract followed by 3 μ l of a solution of RNase A (1 mg/ml). After 30 min incubation at 37°C, 3 μ l of a solution of proteinase K (1 mg/ml) was added and the extract was incubated for additional 30 min at 37°C. Then the extracts were mixed with 12 μ l loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol) and subjected to 1.5% agarose gel electrophoresis. DNA ladders were visualized by ethidium bromide staining under UV light.

JNK immune complex kinase assay. Jurkat E6.1 cells (2×10^6 per ml RPMI 1640 medium) were incubated for 30 min at 37°C with 10 μ M of the JNK inhibitor SP600125. Then the cells were stimulated with 30 μ g/ml gal-1. Cell pellets

were treated with 350 μ l lysis buffer (20 mM HEPES (pH 7.6), 10 mM *p*-nitrophenyl phosphate, 0.1 mM Na₃VO₄, 2 mM DTT, 0.1 mM pefabloc, 1% Triton X-100) on ice for 30 min,⁴⁶ followed by centrifugation at 16 000 \times *g* for 10 min at 4°C. From the supernatants (450 μ g extract protein) JNK1 was immunoprecipitated with 15 μ g JNK1 pAb for 1 h at 4°C followed by incubation with 50 μ l protein G agarose for 1 h. Then the beads were washed three times with 300 μ l lysis buffer and twice with kinase buffer (20 mM HEPES (pH 7.6), 20 mM MgCl₂, 20 mM β -glycerophosphate, 20 mM *p*-nitrophenyl phosphate, 0.1 mM Na₃VO₄, 2 mM DTT). The beads were suspended in 20 μ l kinase buffer supplemented with 5 μ g c-Jun(1-169)-GST as a substrate and 5 μ Ci [γ -³²P]ATP and incubated for 20 min at 30°C. Kinase reactions were terminated by addition of 15 μ l 3 \times SDS sample buffer and treated for 5 min at 100°C. Samples were electrophoretically separated and blotted on PVDF membranes. The phosphorylated substrate at 41 kDa was visualized by autoradiography. To control loading, we electrophoretically separated 50 μ g lysate protein/lane and blotted it on PVDF membranes. After blocking with 5% BSA in PBS (pH 7.4), membranes were probed with a JNK1 pAb followed by incubation with an IgG-HRP conjugate. Detection of JNK1 at 46 kDa was performed by ECL.

JNK assay (nonradioactive). JNK activity was measured by the JNK assay kit (New England Biolabs). The preparation of the cell lysates, pulldown of the JNK enzyme from cell extracts (200 μ g protein) with c-Jun(1-89)-GST fusion protein beads (20 μ l), and the kinase assay were performed according to the manufacturer's protocol. After termination of the kinase reaction with 3 \times SDS electrophoresis sample buffer, probes were electrophoretically separated and blotted on Hybond ECL nitrocellulose membranes. JNK-induced substrate phosphorylation was recorded with a phospho-c-Jun (Ser63) pAb and an IgG-HRP conjugate by ECL.

Conflict of interest

The authors declare no conflict of interest.

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