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Regulation of Activation-induced Fas (CD95/Apo-1) Ligand Expression in T Cells by the Cyclin B1/Cdk1 Complex*

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Fas (CD95/Apo-1) ligand-mediated apoptosis has been recognized as an important mechanism of cell-mediated cytotoxicity and maintenance of immune homeostasis. Chronically activated T cells undergo activation-induced cell death (AICD), which depends on simultaneous Fas and Fas ligand expression. Previous reports have suggested that AICD might be linked to cell cycle progression of T cells and therefore to the expression of cell cycle-related molecules. In particular, cyclin B1 has been implicated in the induction of AICD in T cells. In this study, we have investigated the role of cyclin B1 in AICD and the expression of effector molecules involved in this form of cell death. Our results show that inhibition of cyclin B1 blocks AICD in T cells through specific inhibition of Fas ligand expression but not Fas-induced apoptosis. This effect of cyclin B1 appears to be mediated through the cyclin B1/cyclin-dependent kinase 1 (Cdk1/Cdc2) complex because overexpression of cyclin B1 enhances *FasL* promoter activity, whereas a dominant-negative version of Cdk1 blocks Fas ligand promoter induction. We provide further evidence that cyclin B1/Cdk1 regulates *FasL* transcription through the regulation of NFκB activation because dominant-negative Cdk1 inhibits activation-induced NFκB reporter and Rel A-induced *FasL* promoter activity. In conclusion, our data support a link between cell cycle progression, activation-induced Fas ligand expression, and apoptosis in T cells.

Members of the tumor necrosis factor superfamily play a crucial role in immune homeostasis and cell-mediated cytotoxicity (reviewed in Ref. 1). In particular, Fas ligand (FasL, CD95L) has been implicated in homeostatic T and B lymphocyte apoptosis, and mice lacking either functional FasL (*gld/gld* mice) or Fas receptor (*lpr/lpr* or Fas gene-deficient mice) show accumulation of autoreactive T and B cells and development of autoimmune diseases with increasing age (reviewed in Refs. 2–5). Similarly, human patients with genetic defects in the *Fas* or *FasL* genes suffer from an autoimmune lymphoproliferative syndrome (ALPS), characterized by lymphoproliferative disorder, splenomegaly, and a systemic lupus erythematosus-like disease (6). This strongly supports the idea that Fas-induced apoptosis is important in the maintenance of lymphocyte homeostasis and self-tolerance.

Upon restimulation of previously activated T cells, cells rapidly undergo apoptotic cell death. We and others (7–10) have previously characterized this form of activation-induced cell death (AICD)[1] and have found that it requires the activation-induced expression of Fas and its ligand, and subsequent interaction causes the death of the cell. Most interestingly, AICD even occurs in a cell autonomous manner, indicating that FasL interacts with Fas on the same cell (7, 8). *In vivo*, however, a different mechanism may be responsible for the demise of activated T cells. Upon *in vivo* activation, T cells express the Fas receptor and become sensitized for Fas-induced apoptosis. The release of tumor necrosis factor-α by activated T cells induces the expression of FasL in intestinal epithelial cells and liver macrophages, which upon contact kill the activated T cells (11, 12). Although these data suggest that T cell-expressed Fas receptor, rather than the ligand, is crucial for homeostatic cell death of activated T cells during an immune response, other reports (13) have demonstrated that FasL-expressing T cells are responsible for the elimination of autoreactive B cells through Fas-induced apoptosis. Similarly, FasL is one of the major cytotoxic T cell effector mechanisms, and excessive FasL-mediated apoptosis and tissue destruction has been implicated in the pathogenesis of various diseases, such as hepatitis (14), acute graft-*versus*-host disease (15–17), and multiple sclerosis (18).

These findings strongly support the notion that the regulation of FasL expression on activated T cells is a crucial event in the control of various immune responses. We and others have characterized previously various transcription factors that regulate *FasL* gene expression in T cells. These include NFAT (19, 20), EGR-2 and -3 (21, 22), NFκB (23, 24), and AP-1 (23). More recently, we have found that c-Myc, a transcription factor crucially involved in cell cycle progression, directly regulates *FasL* transcription through interaction with a noncanonical consensus sequence in the *FasL* promoter (25–28). Regulation of *FasL* expression by c-Myc may ensure that only proliferating cycling T cells are capable of efficiently expressing FasL upon stimulation.

Most interestingly, another cell cycle-related molecule has

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[1] The abbreviations used are: AICD, activation-induced cell death; PMA, phorbol myristate acetate; AS, antisense; NS, nonsense; PBS, phosphate-buffered saline; RT, reverse transcriptase; HIV, human immunodeficiency virus.

been implicated in (Fas/FasL-dependent) AICD in T cells. Cyclin B1 associates with cyclin-dependent kinases and regulates their activities in promoting cell cycle progression (reviewed in Refs. 29 and 30). Fotedar *et al.* (31) have found previously that cyclin B1 is required for AICD in T cell hybridomas. In this study, we have investigated the specific role of cyclin B1 in the regulation of AICD. We have found that down-regulation of cyclin B1 by antisense oligonucleotides, or olomoucine, an inhibitor of cyclin-dependent kinases, specifically blocks FasL expression but not Fas-induced apoptosis. Most intriguingly, this effect on activation-induced FasL expression appears to be mediated by the cyclin B1/Cdk1 complex because overexpression of cyclin B1 was found to enhance *FasL* promoter activity, whereas a dominant-negative form of Cdk1 inhibited *FasL* promoter activity. We provide further evidence that the cyclin B1/Cdk1 complex may act on the activation of the *FasL* promoter through the regulation of NFκB activity. Our present data confirm a role for cyclin B1 in AICD and FasL expression, and further support a link between cell cycle regulation and FasL expression.

EXPERIMENTAL PROCEDURES

Reagents and Media—The T cell hybridoma A1.1 has been described previously (7, 19). Jurkat cells were obtained from the ATCC. Cells were cultured in Iscove's modified Dulbecco's medium containing 5% fetal calf serum, 2 mM L-glutamine, 50 mM β-mercaptoethanol, 100 units/ml penicillin, 100 μg/ml streptomycin (complete medium). For transfection of Jurkat cells, serum-free AIM-V medium (Invitrogen) was used. Normal murine T cell blasts were generated by dissociating the spleen of C57BL/6 mice between frosted microscopy glass slides and subsequent hypotonic lysis to remove erythrocytes. After resuspension in complete medium, cells were stimulated for 2 days with 1 μg/ml concanavalin A (Sigma), washed once to remove the lectin, and cultured with 100 units/ml recombinant IL-2 (Proleukin, Chiron) for another 3 days to generate T cell blasts.

Phorbol myristate acetate (PMA) was purchased from Sigma, and ionomycin was from Calbiochem. Olomoucine, iso-olomoucine, and nocodazole were from Alexis (Lausen, Switzerland). Hamster anti-mouse CD3ε (clone 145–2C11) was purified from culture supernatant by protein A affinity chromatography. Anti-human Fas was obtained from MBL (clone CH-11, Labforce, Nuningen, Switzerland). Anti-mouse FasL (clone MFL3) and anti-cyclin B1 (clone GNS-11) were from Pharmingen. Anti-c-Myc has been described previously (25). Phosphorothioate oligonucleotides were obtained from Microsynth (Balgach, Switzerland). The following previously published sequences were used: antisense (AS) cyclin B1, 5′-CAT CGG GCT TGG AGA GGG ATT-3′, and nonsense (NS) cyclin B1, 5′-TGA GGC ATT TGA GCT CGG GAG-3′ (31). The NS cyclin B1 oligonucleotide contains the same nucleotide composition as AS cyclin B1, however, with a random sequence.

Detection of Cyclin B1 Expression by Flow Cytometry—Protein levels of cyclin B1 in AS- or NS-treated cells were assessed by intracellular staining and detection by flow cytometry. Briefly, A1.1 cells were incubated with 10 μM AS or NS cyclin B1 overnight. After that, cells were harvested, fixed in 4% paraformaldehyde in PBS, and permeabilized in PBS containing 0.1% Triton X-100 and 1% bovine serum albumin. Cells were then stained with cyclin B1 antibody or isotype control and phycoerythrin-conjugated secondary antibody. After fixation in 4% paraformaldehyde, cyclin B1 expression was assessed on a FACScan (BD Biosciences) by using Cellquest software. Electronic gates were set around viable cells based on forward-side scatter properties. The inhibition of cyclin B1 expression was analyzed for statistical significance using Student's *t* test. Treatment of cells overnight with AS cyclin B1 resulted in ~27% reduction in cyclin B1-positive cells compared with control cells ($p > 0.005$).

AICD—A1.1 cells (5×10^4/200 μl) were preincubated with medium control or different concentrations of cyclin B1 AS or NS for 4 h or with olomoucine or iso-olomoucine for 30 min, prior to stimulation with medium control or immobilized anti-CD3 (1 μg/ml) in 96-well flat bottom plates. Cells were then incubated for 16 h, and apoptosis was assessed by annexin V staining, as described previously (25, 32).

Assessment of Functional FasL Expression—Functional FasL expression on activated T cells was assessed by the ability to induce DNA fragmentation in Fas-sensitive target cells (7, 19, 25)). A1.1 cells (8×10^4/100 μl) or murine T cell blasts (2×10^5/100 μl) were preincubated with medium control or different concentrations of olomoucine or iso-olomoucine for 30 min, or cyclin B1 AS or NS for 4 h, prior to transfer to control or anti-CD3-coated 96-well flat bottom plates. Fas-sensitive Jurkat cells were labeled with 5 μCi/ml [^{3}H]thymidine for 2 h at 1×10^6/ml in complete medium, washed two times in PBS, and resuspended in medium. Target cells (2×10^4/100 μl) were then added to the activated T cells. After 18 h, 5 mM EDTA in PBS was added, and nonfragmented [^{3}H]thymidine-labeled DNA was harvested on glass fiber filters and counted in a liquid scintillation counter. DNA fragmentation was assessed as follows: % DNA fragmentation = 100 × (1 − cpm experimental group/cpm control group) ± S.D. Specificity of FasL-mediated cytotoxicity was confirmed by neutralization with 10 μg/ml anti-FasL.

RT-PCR for FasL—A1.1 cells were either preincubated with medium control, olomoucine, iso-olomoucine (50 μM), or AS or NS cyclin B1 oligonucleotides (10 μM) for 4 h. After that cells were either left untreated or stimulated with plate-bound anti-CD3 (1 μg/ml) for 4 h. Cells were then harvested, and total RNA was isolated using Trizol reagents per the manufacturer's suggested protocol (Sigma). Two μg of total RNA were reverse-transcribed using a commercial kit and the manufacturer's suggested conditions (Promega, Madison, WI). For real time PCR 1 μl of 1:2 diluted RT product was then added to the PCR mix consisting of 12.5 μl of SYBR Green mix (Applied Biosystems, Foster City, CA), 1.5 μl of forward primer (5 μM), 1.5 μl of reverse primer (5 μM), and 8.5 μl of H_2O. The following primers were used: for murine *FasL*, forward 5′-CAGCAGTGCCACTTCATCTTGG-3′ and reverse 5′-TTCACTCCAGAGATCAGAGCGG-3′; for glyceraldehyde-3-phosphate dehydrogenase, forward 5′-TTCACCACCATGGAGAAGGC-3′ and reverse 5′-GGCATGGACTGTGGTCATGA-3′. The DNA was amplified in a TaqMan PCR machine (Applied Biosystems) using the following conditions: 2 min at 50 °C, 10 min at 95 °C, and 45 cycles for 5 s at 95 °C and 2 min at 60 °C. *FasL* gene expression was calculated by using the Sequence Detection System version 1.7 from Applied Biosystems. Alternatively, *FasL* and actin expression were also assessed by semi-quantitative conventional RT-PCR as described previously (33).

Cell Cycle Analysis and FasL Expression—A1.1 cells were either left untreated or cultured overnight with 100 μg/ml nocodazole to induce a cell cycle arrest in the G_2 phase. Cells were then washed two times with PBS and resuspended in medium without nocodazole. After 0, 2, 4, or 6 h an aliquot of the nocodazole-treated cells or control cells was harvested, and the DNA content, as a measurement of cell cycle progression, was analyzed by propidium iodide staining (7). At the same time points, aliquots were collected and either cultured for 1 h in medium or in anti-CD3-coated tissue culture plates. Cells were then harvested, and total RNA was isolated. *FasL* mRNA expression was analyzed using the assay-on-demand kit and 18 S rRNA as internal control as suggested by the manufacturer (Applied Biosystems, Foster City, CA).

Western Blotting—A1.1 cells were treated with medium control or nocodazole as described above. Cell cycle-synchronized cells were harvested at different time points and lysed in SDS-PAGE sample buffer. After electrophoresis on a 12% gel and transfer to nitrocellulose membrane, cyclin B1 and c-Myc expression were detected by using corresponding primary and secondary antibodies and chemiluminescence. Equal protein loading was confirmed by the detection of tubulin.

FasL Promoter Reporter Assay—FasL promoter activity was assessed as described previously (23, 25, 27). Briefly, Jurkat cells (10^6/ml) were transiently transfected with 3 μg of luciferase reporter construct, containing a 1.2-kb fragment of the human *FasL* promoter (HFLP) (23) or NFκB, AP-1, and NFAT luciferase reporter constructs (24), and 1 μg of pCMV β-galactosidase (Clontech) in AIM-V medium using lipofection or the Amaxa Nucleofection System according to the manufacturer's suggested protocol (Amaxa, Cologne, Germany). After overnight culture, cells were washed in PBS and resuspended in complete medium. Cells were then stimulated for 16 h with medium control or 50 ng/ml PMA and 500 ng/ml ionomycin, washed, and lysed in 100 μl of lysis buffer (0.2% Triton X-100, 92 mM KH_2PO_4, 0.91 mM K_2HPO_4, 1 mM dithiothreitol). Luciferase activity in the cell-free supernatant was assessed in a TD-20/20 luminometer (BioSystems, Sunnyvale, CA) and normalized with β-galactosidase activity to correct for different transfection efficiencies. In some experiments, cells were also co-transfected with human cyclin B1 (pCMV cyclin B1, kindly provided by Ruth J. Muschel, University of Pennsylvania, Philadelphia (34)), dominant-negative Cdk1 or Cdk2 (pCMV DN Cdk1, pCMV DN Cdk2, kindly provided by Ed Harlow, Massachusetts General Hospital Cancer Center, Charlestown, MA (35)), c-Myc (pSP271 Myc (25)), or the NFκB subunit Rel A (24). The different DNA concentrations in the different transfections were corrected with empty vector.

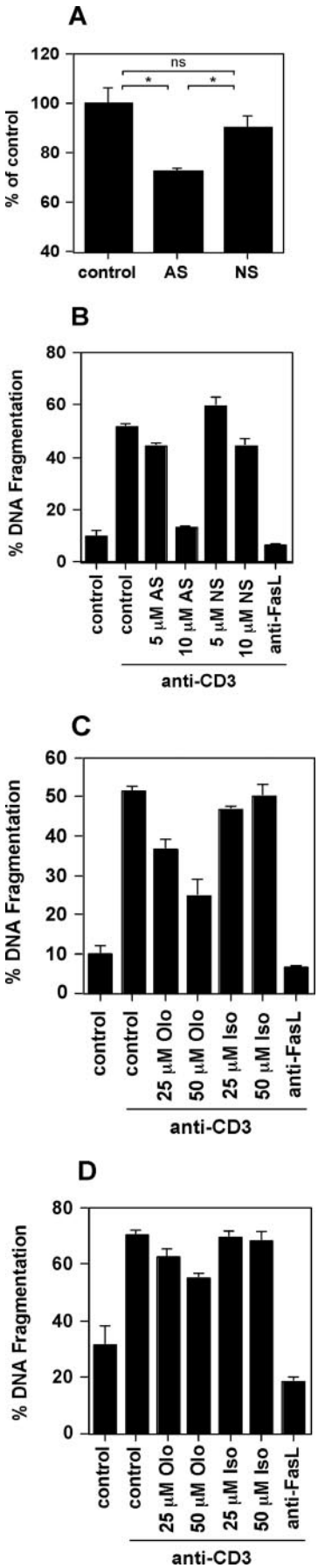

FIG. 1. **AS cyclin B1 and olomoucine block functional FasL expression.** *A*, inhibition of cyclin B1 expression upon treatment with cyclin B1 antisense oligonucleotides. A1.1 cells were treated with antisense (*AS*) or nonsense (*NS*) cyclin B1 oligonucleotides overnight, and the expression of cyclin B1 was assessed by flow cytometry. Results are presented as % positive cells compared with control treatment. Mean values ± S.D. of quadruplicate experiments are shown. An *asterisk* indicates $p < 0.005$ (Student's *t* test), and *ns* indicates not significant. *B*, A1.1 cells were preincubated with different concentrations of antisense (*AS*) or nonsense (*NS*) cyclin B1 oligonucleotides and then stimulated with plate-bound anti-CD3. *C* and *D*, A1.1 cells (*C*) or mouse T cell blasts (*D*) were preincubated with the indicated concentrations of olomoucine or iso-olomoucine prior to stimulation with anti-CD3. Functional FasL expression was assessed by induction of DNA fragmentation in Fas-expressing Jurkat cells. Mean values of triplicate experiments of typical experiments ± S.D. are shown.

RESULTS

Cyclin B1 Antisense Oligonucleotides Down-regulate Cyclin B1 Protein Expression—A previous report by Fotedar *et al.* (31) has shown that treatment of A1.1 T cell hybridomas with cyclin B1 antisense oligonucleotides resulted in inhibition of activation-induced apoptosis. Our next goal was to investigate the molecular basis by which cyclin B1 antisense inhibits this form of T cell apoptosis. In order to assess the activity of the antisense oligonucleotides used, A1.1 T cells were treated with cyclin B1 antisense or as a control nonsense oligonucleotides, and expression levels of cyclin B1 protein were examined by flow cytometry. Fig. 1*A* shows that treatment of cells with antisense oligonucleotides resulted in a clear and significant inhibition of cyclin B1 expression, whereas control nonsense oligonucleotides did not alter cyclin B1 expression.

Role of Cyclin B1/Cdk1 Complex in Activation-induced FasL Expression—Our previous findings have demonstrated that AICD in $CD4^+$ T cells occurs through activation-induced expression of Fas and FasL and subsequent Fas-induced apoptosis (7, 19, 36). In this process, the regulation of FasL expression appears to be a particularly important component because most inhibitors of AICD affect *FasL* transcription (37). We have thus investigated whether inhibition of cyclin B1 expression inhibits FasL expression. A1.1 cells rapidly express FasL on their cell surface upon stimulation with plate-bound anti-CD3 (7). This can be monitored by the ability to induce DNA fragmentation in Fas-sensitive targets, such as Jurkat cells (7, 19, 33). Fig. 1*B* demonstrates that activated A1.1 cells potently induce DNA fragmentation in Jurkat cells in a FasL-dependent manner, because target cell apoptosis was completely blocked by a neutralizing anti-FasL antibody. Most importantly, FasL-mediated DNA fragmentation was clearly reduced when A1.1 cells were preincubated with AS cyclin B1, but not with NS cyclin B1, oligonucleotides. This suggests that cyclin B1 expression is required for activation-induced FasL expression.

Cyclin B1 primarily associates with the cyclin-dependent kinase Cdk1 and regulates its activity (30). Olomoucine is a potent inhibitor of cyclin-dependent kinases, with a preference for Cdk1 and Cdk2. We thus analyzed its effect on activation-induced functional FasL expression. Similarly to the inhibition of cyclin B1 by AS oligonucleotides, olomoucine clearly inhibited the activation-induced expression of functional FasL and thus the DNA fragmentation of the target cells (Fig. 1*C*). No inhibition was seen with the control reagent iso-olomoucine.

Because T cell hybridoma cells, such as A1.1 cells, may behave differently than regular T cells, we confirmed these data using normal mouse T cells. Fig. 1*D* shows that olomoucine, but not iso-olomoucine, also inhibited functional FasL expression in mouse T cell blasts, although to a lesser degree than in A1.1 T cell hybridomas.

Inhibitors of the Cyclin B1/Cdk1 Complex Do Not Block Fas-induced Apoptosis—To ensure that the reagents used did not affect the Fas signaling in the target cells and would thus result in reduced DNA fragmentation, we analyzed the effect of

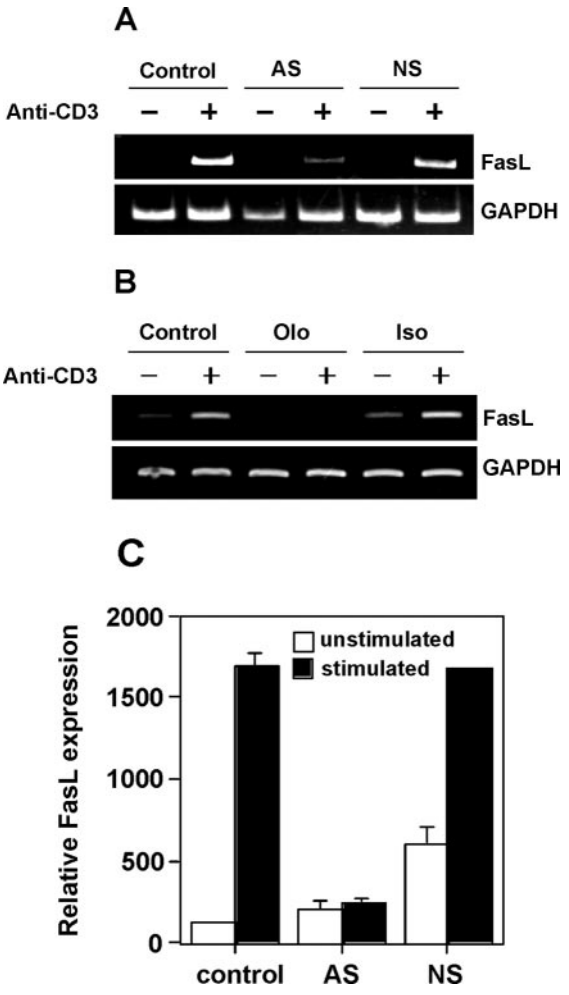

FIG. 2. **Inhibition of *FasL* transcription by antisense cyclin B1 and olomoucine.** A1.1 cells were preincubated with 10 μM antisense (*AS*) or nonsense (*NS*) cyclin B1 oligonucleotides (*A*), or 50 μM olomoucine (*Olo*) or iso-olomoucine (*Iso*) (*B*) and the stimulated with plate-bound anti-CD3 and analyzed for *FasL* or glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA expression by RT-PCR. *C,* analysis of activation-induced *FasL* and inhibition by antisense or nonsense cyclin B1 by real time PCR.

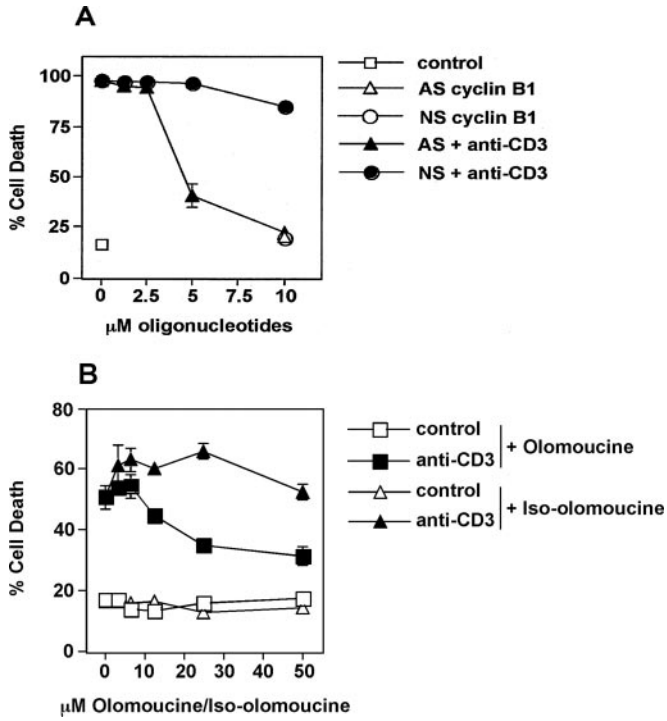

FIG. 3. **Inhibition of AICD by antisense cyclin B1 and olomoucine.** A1.1 cells were preincubated with either increasing concentrations of antisense (*AS*) or control nonsense (*NS*) cyclin B1 oligonucleotides (*A*) or the cyclin-dependent kinase inhibitor olomoucine or its inactive control iso-olomoucine (*B*). AICD was induced by activation with plate-bound anti-CD3 and assessed by annexin V staining. Mean values of triplicates of typical experiments ± S.D. are shown.

olomoucine and AS cyclin B1 on anti-Fas-induced apoptosis in Jurkat cells. Fas cross-linking by the anti-Fas IgM antibody potently induced apoptosis in Jurkat cells, as measured by annexin V staining. However, neither AS cyclin B1, olomoucine, nor their control reagents significantly inhibited apoptosis induction (data not shown). This confirms previous findings by others (38, 39) who showed that Fas-mediated apoptosis is independent of Cdk1 activity.

Antisense Cyclin B1 and Olomoucine Block Activation-induced FasL Transcription—To confirm that inhibition of the cyclin B1/Cdk1 complex by AS cyclin B1 or olomoucine targets *FasL* gene expression, we directly assessed *FasL* transcription by RT-PCR. Fig. 2*A* shows that *FasL* expression was not detected in unstimulated cells but was induced upon stimulation with plate-bound anti-CD3. Activation-induced *FasL* transcription was clearly reduced when cells were preincubated with AS cyclin B1 but not with NS control oligonucleotides. These analyses were also confirmed by quantitative real time RT-PCR (Fig. 2*C*) and RNase protection assay (data not shown). Similarly, preincubation of A1.1 cells by olomoucine, but not iso-olomoucine, inhibited activation-induced *FasL* transcription (Fig. 2*B*).

Inhibition of AICD in T Cells by Cyclin B1 Antisense Oligonucleotides and Olomoucine—Previous results from Fotedar *et al.* (31) have demonstrated that T cells undergo AICD primarily out of the G_2 phase of the cell cycle and that cyclin B1 is required for this form of cell death, because specific inhibition of cyclin B1 expression by antisense oligonucleotides blocked apoptosis. A1.1 T cell hybridomas rapidly undergo a FasL-dependent form of AICD upon stimulation by immobilized anti-CD3 (7, 19). Fig. 3*A* shows that a majority of the cells became apoptotic upon stimulation. Similarly, no inhibition of apoptosis induction was observed when cells were preincubated with control oligonucleotides (NS cyclin B1). In contrast, down-regulation of cyclin B1 by specific antisense oligonucleotides significantly reduced AICD in these cells. Thus, these results confirm the previous findings of Fotedar *et al.* (31).

Because the effect of cyclin B1 down-regulation on AICD in T cells might be related to a reduced activity of Cdk1, we tested whether treatment of T cells with olomoucine could inhibit AICD. Fig. 1*B* demonstrates that olomoucine blocked AICD in a dose-dependent manner and at relatively low concentrations. This effect appeared to be specific because the inactive form of the inhibitor, iso-olomoucine, showed no effect on AICD, even at high concentrations.

Inhibition of the Cyclin B1/Cdk1 Complex Affects the FasL Promoter Activity—We have previously used a human *FasL* promoter reporter construct to investigate the regulation of the *FasL* gene transcription (23, 25, 27). The *FasL* promoter is regulated by multiple transcription factors, and their activation upon T cell stimulation results in increased reporter gene transcription and translation. We have thus employed this system to investigate further the role of the cyclin B1/Cdk1 complex on *FasL* gene activation. Jurkat cells were transiently transfected with the human *FasL* promoter luciferase reporter construct (HFLP-Luc) and stimulated with PMA and ionomycin, simulating TCR stimulation (23, 25, 27). Fig. 4*A* shows that Jurkat T cell activation resulted in a strong induction of

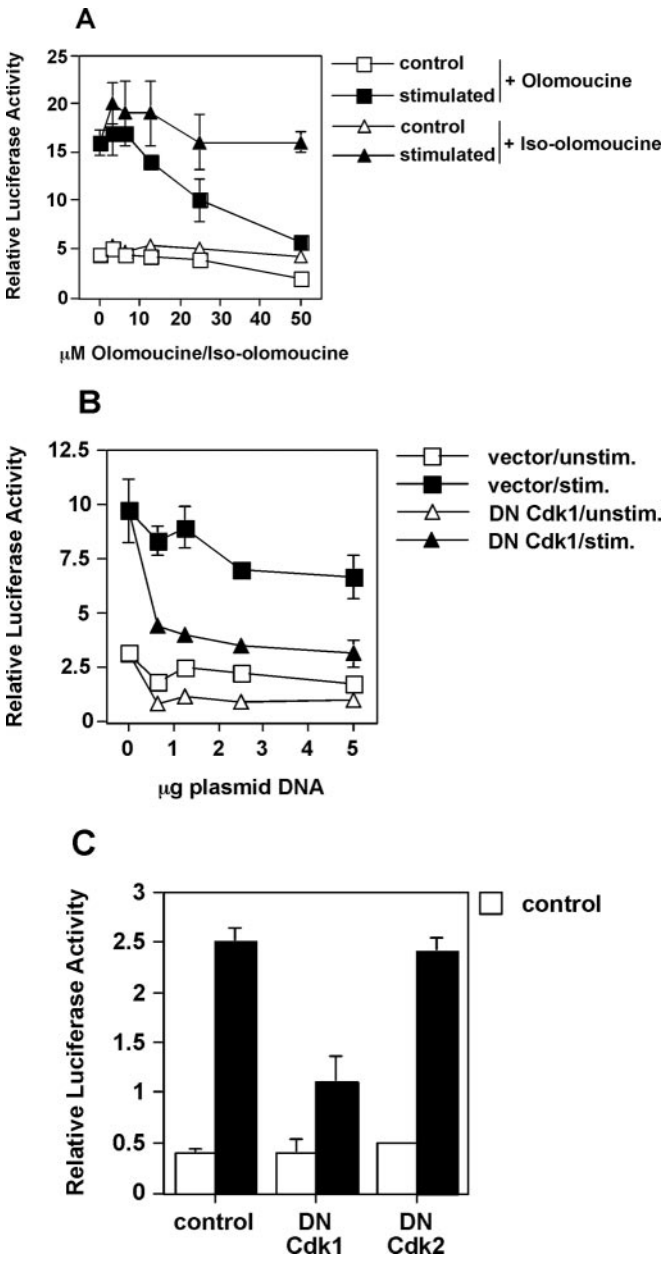

FIG. 4. **Inhibition of the Cdk1/cyclin B1 complex inhibits activation-induced *FasL* promoter activity.** *A*, Jurkat cells, transiently transfected with the human *FasL* promoter reporter construct, were treated with different concentrations of olomoucine or iso-olomoucine. Upon stimulation with PMA and ionomycin, luciferase induction was assessed. *B*, Jurkat cells were transiently transfected with the human *FasL* promoter reporter construct and different concentrations of dominant-negative Cdk1 (*DN Cdk1*) expression plasmid or empty vector control. After stimulation with PMA and ionomycin, luciferase induction was assessed. *C*, Jurkat cells were transfected with the human *FasL* reporter construct and dominant-negative Cdk1 (*DN Cdk1*) or dominant-negative Cdk2 (*DN Cdk2*). Upon stimulation, reporter gene expression was analyzed. Mean values of triplicates ± S.D. of typical experiments are shown.

FasL promoter activity. In agreement with our findings described above, preincubation of the cells with increasing concentrations of olomoucine, but not its control iso-olomoucine, strongly inhibited activation-induced *FasL* promoter activity in a dose-dependent manner. These findings further confirm that olomoucine inhibits AICD at the level of *FasL* transcription.

Cyclin B1 is the regulatory subunit of the cyclin B1/Cdk1 complex and regulates its kinase activity. Cyclin B1 levels are regulated through its transcription and degradation (reviewed in Refs. 29, 30, and 40). We have thus assessed whether overexpression of cyclin B1 may enhance activation-induced *FasL* promoter activity. Co-transfection of Jurkat cells with a cyclin B1 expression plasmid resulted in a 2–3-fold increase in *FasL* promoter activity over that observed in control transfected cells (data not shown). This suggests that cyclin B1 may represent a limiting factor in the regulation of *FasL* transcription.

Cyclin B1 primarily associates with Cdk1, regulating the activity of this complex during the G_2/M transition (reviewed in Refs. 29 and 30). We have further investigated the role of Cdk1 in the regulation of *FasL* promoter activity. For this purpose, we employed a dominant-negative form of the Cdk1 kinase (35). In contrast to cyclin B1, overexpression of dominant-negative Cdk1 resulted in a strong dose-dependent suppression of activation-induced *FasL* promoter activity (Fig. 4*B*). This pronounced inhibition of the *FasL* promoter activity by specific inhibition of the Cdk1 kinase confirms our findings that the Cdk1 kinase inhibitor olomoucine efficiently blocks *FasL* transcription (Fig. 3*B*) and promoter activity (Fig. 4*A*). Because olomoucine is not only specific for Cdk1, but also inhibits Cdk2 with the same ID_{50} (7 μM), we also investigated a potential role for Cdk2 in activation-induced *FasL* promoter activation. Fig. 4*C,* however, shows that overexpression of dominant-negative Cdk1, but not dominant-negative Cdk2, results in strong inhibition of *FasL* promoter activity. In conclusion, our data suggest an important and specific role for the cyclin B1/Cdk1 complex in the regulation of *FasL* promoter activity in T cells.

Cell Cycle Stage-dependent Expression of Activation-induced FasL—Because the cyclin B1/Cdk1 complex is crucially involved in cell cycle progression and, as shown here, also in FasL expression, we addressed the question whether FasL expression is preferentially induced at a given cell cycle stage. A1.1 cells were thus treated with nocodazole to achieve a cell cycle arrest and synchronization of the cells in G_2 (Fig. 5*A*). After that cells were released from the cell cycle block, and the DNA content and activation-induced *FasL* transcription was assessed every 2 h. Fig. 5*A* shows that at 0 h the majority of cells accumulated in G_2/M but rapidly progressed into the G_1 and S phase after release from the cell cycle block. At 0, 2, 4, and 6 h after release from the nocodazole block, cells were stimulated with anti-CD3 for 1 h, after which total RNA was isolated and *FasL* expression was assessed by real time RT-PCR. Whereas activation-induced *FasL* expression was readily detectable in control cells, no activation-induced increase was observed at 0 or 2 h after the nocodazole block (Fig. 5*B*). Most interestingly, however, strongly enhanced basal and activation-induced *FasL* expression was observed 4 h post-nocodazole block, which declined after 6 h. In order to correlate the progression in cell cycle with the expression of specific markers, we assessed the expression of cyclin B1 and c-Myc at these different time points upon release from the nocodazole block. Fig. 5*C* shows that cells arrested in G_2/M accumulated cyclin B1, whereas c-Myc levels were reduced. In contrast, cells that progressed into G_1 rapidly lost cyclin B1 expression, but c-Myc expression became induced. These data confirm the cell cycle stage-specific expression of cyclin B1 in G_2/M and c-Myc in G_1/S and further show that cyclin B1 expression precedes *FasL* expression. This strongly suggests that Cdk1/cyclin B1 activity is required for the expression and/or activation of molecules that regulate *FasL* transcription.

Role of NFκB in Cyclin B1/Cdk1-mediated Regulation of FasL Promoter Activation—In order to investigate the molecular target of cyclin B1/Cdk1 activity, we examined the effect of olomoucine on the activation-induced activity of transcription factors described previously to regulate *FasL* promoter activity, *i.e.* NFAT, AP-1, NFκB, and c-Myc (41). Jurkat cells were thus

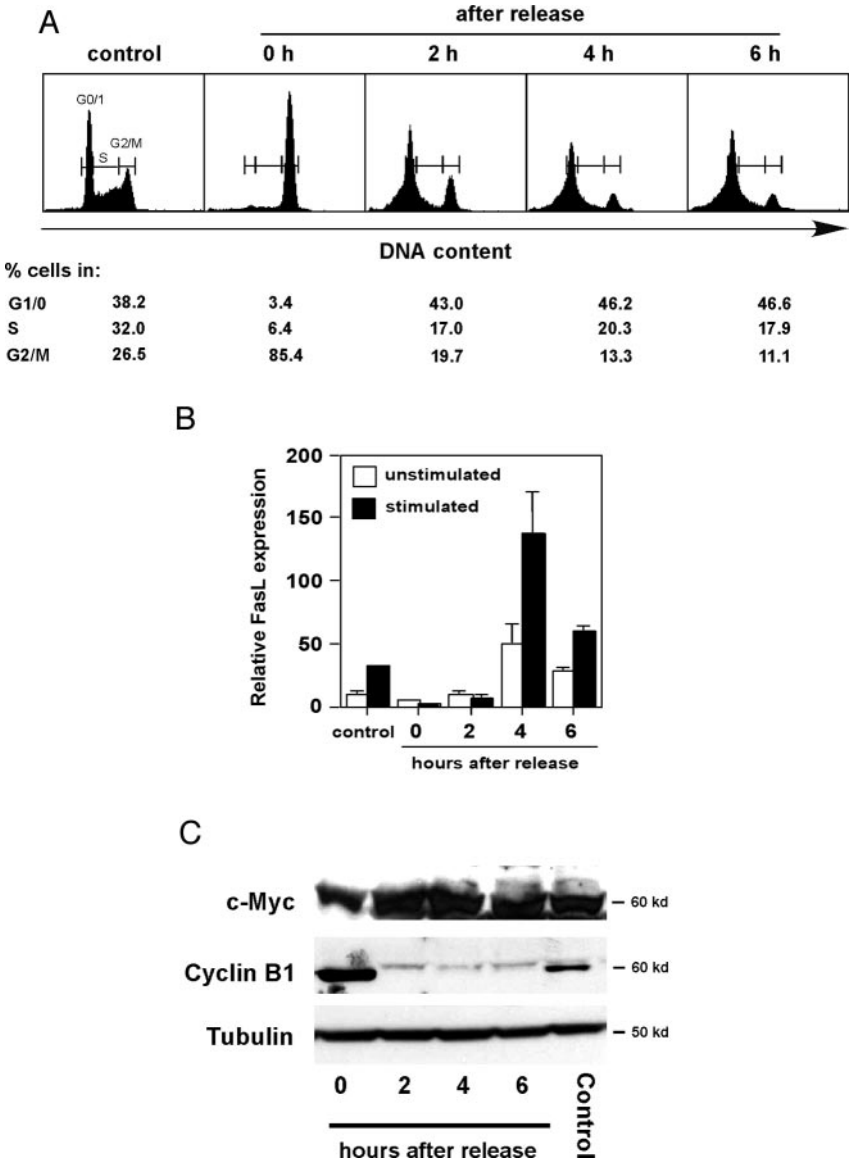

FIG. 5. **Cell cycle stage-dependent expression of *FasL*.** *A*, A1.1 cells were synchronized in G_2 with nocodazole and then simultaneously released. The DNA content was assessed at 0, 2, 4, and 6 h and compared with control cells. *Bars* indicate the different cell cycle stages, and the chart shows the percentage of cells in a given stage. *B*, control cells or synchronized cells were harvested at 0, 2, 4, and 6 h after nocodazole block and stimulated for 1 h with medium control (*unstimulated*) or plate-bound anti-CD3 (*stimulated*). *FasL* expression was then assessed by real time RT-PCR. A typical experiment out of three is shown. *C*, cells were synchronized as described in *A* and *B*, and aliquots harvested after different time intervals were analyzed for c-Myc and cyclin B1 expression by Western blot. Equal loading was confirmed by the detection of tubulin.

transfected with the different luciferase reporter constructs, and the effect of olomoucine on activation-induced luciferase activity was examined. Most interestingly, we found a pronounced inhibition of NFκB reporter activity by olomoucine but not by iso-olomoucine (Fig. 6*B*), suggesting that inhibition of Cdk1 leads to reduced NFκB activation. This result was confirmed by overexpression of dominant-negative Cdk1, which also significantly blocked activation-induced NFκB reporter activity (Fig. 6*C*). Finally, we assessed whether dominant-negative Cdk1 could also inhibit NFκB-induced *FasL* promoter induction. Jurkat cells were thus transiently transfected with the *FasL* promoter construct, and the NFκB subunit Rel A and dominant-negative Cdk1. Fig. 6*D* shows that overexpression of Rel A showed a dramatic increase in activation-induced *FasL* promoter activity, which was significantly inhibited by co-expression of dominant-negative Cdk1. In contrast, activation-induced AP-1 (Fig. 6*E*) and NFAT (Fig. 6*F*) reporter activity was not inhibited by overexpression of dominant-negative Cdk1. Thus, these data indicate that cyclin B1/Cdk1 regulates *FasL* promoter activity through the control of NFκB activation.

DISCUSSION

Cell cycle progression and associated cell growth on the one hand, and cell death on the other hand appear to be fundamentally opposing events. Yet there is accumulating evidence that cell growth and cell death have more things in common than previously thought. For example, the transcription factor c-Myc is induced by mitogenic stimuli and is crucial for the transition from the G_1 to the S phase in the cell cycle (reviewed in Ref. 42). However, inappropriate expression of c-Myc is also a potent inducer of apoptosis (43, 44). Similarly, various studies have found differential requirements of distinct cell cycle stages for the induction of apoptosis. Of particular interest in this regard is the link between cell cycle stage and the induction of AICD in T cells. Originally stimulated by the observation that resting T cells are resistant to AICD, but become sensitive upon entry into cell cycle (45), several research groups have investigated this issue, however, with quite conflicting results. Although some studies have found that T cells can undergo AICD from every stage of the cell cycle (46), others have found a requirement for the G_1, S, or G_2/M phase, respectively (31, 47–51). Thus, depending on the model system and the technique used to synchronize T cells in a given cell cycle stage, completely opposing results were obtained. However, although it is currently clear that AICD in $CD4^+$ T cells proceeds primarily via Fas/FasL interaction (7–10, 19, 36), most studies have not investigated the individual requirements of the expression of

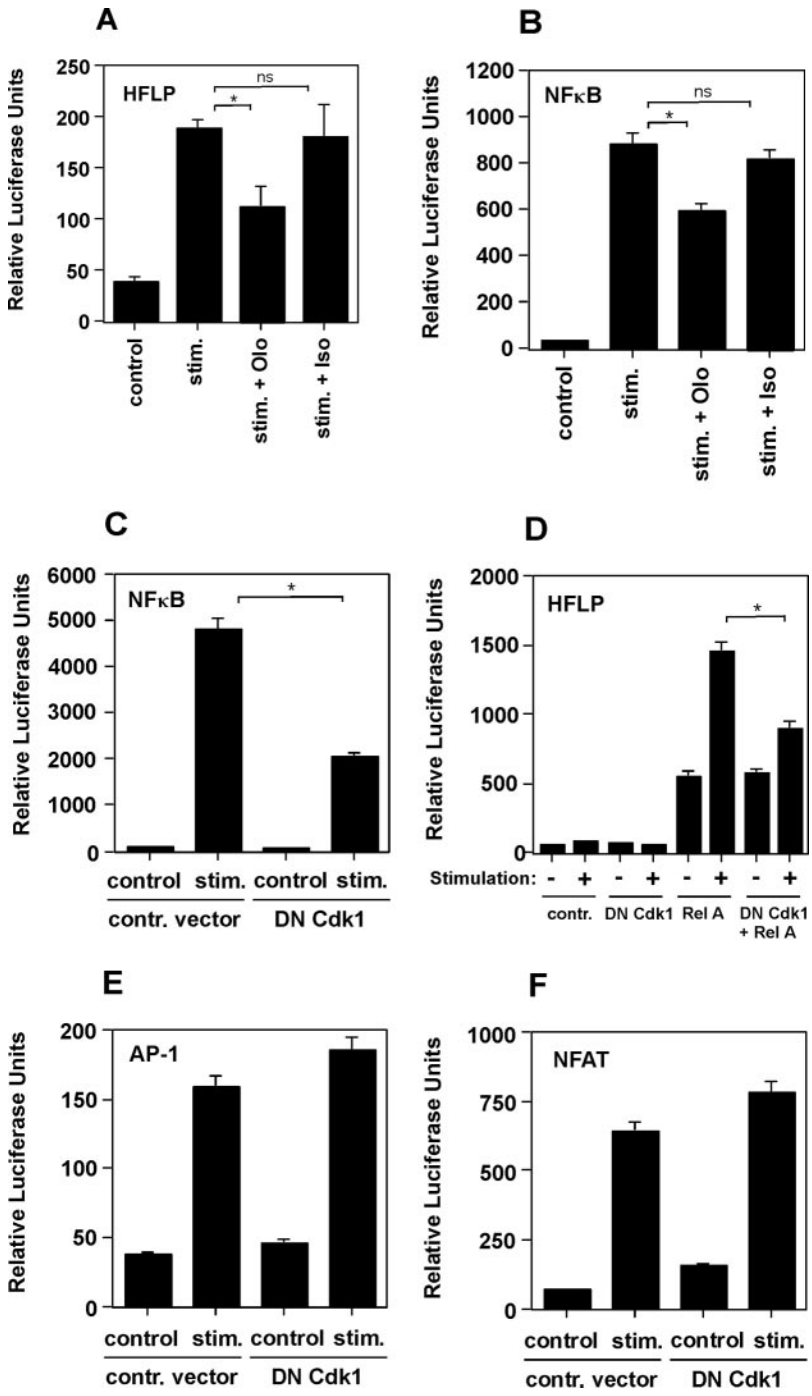

FIG. 6. **Role of NFκB in cyclin B1/Cdk1-regulated *FasL* promoter activation.** Jurkat cells were transfected with the human *FasL* promoter construct (*HFLP*) (*A*) or an NFκB reporter construct (*NFκB*) (*B*). Cells were the treated with olomoucine (*Olo*) or iso-olomoucine (*Iso*) and stimulated (*stim.*) with PMA and ionomycin, and luciferase activity was assessed. *C*, cells were transfected with the NFκB reporter construct and vector control or dominant-negative Cdk1 (*DN Cdk1*). Upon stimulation, luciferase activity was measured. *D*, cells were transfected with the human *FasL* reporter construct and either DN Cdk1, Rel A, or both, and luciferase activity of stimulated and unstimulated cells was assessed. *E* and *F*, cells were transfected with the AP-1 reporter (*E*) or NFAT reporter (*F*) construct, and vector control or dominant-negative Cdk1 (*DN Cdk1*). Upon stimulation, luciferase activity was measured. Mean values of triplicate or quadruplicate experiments ± S.D. of typical experiments are shown. An *asterisk* indicates $p < 0.05$ (Student's *t* test).

these key molecules involved in mediating AICD for distinct cell cycle stages. We have reported previously (25–28) a link between cell cycle and FasL expression. c-Myc is an essential transcription factor for the induction of *FasL* transcription in T cells. Similarly, in this study we have now characterized a role of the cyclin B1/Cdk1 complex in the expression of FasL. Thus, our study confirms a previous report by Fotedar *et al.* (31) who have found a requirement for cyclin B1 in the induction of AICD in T cells.

The discrepancies between different studies in the relative importance of distinct cell cycle stages for the induction of AICD are difficult to reconcile but may be related to our own findings. Cyclin B1 expression peaks in the G_2/M transition and thus contributes to the regulation of the cyclin B1/Cdk1 complex (reviewed in Refs. 29 and 30). However, inappropriate expression of cyclin B1 and activation of the cyclin B1/Cdk1 complex has been reported in various systems (52–56). Thus, if cell cycle dependence is linked to the expression of cyclin B1 and activation of the cyclin B1/Cdk1 complex, AICD and apoptosis may be induced at different stages of the cell cycle, depending on the cell type used. A G_2/M requirement for the induction of AICD, as reported by Fotedar and colleagues (31), is certainly in agreement with our own findings, because cyclin B1 is primarily expressed in G_2/M and found to be required for activation-induced FasL expression and AICD.

Another pitfall in the interpretation of the different studies is the fact that FasL is not only regulated at a transcriptional level but is often stored as preformed protein in granule-like vesicles (33, 57) and rapidly transported to the cell surface upon T cell activation. We have found previously that FasL cell surface expression upon TCR ligation of T cell blasts is only partially blocked by protein synthesis inhibitors (33). This may

also explain why olomoucine only partially inhibited functional cell surface FasL expression on mouse T cell blasts (Fig. 2*C*), whereas olomoucine effectively blocked *FasL* transcription and functional FasL expression in A1.1 T cell hybridomas (Figs. 2*B* and 3*B*). Under these circumstances (*i.e.* preformed FasL), it is unlikely that distinct cell cycle stage and associated molecules are required for the activation-induced release of FasL and subsequent Fas-induced apoptosis. Thus, we have found that activation-induced *FasL* transcription is dependent on the activation of the cyclin B1/Cdk1 complex; however, Fas-induced apoptosis is not.

Fas/FasL interactions do not only seem to play a role in normal T cell homeostasis but may also be the underlying mechanism of different immunopathologies. In particular, Fas/FasL-mediated apoptosis has been suggested to be at least in part responsible for the loss of $CD4^+$ T cells during HIV infection (reviewed in Ref. 58). HIV infection of T cells can induce *FasL* transcription, and the HIV Tat protein has been found to support EGR-2/-3- and NFκB-mediated *FasL* transcription (59, 60). It is thus of interest to note that T cells from HIV-infected patients express inappropriate levels of cyclin B1 and show elevated Cdk1 kinase activity (54, 55). Thus, enhanced cyclin B1/Cdk1 complex activity may contribute to HIV-induced FasL expression and thus the selective loss of $CD4^+$ T cells in HIV patients.

The questions remains why FasL expression in T cells is linked to cell cycle-associated molecules. It is clear that resting T cells only inefficiently express FasL upon TCR stimulation; however, primed T cells respond to restimulation with the rapid and abundant expression of FasL. Primary T cell stimulation occurs predominantly in lymph nodes and spleen, at a place where FasL expression would cause severe harm and probably would rather inhibit the induction of an immune response than support it. In contrast, stimulation of primed T cells occurs directly in the target tissue where FasL expression is required for the induction of cell-mediated cytotoxicity. A requirement for transcription factors or other molecules only expressed in cycling T cells may thus ensure appropriate expression of FasL only in the effector phase of T cell activation. The cyclin B1/Cdk1 complex can regulate target gene expression through the phosphorylation of regulatory proteins (reviewed in Refs. 29 and 30). However, it cannot directly induce gene expression, such as *FasL*. It is thus likely that the cyclin B1/Cdk1 complex is required for the activation or expression of a transcription factor involved in *FasL* expression. A possible candidate is certainly c-Myc. c-Myc is a cell cycle-linked transcription factor and is expressed predominantly in the G_1/S transition, thus shortly after the cyclin B1/Cdk1 complex activation. In addition, we have previously found that c-Myc is required for AICD and activation-induced FasL expression in T cells (25–28). In support of this hypothesis, we observed that in cell cycle-synchronized cells FasL expression was preferentially induced in cells that had progressed into the G_1/S stage (Fig. 5*A*), where c-Myc expression was also most abundant (Fig. 5*C*). Although it is intriguing to suggest that the cyclin B1/Cdk1 complex might be implicated in c-Myc expression or activation, and thus FasL induction, we failed so far to demonstrate a definitive link between the cyclin B1/Cdk1 complex, c-Myc and FasL. In contrast, we observed that both olomoucine and transient overexpression of dominant-negative Cdk1 significantly blocked stimulation-induced NFκB activation but not AP-1 or NFAT activation, suggesting that cyclin B1/Cdk1 may regulate *FasL* transcription through the control of NFκB activity. These findings were further confirmed by the demonstration that overexpression of Rel A, an NFκB subunit and important regulator of *FasL* gene expression (24), strongly enhanced basal and activation-induced *FasL* promoter activity, which was significantly attenuated upon co-expression of dominant-negative Cdk1. This shows that cyclin B1/Cdk1 inhibition can inhibit both activation-induced as well as Rel A-driven *FasL* promoter activity and suggests an important role for cyclin B1/Cdk1 in the regulation of NFκB activity and subsequent *FasL* promoter induction.

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