Coupling of the Cell Cycle and Apoptotic Machineries in Developing T Cells

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Proliferation and apoptosis are diametrically opposite processes. Expression of certain genes like c-Myc, however, can induce both, pointing to a possible linkage between them. Developing CD4+/CD8+ thymocytes are intrinsically sensitive to apoptosis, but the molecular basis is not known. We have found that these noncycling cells surprisingly express many cell cycle proteins. We generated transgenic mice expressing a CDK2 kinase-dead (CDK2-DN) protein in the T cell compartment. Analysis of these mice showed that the CDK2-DN protein acts as a dominant negative mutant in mature T cells as expected but surprisingly, it acts as a dominant active protein in CD4+/CD8+ thymocytes. The levels of CDK2 kinase activity, cyclin E, cyclin A, and other cell cycle proteins in transgenic CD4+/CD8+ thymocytes are increased. Concurrently, caspase levels are elevated, and apoptosis is significantly enhanced in vitro and in vivo. E2F-1, the unique E2F member capable of inducing apoptosis when overexpressed, is specifically up-regulated in transgenic CD4+/CD8+ thymocytes but not in other T cell populations. These results demonstrate that the cell cycle and apoptotic machineries are normally linked, and expression of cell cycle proteins in developing T cells contributes to their inherent sensitivity to apoptosis.

Proliferation and apoptosis are two opposing processes. In the first instance, cells overcoming the G1 checkpoint will enter the S phase, replicate their DNA and undergo mitosis, resulting in separation of their daughter cells. Different cell cycle phases have specific cell cycle protein expression profiles (1, 2). Cyclin-dependent kinases (CDK)2 are master regulators of cell cycle progression. Their activities are in turn regulated by CDK activating kinases, cyclins, and the CDK inhibitors. CDK2 is crucial for cell cycle progression in cell lines and functions in the G1/S transition and the S and G2 phases of the cell cycle. In most cells, mitogenic stimulation leads to increased transcription of the first G1 kinases, CDK4 or CDK6 and cyclin D. The increased protein levels of CDK4-CDK6-cyclin D titrate p27 away from the inactive CDK2-cyclin E/p27 complex, resulting in activation of the CDK2-cyclin E complex. CDK2 mediates its functions through phosphorylation of many substrates, including Rb and p27. Rb phosphorylation releases E2F from its pocket, allowing E2F to activate transcription of S phase-related genes (3, 4). p27 phosphorylation initiates its own eventual degradation through the Skp2 ubiquitin E3 ligase (1, 2). Overexpression of a dominant negative CDK2 mutant (D146N) in cell lines and in primary neural cells induces a cell cycle block (5–8). Similarly, overexpression of a CDK2 inhibitor like p27Kip1 causes cell cycle arrest and inhibits initiation of DNA synthesis in mammalian cells (9).

Apoptosis is mostly mediated through either the extrinsic or the intrinsic pathway (10–12). The extrinsic pathway is mediated by the tumor necrosis factor family of death receptors including Fas and tumor necrosis factor receptor. The intrinsic pathway involves mitochondria and is mediated by the Bcl-2 family members (13). Activation of Bim, a BH3-only protein, for example, can result in the release of cytochrome c and Smac from mitochondria (14–16). Overexpression of Bcl-2 or Bcl-x can block this process. Caspases are essential components of the apoptotic machinery, including initiator caspases (caspase-8 and -9) and effector caspases (caspase-3, -6, and -7). Both pathways lead to the activation of the effector caspases, resulting in apoptosis.

Although the proteins participating in proliferation and apoptosis are clearly distinct, these two processes must be linked at some levels. During development, the cells that do not proliferate well (e.g. under a growth factor limiting condition) are purged actively through apoptosis (17, 18). Enforced expression of E2F-1 can result in proliferation, but it also increases transcription of several caspases, resulting in apoptosis (19–21). c-Myc, a protein required for normal proliferation, is an oncogenic and pro-apoptotic molecule when it is overexpressed and deregulated (22–24). One of the possible consequences for the coupling of proliferation and apoptotic machineries is for proliferating cells to die by default. For a cell to replicate successfully, it needs to suppress its inherent apoptotic program (18, 25). Alternatively, proliferating cells might acquire “addiction” to proliferative signals to gain resistance to apoptosis (25). The coupling of cell cycle activities and apoptosis in the first scenario is thought to prevent widespread spontaneous tumorigenesis in mammals (22). However, whether and how this coupling operates, especially under physiological conditions, is not completely understood.

T cell development is a complicated process with many decision steps involving proliferation and apoptosis (26, 27).
CD4\(^-\)CD8\(^-\) (DN) thymocytes, the early T cell progenitors, will only proliferate to become CD4\(^+\)CD8\(^+\) thymocytes (double positive (DP)) if they successfully rearrange their T cell receptor (TCR) \(\beta\) gene segments. TCR\(\alpha\) rearrangements take place at the DP stage. Positive selection then selects the expressing TCR\(\alpha\)\(\beta\) receptors with the proper affinity for the major histocompatibility complex-antigen complex and allows them to differentiate into mature thymocytes (CD4\(^-\)CD8\(^-\) or CD4\(^-\)CD8\(^+\) SP cells). Cells expressing TCR that bind too strongly to the major histocompatibility complex-antigen complex undergo negative selection and die by apoptosis (28). DP cells also have a finite lifespan in vivo of \(~4\)–\(~5\) days (29). Because of the lack of allelic exclusion, the TCR\(\alpha\) gene keeps rearranging until DP cells die (30). \(\gamma\alpha\) gene segments first rearrange to the 5\(^-\)-most \(\alpha\) segments (31). Rearrangements will continue to the more 3\(^-\)-most \(\alpha\) segments until DP cells undergo positive selection or die (32). In Bcl-xL transgenic mice where DP thymocytes live for a longer period, most of the TCR\(\alpha\) rearranged genes utilize the 3\(^-\)-most \(\alpha\) segments (33, 34). In contrast, in mice with short-lived DP thymocytes like ROR\(\gamma\)\(^-/-\) mice (35), the TCR\(\alpha\) repertoire consists of mostly 5\(^-\)-most \(\alpha\) (33, 34). Wild-type DP cells with an average lifespan of \(~4\)–\(~5\) days have the most diverse and balanced \(\alpha\) usage. Thus, the inherent sensitivity of DP cells to apoptosis is immunologically important.

Consistent with their short lifespan in vivo, DP cells are inherently sensitive to apoptosis in vitro. DP cells die quickly in vitro, and the addition of stimuli like anti-CD3/CD28 antibodies, phorbol 12-myristate 13-acetate and ionomycin, glucocorticoid, or irradiation leads to accelerated apoptosis (36–39). The molecular basis for the inherent sensitivity of DP to cell death is not clear. We have observed recently that DP cells express many of the cell cycle-specific proteins (40), which are down-regulated upon positive selection. Intracellular staining studies showed that these cell cycle proteins are uniformly distributed in all of the DP cells. Based on the proliferation-apoptosis coupler hypothesis (18, 25), we hypothesized that expression of these cell cycle proteins in DP thymocytes, although it does not result in proliferation, contributes to their sensitivity in apoptosis. To test this hypothesis, we altered the CDK2 kinase activity in DP cells and assessed their state of apoptosis.

**EXPERIMENTAL PROCEDURES**

**Generation of Dominant Negative CDK2 Transgenic Mice—**The pTG4 vector was constructed based on the pTG2 vector that we generated before (41). We cloned the murine stage-specific thymocyte enhancer (TE) and the silencer into the pTG4 vector at the HpaI site located between the CD4\(^+\) and silencer was cloned into the same vector at the 3\(^-\)end of the TE sequence. The CD4\(^+\) gene keeps rear-rangings until DP cells die (30). V\(\alpha\) gene segments first rearrange to the 5\(^-\)-most \(\alpha\) segments (31). Rearrangements will continue to the more 3\(^-\)-most \(\alpha\) segments until DP cells undergo positive selection or die (32). In Bcl-xL transgenic mice where DP thymocytes live for a longer period, most of the TCR\(\alpha\) rearranged genes utilize the 3\(^-\)-most \(\alpha\) segments (33, 34). In contrast, in mice with short-lived DP thymocytes like ROR\(\gamma\)\(^-/-\) mice (35), the TCR\(\alpha\) repertoire consists of mostly 5\(^-\)-most \(\alpha\) (33, 34). Wild-type DP cells with an average lifespan of \(~4\)–\(~5\) days have the most diverse and balanced \(\alpha\) usage. Thus, the inherent sensitivity of DP cells to apoptosis is immunologically important.

Add to the 3\(^-\)end of the silencer to facilitate excision of the entire insert for transgenic injections. To generate the dominant negative CDK2 transgenic construct, the human dominant negative CDK2 was obtained by digesting the pCMV-CDK2-DN plasmid (Addgene) using BamHI and subcloned into the pTG4 vector at the HpaI site located between the CD4\(^+\) and silencer similar to those reported by Adlam and Siu (45). A truncated human growth hormone (hGH) gene provides a poly(A) site and introns for efficient transgenic expression. 

**Cell Cycle and Apoptosis in Thymocytes**

**Flow Cytometric Analysis—**The cells were prepared from lymphoid organs isolated from mouse littermates. After red blood cell lysis, they were stained with the indicated antibodies. Anti-CD4, anti-CD8, anti-TCR\(\beta\), anti-CD44, anti-CD25, and anti-heat stable antigen or CD24 antibodies were purchased from BD Pharmingen. Intracellular staining was performed as described previously (42). Briefly, formaldehyde was added directly to culture medium to a final concentration of 2% and incubated for 10 min at room temperature. The cells were pelleted, resuspended in ice-cold methanol, and incubated for 15–30 min on ice. The cells were then washed three times with staining buffer (0.5% bovine serum albumin in phosphate-buffered saline) and stained with antibodies of interest. Fresh isolated thymocytes and peripheral T cells were fixed and stained with anti-CDK2 and anti-

![FIGURE 1. Generation of the CDK2-DN transgenic mice.]
Ki-67 antibodies. Column-purified peripheral T cells were stimulated with plate-bound anti-CD3 (2 μg/ml) and anti-CD28 (2 μg/ml) for 48 h, fixed, and then stained with anti-Ki-67 antibody. Anti-CDK2 antibody was purchased from Santa Cruz Biotechnology, and anti-Ki-67 antibody was purchased from BD Bioscience. BrdUrd incorporation was performed as follows: fresh isolated thymocytes were stimulated with plate-bound anti-CD3 (2 μg/ml) and anti-CD28 (2 μg/ml) for 8 h, and 10 μM of BrdUrd was added into cell culture medium for another 16 h. The BrdUrd staining was measured after the cells were fixed. The anti-BrdUrd antibody was purchased from Caltag.

Carboxyl Fluorescein Diacetate Succinimidyl Ester (CFSE) Staining—Column-purified peripheral T cells were labeled with 10 μM CFSE (Invitrogen) in phosphate-buffered saline, 0.1% bovine serum album at 37 °C for 10 min. After washing twice with 10% fetal calf serum/RPMI 1640, T cells were stimulated with 1 μg/ml plate-bound anti-CD3 and anti-CD28 antibodies for 3 days. The stimulated cells were harvested, stained with phycoerythrin-cy5 anti-CD4 and phycoerythrin anti-CD8, and then analyzed for CFSE fluorescence by flow cytometry.

Western Blotting—Cell lysates were prepared from sorted DP thymocytes, CD4+CD8− SP thymocytes, CD4+ peripheral T cells, or CD8+ peripheral T cells. The following antibodies were used in this study: anti-CDK2, anti-cyclin E, anti-cyclin A, anti-p130, anti-p27, anti-p107, and anti-Rb antibodies (purchased from Santa Cruz Biotechnology) and affinity-purified anti-ERK5 antibody (43). Total thymocytes isolated from either wild-type or transgenic mice were put in culture for 4 or 8 h, and cell lysates were prepared. Anti-FoxM1, anti-c-Myc, anti-caspase-3, and anti-caspase-8 antibodies were purchased from Santa Cruz Biotechnology; anti-p53 antibody was purchased from Cell Signaling; anti-actin and anti-p19 antibodies were purchased from Abcam.

Co-immunoprecipitation—Whole cell lysates were prepared from total thymocytes, lymph node cells, and activated lymph node cells, which were stimulated with soluble anti-
CD3/CD28 (1 μg/ml) for 16 h. Co-immunoprecipitation was performed using rabbit anti-p27 antibody (Santa Cruz Biotechnology). Co-immunoprecipitates were blotted with mouse anti-p27, mouse anti-cyclin E, and mouse anti-CDK2 antibodies (Santa Cruz Biotechnology).

CDK2 Kinase Assay—Whole cell extracts were prepared from sorted DP thymocytes. CDK2 kinase activity was measured as described previously (44). Briefly the complexes were immunoprecipitated overnight at 4 °C using anti-CDK2, anti-cyclin E, or anti-cyclin A antibody (Santa Cruz Biotechnology) and captured with protein G beads (Pierce). Kinase assays were performed using histone H1 as the substrate (Sigma).

Apoptosis Assay—After cells was isolated and cultured with or without anti-CD3/CD28 for the indicated time, the thymocytes were collected and stained with cell surface markers, CD4 and CD8, as well as annexin V (BD Biosciences) and 7-aminoactinomycin-D for flow cytometric analysis. For TUNEL assay, freshly isolated thymocytes were fixed and labeled using the manufacturer’s suggested protocol (R & D Systems). DNA fragmentation was detected by flow cytometry.

RT-PCR Analysis of Jα Usage—RNA was isolated from column-purified peripheral T cells, and cDNA was synthesized using oligo(dT) primers. PCR products were amplified by using primers Va8 (5′-CAGACAGAGGCTTGGTAC) and Caα (5′-TGGGTTGTCTTCTTGAAG) and cloned for sequencing using the Caα primer (5′-GGCGCATCATTGGGAGTGC).

Quantitative Real Time RT-PCR—Total RNAs were extracted from sorted DP thymocytes, CD4+ CD8− SP thymocytes, and column-purified mature splenic/lymph node T cells. The primer sequences used in RT-PCR are: E2F-1 forward (5′-GCAGATCCATACCCCTCGT) and reverse (5′-CCCATT- TTGGTCTGTCTCAAT), E2F2 forward (5′-GATGGAGTCCTTTGGACCTGAA) and reverse (5′-GATGAGCTGGATACCCCT- CCA), E2F3 forward (5′-TGGAGCTGTCTGAGGATGG) and reverse (5′-GCCAGGGTGACGGTCTCC), E2F4 forward (5′-CCAAGGACGGAATGTACG) and reverse (5′-CCAGAAGCTCAGGGAGCAT), endogenous CDK2 forward (5′-GCCGGAGGTCCAGGTGTG) and reverse (5′-GGACCTGACAGGAAGC). cyclin E forward (5′-CTCTCAAGTGCCAGATTG) and reverse (5′-CCA-

RESULTS

Generation and Analysis of Transgenic Mice Expressing a CDK2 Dominant Negative Protein—To see whether disruption of one or more of the cell cycle proteins in DP thymocytes might affect their sensitivity to apoptosis, we expressed a known CDK2 kinase-dead dominant negative protein (D146N) (5) in DP and CD4+ SP/mature T cells (henceforth termed CDK2-DN). A combination of CD4 regulatory elements (distal and proximal CD4 enhancers plus the CD4 silencer; Fig. 1A) was used for the transgenic construct (45). These elements allow expression of the transgene in DP and CD4+ SP cells but not in DN thymocytes, thus allowing us to bypass the proliferative transition stage between DN and DP cells. Several transgenic founders were obtained. Two of them (lines 1 and 3) were chosen for analysis and yielded similar results. Intracellular staining with CDK2-specific antibody, which detects both the endogenous and transgenic proteins, showed a higher expression level of CDK2 in the transgenic DP, CD4+ SP, and mature CD4+ T cell populations but not CD8+ SP and mature CD8+ T cell populations (Fig. 1B). CD8+ intermediate single positive cells, an intermediate stage between DN and DP thymocytes, also expressed some transgenic proteins, but DN thymocytes did not, including DN1 and DN2/3 populations (Fig. 1B).

Thymus, spleen, and lymph nodes from both CDK2-DN transgenic lines were analyzed for their cellularities and cell composition by flow cytometric analysis. Transgenic CDK2-DN mice have a 1.5−2-fold decrease of thymocyte cellularities (CDK2-DN transgenic (Tg); line 1, 160 ± 18 × 106; non-Tg, 252 ± 41 × 106, n = 5; line 3, 128 ± 51 × 106; non-Tg, 216 ± 54 × 106, n = 7). Flow cytometry showed a decrease of DP, CD4+, and CD8+ SP thymocytes but no change in the absolute cell number of DN thymocytes (Fig. 2, A and C). Analysis of DN population with a combination of CD44- and CD25-specific antibodies showed a normal progression of early T cell development from DN1 (CD44+CD25−) to DN2 (CD44+CD25+) to DN3 (CD44+CD25+) to DN4 (CD44−CD25−) (Fig. 2B). Positive selection occurred normally as indicated by high levels of heat stable antigen or CD24 expression in DP cells followed by its down-regulation in SP thymocytes (Fig. 2B), suggesting that the lower cell number of transgenic SP thymocytes is the direct result of the decreased number of DP cells.
CDK2-DN Is a Dominant Negative Mutant in Mature T Cells but Surprisingly Acts as a Dominant Active Protein in DP Thymocytes—To see whether the CDK2-DN transgenic protein serves as a dominant negative protein as expected (5), we isolated mature CD4+ T cells from transgenic and nontransgenic littermates. Stimulation of the wild-type mature T cells with plate-bound anti-CD3/CD28 antibodies led to proliferation as evidenced by the increased intracellular staining of Ki-67, a nuclear protein that expression correlates with non-G0 cells (46). However, stimulated CDK2-DN transgenic T cells expressed a lower level of Ki-67 (Fig. 3A). To examine cell division, we loaded mature T cells with CFSE. Upon cell division, CFSE is evenly distributed among the daughter cells, leading to decreased fluorescence. Although nontransgenic (non-Tg) T cells underwent multiple divisions when stimulated, the CDK2-DN transgenic T cells hardly divided by 48 h poststimulation and showed only a small shift of CFSE staining by 72 h. This is consistent with the known CDK2-DN function in blocking S phase progression (Fig. 3B). We conclude that the transgenic CDK2-DN protein acts as a dominant negative protein in mature T cells.

In contrast to the dominant negative effect of CDK2-DN seen in mature T cells, however, we observed an increased Ki-67 staining in DP thymocytes of CDK2-DN mice (Fig. 4A). As others and we have reported, although DP thymocytes do not proliferate, they exhibit a high level of Ki-67 staining (40, 47). We showed that DP thymocytes exist in an unusual state of cell cycle, expressing all of the cell cycle proteins examined, including Cdc2, cyclin A, cyclin B, p27, p21, survivin, and the Rb family members p130 and p107 (40). Positive selection leads to down-regulation of all of these cell cycle proteins (40, 48), including Ki-67. DP thymocytes from both lines of CDK2-DN transgenic mice exhibited higher levels of Ki-67 when compared with their wild-type DP cells, but down-regulation of Ki-67 was observed normally following positive selection (Fig. 4A). These transgenic DP thymocytes also exhibited spontaneous entry into the cell cycle in vitro as measured by BrdUrd incorporation. Although very few BrdUrd+ cells were seen in wild-type DP and CD4+ SP thymocytes, transgenic thymocytes contained a large fraction of BrdUrd+ cells (Fig. 4B). Although it is formally possible that the spontaneous increase of BrdUrd could reflect increased turnover of DP cells from their precursors, we deem this unlikely because this experiment was conducted in vitro in the absence of any supporting stromal cells. Stimulation with anti-CD3/CD28 did not lead to a further increase of this BrdUrd+ population in DP cells, although a slight increase was seen in transgenic CD4+ SP cells. Examination of the side/forward scattering also showed that DP cells from transgenic CDK2-DN mice have increased their cell size (Fig. 4C). This was less evident for the transgenic CD4+ SP thymocytes. We conclude that the CDK2-DN protein acts as a dominant active protein in DP thymocytes, increasing instead of down-regulating the cell cycle activities of the transgenic DP cells.

CDK2-DN DP Thymocytes Show Increased Cell Cycle Activities—To see whether the increased Ki-67 level in CDK2-DN DP thymocytes was accompanied by other changes in their cell cycle activities, we performed Western blot analysis and CDK2 kinase assay. As reported previously, CDK2 is expressed in DP thymocytes but is down-regulated upon positive selection. CDK2-specific antibody can recognize both endogenous CDK2 and CDK2-DN proteins. As expected, the total level of CDK2 and CDK2-DN was elevated to a higher level in transgenic DP and CD4 SP thymocytes (Fig. 5A). This is consistent with the data from the intracellular staining (Fig. 1B). In addition, we also observed increased levels of cyclin E, cyclin A, and the Rb family mem-

**FIGURE 4.** CDK2-DN is a dominant active protein in DP thymocytes. A, increased cell cycle activities in DP but not SP thymocytes of CDK2-DN transgenic mice (lines 1 and 3, solid lines) were measured by intracellular staining with Ki-67-specific antibody. Signals for wild-type thymocytes are denoted by the dotted line. The gray areas represent signals from the isotype control staining. B, thymocytes from CDK2-DN Tg or wild-type (wt, dotted lines) and BrdUrd was added during the last 16 h to measure cell cycle entries. Stimulation of the wild-type (wt, dotted lines) and BrdUrd+ population in DP cells, although a slight increase was seen in transgenic CD4+ SP cells. Examination of the side/forward scattering also showed that DP cells from transgenic CDK2-DN mice have increased their cell size (Fig. 4C). This was less evident for the transgenic CD4+ SP thymocytes. We conclude that the CDK2-DN protein acts as a dominant active protein in DP thymocytes, increasing instead of down-regulating the cell cycle activities of the transgenic DP cells.
ber p107 in the transgenic DP thymocytes. In contrast, p27 cell cycle inhibitor, Rb, and its family member p130 were down-regulated (Fig. 5A).

To assess the CDK2 kinase activity, we isolated the CDK2/cyclin complex directly by immunoprecipitation using the anti-CDK2 antibody or indirectly by co-immunoprecipitation using anti-cyclin E- or anti-cyclin A-specific antibodies. Immunoprecipitates were incubated with or without a CDK2 kinase substrate (purified histone H1) in the presence of $\gamma^{32}$P. As seen in Fig. 5B, wild-type DP thymocytes contained active CDK2 kinase activity. However, this was further enhanced in the CDK2-DN transgenic cells. Thus, the presence of the CDK2-DN protein in DP thymocytes enhances their cell cycle activities.

To explore the mechanism of differential responses of CDK2-DN protein in DP and mature T cells, we performed co-immunoprecipitation experiments with anti p27 antibodies. Co-immunoprecipitates from anti-p27 were blotted with anti-CDK2 and anti-cyclin E to detect the existence of the p27-CDK2-cyclin E complex. In wild-type thymocytes, a high level expression of p27 allows it to interact with both CDK2 and cyclin E (Fig. 5C), presumably resulting in the reduced cell cycle activities in DP thymocytes. In CDK2-DN transgenic thymocytes, however, cyclin E, despite the large amount of protein expression detected in the lysates, could not be seen in the p27 co-immunoprecipitates. This is most likely due to the increased expression of CDK2-DN, which soaks up most of p27, releasing cyclin E to bind to the endogenous CDK2 with the ensuing increase of cell cycle activities. In contrast to thymocytes, wild-type mature T cells contain less p27 and very little CDK2. No cyclin E could be seen in the p27 immunoprecipitates. Expression of CDK2-DN would thus be expected to block the endogenous CDK2-cyclin E activities in activated T cells. We also performed real time PCR to examine transcription of endogenous CDK2 levels and cyclin E. No changes were detected between mRNA levels of endogenous CDK2 between wild-type and transgenic thymocytes and mature T cells (Fig. 5D). In contrast, transcription of cyclin E is significantly increased in transgenic DP thymocytes but not in SP thymocytes or mature T cells (Fig. 5D). This datum is consistent with the idea that increased CDK2-cyclin E kinase activity in DP thymocytes can turn on transcription of cyclin E in a positive feedback loop (49).

**CDK2-DN Transgenic DP Thymocytes Exhibit Increased Apoptosis**—We hypothesized that the cell cycle activities in DP thymocytes confer to them the sensitivity to apoptosis. If
so, increasing their cell cycle activities should lead to increased cell death. Our data indicated that this is true in the CDK2-DN transgenic mouse model. When cultured in vitro, DP thymocytes from transgenic mice died faster than their nontransgenic counterparts (Fig. 6A). As expected, no difference was found between transgenic and nontransgenic CD4+ SP thymocytes. The addition of cross-linking anti-CD3/CD28 to DP thymocytes, which mimics negative selection, increased the number of apoptotic cells further (Fig. 6B). We also assessed TCR Jα usage in peripheral T cells as a measurement of the survival window of DP cells in vivo. TCRα rearrangements begin in the 5′ end of the Jα segments and progressively switch to the downstream Jα segments until DP thymocytes die (or until DP cells are positively selected) (31, 32). In short-lived DP cells (e.g. RORγt-/- cells), the peripheral TCR repertoire consists of primarily the 5′ Jα gene segments (34). Conversely, long-lived DP cells (e.g. Bcl-xL transgenic thymocytes) employ mostly the 3′ Jα gene segments (34). We isolated Jα sequences from CDK2-DN Tg and non-Tg peripheral T cells by PCR using a primer corresponding to Va8 sequence and a Ca primer. Sequencing analysis of 71 of them showed that the CDK2-DN transgenic mice used more 5′ Jα (32 versus 16) and fewer 3′ Jα gene segments (5 versus 24) when compared with the wild-type littermate repertoire (Fig. 6C). These data indicate that the transgenic DP thymocytes have shorter lifespans in vivo than their wild-type counterparts. This was further supported by direct TUNEL staining, which showed a consistent 1.8–4-fold increase of apoptotic cells in nonstimulating conditions (Fig. 7). We conclude that increasing the cell cycle activities in noncycling DP thymocytes affects their propensity to undergo apoptosis in vitro and in vivo.

CDK2-DN Thymocytes Exhibit Increased Levels of Cell Cycle and Apoptotic Proteins—To see which proteins might mediate the increased cell cycle activities and apoptosis in CDK2-DN transgenic thymocytes, we performed Western blot analysis with antibodies specific for proteins involved in proliferation or apoptosis. As expected, CDK2-DN DP thymocytes exhibit increased expression of FoxM1 (Fig. 8A), which has been implicated in the regulation of the cell cycle proteins involved in all phases of the cell cycle (50–52). We also observed elevated levels of caspase-3 and caspase-8 (Fig. 8B), both of which are essential components of the apoptotic machinery.

To investigate which proteins are the direct linkage of cell cycle and apoptosis in CDK2-DN DP thymocytes, e-Myc
and p53 (22, 54, 55), two proteins known to be involved in cell cycle and apoptosis, were examined. A slight increase of both c-Myc and p53 protein levels was observed in CDK2-DN DP cells (Fig. 8A). Analysis of another component of the c-Myc/p53 network, p19arf (56), showed very little difference between transgenic and nontransgenic DP cells. Previous publications have shown that increasing c-Myc expression does not affect T cell development (57) and thymocyte apoptosis other than irradiation-induced death is also normal in p53−/− mice (36). Therefore, the slight difference of c-Myc and p53 observed in CDK2-DN DP thymocytes may not be the key molecules responsible for the linkage of cell cycle and apoptosis. We thus examined another possible candidate, E2F-1, which has also been shown to be involved in both cell cycle and apoptosis (3, 4, 58, 59). The interaction between members of the E2F family and the retinoblastoma proteins is regulated by the CDK2 kinase activity. As cells progress into the S phase, CDK kinases phosphorylate Rb and release E2Fs, which transactivate genes required for replication. E2F-1, in particular, is the only one thought to possess pro-apoptotic activities when overexpressed through direct transcription regulation of caspase genes (19–21). We performed quantitative RT-PCR analysis of E2F-1, E2F-2, E2F-3, and E2F-4 using mRNAs from sorted DP and CD4 SP thymocytes as well as purified naive T cells. As shown in Fig. 8C, no changes in E2F-2, -3, or -4 were found in all of the T cell populations between transgenic and nontransgenic mice. Interestingly, however, the E2F-1 transcript was consistently up-regulated in DP cells from CDK2-DN transgenic mice. This difference was not observed in CD4+ SP thymocytes or naive T cells. Thus, E2F-1 is likely one of the key mediators responsible for the increased cell cycle and apoptosis in CDK2-DN DP thymocytes.

DISCUSSION

One of the mysteries surrounding CD4+CD8+ (DP) thymocytes is their state of the cell cycle. DP cells are not proliferating, but they express many cell cycle proteins specific for G1, S, and G2/M cell cycle phases. Intracellular staining with antibodies for the cell cycle proteins indicates that DP cells uniformly express these proteins (see Fig. 3 in Ref. 40). E2F gel shift analysis with DP cell extracts showed that E2F binds to both cycling-specific Rb family member p107 and the quiescent-specific Rb family member p130 (40). Most strikingly is the highly active CDK2 kinase activity in DP thymocytes that is almost as high as those found in proliferating mature T cells (as shown by the results of this paper and in Fig. 4 in Ref. 40). However, the cell cycle inhibitors such as p27 and p21 are also highly expressed in DP thymocytes. They might serve to inhibit DP cells from entering into the S phase. Expression of all of the cell cycle proteins, including p27, decreases during positive selection. We hypothesized that the cell cycle machinery and apoptosis are linked in developing thymocytes, and the high level cell cycle activities contribute to their inherent sensitivity to apoptosis, an attribute important for negative selection and TCRα chain diversity.

In this paper, we showed that enforced expression of a CDK2 dominant negative mutant in DP thymocytes increases their cell cycle activity as well as apoptosis in vitro and in vivo. It is interesting to see that a kinase-dead CDK2 protein, which acts as a dominant negative protein in most situations (5–8), including mature T cells (this paper), serves as a dominant active protein in DP thymocytes. Our data supported the scenario that this CDK2-DN protein binds to p27 and p21, which are expressed highly in DP thymocytes, titrating them away from the endogenous CDK2-cyclin E and CDK2-cyclin A complexes, resulting in higher CDK2 kinase activities. This in turn leads to an augmented cyclic E level, decreased p27 expression, and increased E2F-1 activity. E2F-1 represses Rb through an autoregulatory loop (60), leading to decreased Rb protein level. An active E2F-1 also
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The role of CDK2 in T cell development is highly controversial. In CDK2-null mice, T cell development and thymocytes apoptosis are normal (62), indicating that CDK2 is not an essential gene. However, induced CDK2 kinase activity was reported in apoptotic DP cells stimulated by glucocorticoid, irradiation, Fas ligation, or TCR signaling (63–65). Roscovitine, a chemical inhibitor of the CDK2 activity, rescues DP cells from cell death (63–65). However, roscovitine was reported recently to be capable of inhibiting apoptosis of CDK2−/− thymocytes, suggesting that this inhibitor can work through other redundant CDK2 family kinases, such as CDK5 or CDK9. In this study, we demonstrate that CDK2 plays a very important role on both peripheral T cells and DP thymocytes but with a drastically different function. As expected, proliferation of peripheral T cells is severely impaired by overexpressing a CDK2 dominant negative protein. However, overexpression of the same CDK2-DN protein serves as a dominant active protein in DP thymocytes and enhances apoptosis. We discovered that E2F-1 transcription but not that of the three other E2F family members (E2F-2, E2F-3, and E2F-4) is highly elevated in CDK2-DN transgenic DP thymocytes. Interestingly, E2F-1 is the only E2F family member that has been reported to possess a pro-apoptotic activity when overexpressed (19–21). Enforced expression of E2F-1 can result in increased transcription of caspase-3, -7, -8, and -9 (21). Indeed, higher levels of expression of caspase-3 and -8 were observed in the CDK2-DN DP thymocytes. E2F-1-deficient thymocytes are more resistant to apoptosis than their wild-type counterparts when cultured in vitro (66), and E2F-1−/− mice exhibit defective negative selection (67). Thus, the steady state CDK2 (or CDK2-like) kinase activity in DP thymocytes might contribute to the constitutive activation of the E2F-1 transcription factor, resulting in the propensity of DP to undergo apoptosis. How DP thymocytes express all of these cell cycle proteins and whether there is a master cell cycle gene that regulates the expression of cell cycle proteins in DP cells await further studies in the future.

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REFERENCES

1. Sherr, C. J., and Roberts, J. M. (1999) Genes Dev. 13, 1501–1512
2. Sherr, C. J., and Roberts, J. M. (2004) Genes Dev. 18, 2699–2711
3. DeGregori, J. (2002) Biochem. Biophys. Acta 1602, 131–150
4. Dimova, D. K., and Dyson, N. J. (2005) Oncogene 24, 2810–2826
5. van den Heuvel, S., and Harlow, E. (1993) Science 262, 2050–2054
6. Hofmann, F., and Livingston, D. M. (1996) Genes Dev. 10, 851–861
7. Hu, B., Mitra, J., van den Heuvel, S., and Enders, G. H. (2001) Mol. Cell Biol. 21, 2755–2766
8. Ferguson, K. L., Callaghan, S. M., O’Hare, M. J., Park, D. S., and Slack, R. S. (2000) J. Biol. Chem. 275, 33593–33600
9. Polyak, K., Lee, M. H., Erdjument-Bromage, H., Koff, A., Roberts, J. M., Tempst, P., and Massague, J. (1994) Cell 78, 59–66
10. Nagata, S. (1999) Annu. Rev. Genet. 33, 29–55
11. Green, D. R., and Reed, J. C. (1998) Science 281, 1309–1312
12. Strasser, A., O’Connor, L., and Dixit, V. M. (2000) Annu. Rev. Biochem. 69, 217–245
13. Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomasselli, K. L., Debatin, K. M., Kramer, P. H., and Peter, M. E. (1998) EMBO J. 17, 1675–1687
14. Green, D. R. (2000) Cell 102, 1–4
15. Verhagen, A. M., Eckert, P. G., Pakusch, M., Silke, J., Connolly, L. M., Reid, G. E., Moritz, R. L., Simpson, R. J., and Vaux, D. L. (2000) Cell 102, 43–53
16. Du, C., Fang, M., Li, Y., Li, L., and Wang, X. (2000) Cell 102, 33–42
17. Raff, M. C. (1992) Nature 356, 397–400
18. Abrams, J. M. (2002) Cell 110, 403–406
19. DeGregori, J., Leone, G., Miron, A., Jakoi, L., and Nevins, J. R. (1997) Proc. Natl. Acad. Sci. U.S.A. 94, 7245–7250
