CD28 Costimulation Mediates T Cell Expansion Via IL-2-Independent and IL-2-Dependent Regulation of Cell Cycle Progression

Leonard J. Appleman, Alla Berezovskaya, Isabelle Grass and Vassiliki A. Boussiotis

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CD28 Costimulation Mediates T Cell Expansion Via IL-2-Independent and IL-2-Dependent Regulation of Cell Cycle Progression

Leonard J. Appleman, Alla Berezovskaya, Isabelle Grass, and Vassiliki A. Boussiotis

In the presence of TCR ligation by Ag, CD28 pathway mediates the most potent costimulatory signal for T cell activation, cytokine secretion, and T cell expansion. Although CD28 costimulation promotes T cell expansion due to IL-2 secretion and subsequent signaling via the IL-2 receptor, recent studies indicate that the dramatic T cell expansion mediated through the unopposed CD28 stimulation in CTLA4-deficient mice is IL-2 independent. Therefore, we sought to dissect the effects of CD28 and IL-2 receptor pathways on cell cycle progression and determine the molecular mechanisms by which the CD28 pathway regulates T cell expansion. Here we show that CD28 costimulation directly regulates T cell cycle entry and progression through the G1 phase in an IL-2-independent manner resulting in activation of cyclin D2-associated cdk4/cdk6 and cyclin E-associated cdk2. Subsequent progression into the S phase is mediated via both IL-2-dependent and IL-2-independent mechanisms and, although in the absence of IL-2 the majority of T cells are arrested at the G1/S transition, a significant fraction of them progresses into the S phase. The key regulatory mechanism for the activation of cyclin-cdk complexes and cell cycle progression is the down-regulation of p27kip1 cdk inhibitor, which is mediated at the posttranscriptional level by its ubiquitin-dependent degradation in the proteasome pathway. Therefore, CD28 costimulation mediates T cell expansion in an IL-2-independent and IL-2 dependent manner and regulates cell cycle progression at two distinct points: at the early G1 phase and at the G1/S transition. The Journal of Immunology, 2000, 164: 144–151.

The most critical costimulatory signal for the productive outcome of the immune response is provided by the members of B7 family, B7-1 (CD80) and B7-2 (CD86) (1–4). B7 costimulation in the presence of a suboptimal TCR signal results in increased transcription and translation of multiple cytokines, up-regulation of IL-2 receptor α-, β-, and γ-chains (5–7), T cell cell expansion, and effector function (8). The biologic significance of this pathway has been well established in multiple in vivo murine models, clearly demonstrating an active role of B7 in the generation of autoimmunity (9–11), tumor immunity (12–14), and allograft rejection (15). Moreover, blockade of the B7:CD28 costimulatory pathway has been shown to ameliorate autoimmune diseases (16), and inhibit humoral immunity (17) and alloreactivity (18, 19).

Although the functional role of B7:CD28 costimulation is well established, the molecular mechanisms by which this pathway regulates T cell expansion have not been determined. Because CD28 costimulation induces increased IL-2 secretion, it has been hypothesized that CD28 mediates clonal expansion through accumulation of IL-2 and subsequent signaling via the IL-2 receptor pathway (20). However, several lines of evidence accumulated from studies on CD28-, IL-2-, and CTLA4-deficient mice suggest that additional IL-2-independent cell cycle regulatory mechanisms may also be mediated via CD28 costimulation. T cells from IL-2-deficient mice have reduced, but significant, proliferative T cell responses to T cell lectin Con A, which can be fully restored by addition of IL-2 (21). In contrast, T cells from CD28-deficient mice have dramatically impaired proliferative response and IL-2 secretion in response to Con A, which is only partially restored by the addition of exogenous IL-2 (22), suggesting that the profound loss of the ability for T cell expansion in CD28-deficient mice is not simply due to the lack of IL-2 production. More importantly, the dramatic proliferation and activation of T cells in the CTLA4-deficient mice, which illustrates the physiologic consequences of unrelenting B7/CD28-mediated T cell activation, is not due to increased production of IL-2 protein or mRNA as compared with normal control mice (23). These observations strongly suggest that IL-2-independent mechanisms are involved in CD28-mediated T cell expansion. In light of the above observations, we sought to dissect the effects of CD28 and IL-2 receptor-mediated pathways on the cell cycle and determine the mechanisms by which CD28 costimulation regulates T cell expansion.

Cell cycle progression is a complex process that is activated by cyclins that associate with catalytically active cyclin-dependent kinases (cdks)3 to form active holoenzymes and is inhibited by cdk inhibitors (24). Induction of D-type cyclins occurs during G1 phase, induction of cyclin E at the late G1 restriction point, and induction of cyclin A at the S phase entry (24, 25). The orderly progression of the cells through the cell cycle is controlled by the timely expression of cyclins, the activation of cdk enzymatic activity, and the subsequent phosphorylation of the relevant

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3 Abbreviations used in this paper: cdk, cyclin-dependent kinase; Rb, retinoblastoma; PVDF, polyvinylidene difluoride; Ubal, ubiquitin aldehyde; MAPK, mitogen-activated protein kinase; PTK, protein tyrosine kinase; PI-1, proteinase inhibitor-1.
substrates, one of which is the retinoblastoma (Rb) gene product (26). Hyperphosphorylation renders Rb protein incapable of binding E2F-type transcription factors, resulting in activation of transcription of S phase genes (27–29).

In the results to be reported below, we demonstrate that CD28 costimulation directly regulates T cell cycle entry and progression through the G1 phase in an IL-2-independent manner by down-regulating p27Kip1 cdk inhibitor, resulting in activation of cyclin D2-associated cdk4/cdk6 and cyclin E-associated cdk2. Subsequent progression into the S phase is mediated via both IL-2-dependent and IL-2-independent mechanisms, indicated by the fact, although in the absence of IL-2 the majority of T cells are arrested at the G1/S transition, a significant fraction of them progresses into the S phase. These results show that CD28 costimulatory signals mediate entry of T cells into the cell cycle and render them competent for progression into the S phase and clonal expansion in an IL-2-dependent and IL-2-independent manner.

Materials and Methods

T cell preparation and culture

Leukocytes were obtained from normal healthy volunteers by leukapheresis. Mononuclear cells were isolated by Ficol/Hypaque gradient centrifugation (Pharmacia LKB Biotechnology, Piscataway, NJ). Monocytes were depleted by adherence on plastic. The CD28+ T cell population was further enriched by separation from residual monocytes, B cells, and NK cells by mAb and magnetic bead depletions using mAbs anti-CD14 (Mo2), anti-CD11b (Mo1), anti-CD20 (B1), anti-CD16 (3G8), and anti-IL-2Ra mAb (CD25), which have been previously described and produced in our laboratory (30). The efficiency of purification was analyzed in each case by flow cytometry (Epics Elite; Coulter Electronics, Hialeah, FL), using anti-CD3, anti-CD4, and anti-CD8 mAbs, followed by FITC-conjugated goat anti-mouse Ig (Fisher Scientific, Pittsburgh, PA). After separation, T cells were cultured in 24-well plates at 2 × 10^6 cells/ml in complete medium consisting of RPMI 1640 supplemented with 10% heat inactivated FCS, 2% glutamine, 1% penicillin/streptomycin at 37°C with 5% CO2. Anti-CD3 (OKT3, IgG1; American Type Culture Collection, Manassas, VA) mAb was precoated on plastic at a concentration of 0.5 µg/ml. Anti-CD28 (CD152, IgG1) mAb (PharMingen, San Diego, CA) was added at a final concentration of 1:10,000. IL-2 was used at 50 U/ml, and anti-IL-2-neutralizing mAb (CD28) were cultured in 24-well plates at 2 × 10^6 cells/ml in complete medium.

Immunoblotting, immunoprecipitation, and in vitro kinase reactions

Following the indicated conditions and time intervals of culture, cell lysates were prepared, and equal amounts of protein (50 µg/sample) were analyzed by 10% SDS-PAGE, transferred onto nitrocellulose membranes, and immunoblotted with the indicated mAbs or antisera. Cyclin A, cyclin D2, cyclin E, cdk2, cdk4, cdk6, p15INK4b, and p16INK4a antisera were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), p21WAF1/Cip1 mAb from Upstate Biotechnology (Lake Placid, NY), and p27Kip1 mAb from Transduction Laboratories (Lexington, KY). To examine the phosphorylation status of Rb, proteins were analyzed by 6% SDS-PAGE, transferred onto nitrocellulose membrane, and blotted with Rb-specific mAb (PharMingen, San Diego, CA). After immunoblotting with mAbs or antisera, immunodetection was performed by incubation with HRP-conjugated anti-mouse IgG (1:5000) or anti-rabbit IgG (1:10,000) (Promega, Madison, WI) as indicated by the host origin of the primary Ab and developed by chemiluminescence (NEN, Boston, MA). Stripping and reprobing of the immunoblots were done as described (31). For this work, quantitation of the proteins was performed by computer densitometry using AlphaImager Software (San Leandro, CA).

For in vitro kinase reactions, immunoprecipitations were done using equal amounts of protein (500 µg/sample) with anti-cdk2-specific anti-serum agarose conjugate (Santa Cruz Biotechnology), and in vitro kinase reactions were performed using histone H1 (Sigma, St. Louis, MO) as exogenous substrate, according to described protocol (32). After immunoprecipitation with anti-cdk4-specific antisera, in vitro kinase reactions were performed using Rh-GST (Santa Cruz Biotechnology) as exogenous substrate. Reactions were analyzed by 10% SDS-PAGE, transferred to PVDF membrane, and exposed to x-ray film.

Ubiquitin aldehyde (Ubal) and the proteasomal inhibitors MG-132 and proteinase inhibitor-1 (PI-1) were reconstituted in DMSO according to the manufacturer’s instructions (Calbiochem, La Jolla, CA) and used at the following concentrations: Ubal at 5 µM, MG-132 at 10 µM, and PI-1 at 15 µM.

RT-PCR

T cells were cultured in 24-well plates with anti-CD3 and anti-CD28 mAbs as described above, and, after 12 and 24 h of culture, T cells were isolated and used for RNA preparation. Two micrograms of RNA were used for reverse transcription, and PCR amplification of cdna from 2 µg of mRNA was performed as previously described (7). Quantitative PCR (multiplex) was conducted as previously described (33, 34) with two sets of primers with sequences as follows: p27Kip1: 5'-CCATGTCAAACTGCGGATG-3' and 3'-CTTTGGACCTCTCCAGGG-5'. G3PDH: 5'-TGAAGGCTCGAGTCAACGATTGTT-3' and 5'-CATGTGGGCGATGTCACCAC-3'. PCR products were electrophoresed through 2% agarose gels containing ethidium bromide and visualized under UV light. Images were digitized and quantitated with image analysis software. Intensity of the PCR product derived from p27Kip1 was divided over G3PDH, which was the internal control for the quantitative PCR.

Cell cycle analysis by flow cytometry

Cell cycle analysis was performed as described before (35). Briefly, after culture under various conditions, cells were harvested at the indicated time points, and 5 × 10^5 cells per sample were resuspended in 1 ml PBS. Cells were fixed with ethanol, propidium iodide was added at a final concentration of 2.5 µg/ml, ribonuclease was added at 50 µg/ml, and samples were incubated for 30 min at 37°C in the dark. Analysis was performed by FACS (Lysis II software; Becton Dickinson, Mountain View, CA).

Results

CD28 costimulation mediates IL-2-independent entry to the G1 phase of the cell cycle

Early stages of G1 phase are controlled by D-type cyclins and their cdks, cdk4 and its homologue cdk6 (25, 36). Among the D-type cyclins, T cells constitutively express very low levels of cyclin D2, which is rapidly up-regulated after entry into the G1 phase of the cell cycle (25). To examine whether CD28 and IL-2 receptor pathways could independently induce entry into the G1 phase, expression of cyclin D2 was examined. T cells were stimulated with submitogenic concentrations of anti-CD3 mAb alone or with either exogenous IL-2 or anti-CD28 mAb. Expression of cyclin D2 was induced at low levels (2- to 3-fold) by submitogenic anti-CD3 mAb (Fig. 1 A, top panel, lanes 2-4) and was significantly augmented (8- to 12-fold) in the presence of IL-2 (Fig. 1 A, top panel, lanes 5-7). Increase of cyclin D2 by IL-2 peaked at 72 h of culture, and the augmentation was retarded in the presence of anti-IL-2-neutralizing mAb (Fig. 1 A, top panel, lanes 8-10). Cyclin D2 was also up-regulated (7- to 10-fold) by costimulation with anti-CD28 mAb (Fig. 1 A, top panel, lanes 11-13), which was only slightly inhibited in the presence of anti-IL-2-neutralizing Ab (Fig. 1 A, top panel, lanes 14-16). Neither IL-2 nor anti-CD28 mAb alone induced cyclin D2 expression in the absence of anti-CD3 mAb (data not shown). These results show that, in the presence of submitogenic stimulation via the TCR/CD3 complex, additional signals mediated independently through the IL-2 and the CD28 pathways contribute to G1 phase entry, as indicated by synthesis of cyclin D2.

To drive cell cycle progression, cyclins have to associate with the activated isoforms of specific cdks to form active holoenzymes (24). Cdks are constitutively expressed at low levels in unstimulated T cells and are up-regulated after activation (37). D-type cyclins associate with cdk4 and its homologue cdk6. In light of the results described above, we sought to determine whether CD28 costimulation could mediate IL-2-independent signals to up-regulate and activate cdk4 and cdk6, which could then facilitate progression through the G1 phase of the cell cycle. Up-regulation of...
Materials and Methods

Cells were analyzed by 10% SDS-PAGE and blotted with cyclin D2-specific antibodies. Equal amounts of protein were analyzed by 10% SDS-PAGE and blotted with cyclin D2-specific antibodies. Blots were stripped and reblotted with antisera specific for cyclin D2 and cdk6. Results are representative of four experiments. Equal amounts of protein (500 µg/sample) were immunoprecipitated with antisera specific for cyclin D2, and in vitro kinase reactions were performed using GST-Rb as exogenous substrate. Samples were analyzed by 10% SDS-PAGE, transferred to PVDF membrane, and exposed to x-ray film. Results are representative of three experiments.

cdk4 (2- to 3-fold) and cdk6 (2- to 3-fold) protein expression was induced by submitogenic concentration of anti-CD3 mAb (Fig. 1A, middle and bottom panels, lanes 2-4). However, under these conditions, although cdk4 expression was up-regulated, its kinase activity was not activated (Fig. 1B, lanes 2-4). Similarly, cdk6 kinase activity was not activated (data not shown). Addition of IL-2 slightly augmented the expression of cdk4 (2- to 3-fold) and cdk6 (≤2-fold), which peaked at 72 h of culture (Fig. 1A, middle and bottom panels, lane 7) but more importantly, induced cdk4 enzymatic activity (Fig. 1B, lanes 6 and 7), which peaked at 72 h of culture. Addition of anti-IL-2-neutralizing mAb markedly reduced IL-2-induced cdk4 and cdk6 up-regulation (Fig. 1A, middle and bottom panels, lanes 8-10) but more importantly abolished cdk4 activation (Fig. 1B, lanes 8-10). Similar results were observed when cdk6 activation was examined (data not shown).

CD28 costimulation not only up-regulated (4- to 5-fold) cdk4 expression (Fig. 1A, middle panel, lanes 11-13) but also induced its activation (Fig. 1B, lanes 11-13), which peaked at 48 h of culture. Anti-IL-2 mAb only slightly inhibited CD28-mediated up-regulation of cdk4 expression (Fig. 1A, middle panel, lanes 14-16) and activation (Fig. 1B, lanes 14-16). Similar results were observed when expression (Fig. 1A, bottom panel, lanes 11-16) and activation (data not shown) of cdk6 were examined, suggesting that IL-2-independent mechanisms that augment the expression and regulate the enzymatic activation of these cdks are mediated by CD28 costimulation. Taken together, these results indicate that CD28-mediated signals can induce IL-2-independent entry to the cell cycle characterized by synthesis of cyclin D2. Moreover, CD28-mediated signals can induce IL-2-independent enzymatic activation of cyclin D2-associated cdks, cdk4, and cdk6, thereby inducing progression of the T cells through the early stages of G1 phase of the cell cycle.

CD28 costimulation induces IL-2-independent passage of T cells through the G1 restriction point into late G1

Although cyclin D2 and its associated cdks, cdk4 and cdk6, regulate entry and progression through the early stages of the G1 phase of the cell cycle, passage through the G1 restriction point into late G1 requires synthesis of cyclin E and activation of cdk2, resulting in the formation of enzymatically active cyclin E-cdk2 complexes (38). Expression of cyclin E was induced by prolonged incubation (72 h) with submitogenic concentration of anti-CD3 mAb (Fig. 2A, top panel, lane 4). Induction of cyclin E was significantly augmented in the presence of IL-2 and peaked at 72 h of culture (Fig. 2A, top panel, lanes 5-7). Expression of cdk2 (Fig. 2A, bottom panel, lanes 2-4) but not activation (Fig. 2B, lanes 2-4) was induced by 72 h of culture with submitogenic concentration of anti-CD3 mAb. IL-2 augmented (2- to 4-fold) the expression (Fig. 2A, bottom panel, lane 7) and induced the activation (Fig. 2B, lanes 5-7) of cdk2, which peaked at 72 h of culture. CD28 costimulation up-regulated expression of cyclin E (Fig. 2A, top panel, lanes 11-13) as well as expression (3- to 5-fold) and activation of cdk2 (Fig. 2A, bottom panel, lanes 11-13 and Fig. 2B, lanes 11-13) within a shorter time interval of culture and resulted in a peak at 48 h.

Expression of cyclin E and cdk2 as well as activation of cdk2 induced by IL-2 were inhibited in the presence of anti-IL-2 mAb (Fig. 2A and B, lanes 8-10). In contrast, anti-IL-2 mAb had almost no effect on CD28-mediated expression of cyclin E and cdk2 (Fig. 2A, top and bottom panels, compare lanes 11-13 with lanes 14-16), but induced a partial but reproducible inhibition on CD28-mediated activation of cdk2 (Fig. 2B, compare lanes 11-13 with lanes 14-16). The induction of cyclin E and enzymatically active cdk2 by CD28 costimulation, which is only partially inhibited by anti-IL-2 neutralizing mAb, is consistent with the ability of CD28 pathway to provide an IL-2-independent signal, sufficient to induce T cells to transit through the G1 restriction point into the late G1 phase.

CD28 costimulation is capable of inducing hyperphosphorylation of Rb in vivo

Activated cdk2 synergizes with cdk4 and cdk6 to hyperphosphorylate and inactivate Rb, thereby releasing E2F-type transcription factors and allowing synthesis of S phase genes (26-29). To determine whether our in vitro findings on cdk4, cdk6, and cdk2 activation correspond to the in vivo events, the independent effect of CD28 and IL-2 receptor pathways on phosphorylation of endogenous Rb was examined. Submitogenic concentration of anti-CD3 up-regulated Rb protein expression but did not induce its activation.
phosphorylation (Fig. 3, lanes 2–4). In the presence of submitogenic concentration of anti-CD3, IL-2 induced a significant phosphorylation of Rb, as determined by the shift of its electrophoretic mobility (Fig. 3, lanes 5–7), which was prevented by neutralizing anti-IL-2 mAb (Fig. 3, lanes 8–10). The effect of IL-2 on Rb hyperphosphorylation peaked at 72 h of culture (Fig. 3, lane 7), which temporally coincided with the maximum simultaneous expression and activation of cdks, cdk2 (Fig. 1, A and B, and Fig. 2, A and B1), and cdk6 (data not shown), induced by IL-2.

CD28 costimulation also induced hyperphosphorylation of Rb, which peaked at 48 h of culture (Fig. 3, lanes 11–13), which temporally coincided with the simultaneous expression and activation of all cdks induced by CD28 costimulation (Fig. 1, A and B, and Fig. 2, A and B) and was significantly but not completely inhibited by the presence of the anti-IL-2-neutralizing Ab (Fig. 3, lanes 14–16). It is of note that the most potent inhibitory effect of anti-IL-2-neutralizing mAb on CD28-mediated phosphorylation of Rb was detected at 72 h of culture (Fig. 3, compare lanes 13 and 16), which temporally coincided with the inhibitory effect of anti-IL-2-neutralizing mAb on CD28-mediated activation of cdk2 (Fig. 2B, lane 16). Taken together, the above results indicate that CD28 costimulation is capable of providing a signal sufficient to induce hyperphosphorylation of Rb and, therefore, primes T cells for entry into the S phase of the cell cycle.

CD28 costimulation induces down-regulation of p27\(^{kip1}\) cdk inhibitor

Activation of cyclin/cdk holoenzyme is regulated by the presence of specific cdk inhibitors that associate with the cyclin/cdk complex (39). The major inhibitors of cyclin/cdk complexes are the members of the INK family (p15\(^{INK4b}\), p16\(^{INK4a}\), p18\(^{INK4c}\), and p19\(^{INK4d}\)) and the cip/kip (p21\(^{cip1}\), p27\(^{kip1}\), and p57\(^{kip2}\)) families (40–46). INK family members specifically bind to and inhibit D-type cyclins complexed with cdk4 and cdk6, whereas p21\(^{cip1}\) and p27\(^{kip1}\) can inhibit many cyclin/cdk complexes. Because mitogenic anti-CD3 stimulation induced expression but not activation of cdks, whereas IL-2 receptor and CD28 pathway induced expression and also activation of these cdks (Figs. 1 and 2), we sought to determine whether the expression of cdk inhibitors under the various conditions would account for this difference. The representative members of the INK family, p15\(^{INK4b}\) and p16\(^{INK4a}\), were not detected in any of the conditions tested (Fig. 4, first and second panels). Both IL-2 receptor and CD28-mediated signals in the presence of mitogenic TCR stimulation resulted in increased levels of p21\(^{cip1}\) expression (Fig. 4, third panel, lanes 5–7 and 11–13), which temporally coincided with the maximum activation of cdks and phosphorylation of Rb (Fig. 1, A and B, and Fig. 3). The paradoxical increase in the cell cycle inhibitor p21\(^{cip1}\) during T cell expansion is consistent with results from other experimental systems. Because p21\(^{cip1}\) also interacts with the proliferating cell nuclear Ag (PCNA), leading to inhibition of DNA elongation via polymerase \(\beta\), the increase of p21\(^{cip1}\) in proliferating cells has been hypothesized to represent a brief arrest of the cycling cells to allow DNA repair (47–50). Neutralizing anti-IL-2 mAb induced complete blockade of IL-2 (Fig. 4, third panel, compare lanes 5–7 with lanes 8–10) but only partial blockade of CD28-mediated induction of p21\(^{cip1}\) expression (Fig. 4, third panel, compare lanes 11–13 with lanes 14–16).

Submitogenic stimulation by anti-CD3 did not affect the expression of p27\(^{kip1}\) (Fig. 4, third panel, lanes 2–4). Addition of IL-2 under these conditions resulted in down-regulation of p27\(^{kip1}\) protein expression, which was most profound at 72 h of culture (Fig. 4, fourth panel, lanes 5–7), and this down-regulation was prevented by anti-IL-2-neutralizing mAb (Fig. 4, fourth panel, compare lanes 5–7 with lanes 8–10). Costimulation through CD28 induced a more rapid reduction of p27\(^{kip1}\) protein expression (Fig. 4, fourth panel, lanes 11–13), which was detectable by 24 h of culture, and was only partially inhibited by anti-IL-2-neutralizing Ab (Fig. 4, fourth panel, compare lanes 11–13 to lanes 14–16).

These results indicate that, although submitogenic anti-CD3 stimulation induces expression of cdk4, cdk6, and cdk2, it fails to induce enzymatic activity of these cdks, because the expression of p27\(^{kip1}\) remains high. IL-2- and CD28-mediated enzymatic activation of cdks, which is required for the formation of active cyclin/cdk complexes and cell cycle progression, is not simply due to the up-regulation of the expression of cyclins and cdks but also due to the down-regulation of p27\(^{kip1}\) cdk inhibitor, which releases cyclin D2-cdk4/6 and cyclin E/cdk2 complexes from this constraint, resulting in their enzymatic activation. Moreover, these results show that, in addition to the well known IL-2-mediated down-regulation of p27\(^{kip1}\), CD28 pathway activates IL-2-independent mechanisms that result in down-regulation of this cell cycle inhibitor.

Down-regulation of p27 by CD28 costimulation is controlled at posttranscriptional level and is mediated via its ubiquitination and degradation in the proteasome pathway

There have been conflicting reports as to whether expression of p27\(^{kip1}\) during the cell cycle is regulated at the level of protein or mRNA (51–55). It is possible that distinct regulatory mechanisms are operative in different cell types. Therefore, we sought to determine the mechanism by which CD28 costimulation regulates...
the expression of p27kip1 protein in T cells. Quantitative RT-PCR analysis demonstrated that p27kip1 mRNA was not reduced (Fig. 5A) by CD3- and CD28-mediated stimulation although this caused significant decrease in p27kip1 protein expression. These data suggest that reduction in p27kip1 protein by CD28 costimulation is mediated through posttranscriptional mechanisms.

In other experimental systems, posttranscriptional regulation of p27kip1 has been shown to occur through enzymatic ubiquitination followed by targeted proteolysis in the proteasome pathway (54, 56). To examine whether CD28-mediated down-regulation of p27kip1 involves ubiquitin-targeted proteolysis, cultures of T cells with anti-CD3 and anti-CD28 were established in either media alone or in the presence of Ubal. Ubal is a potent and specific inhibitor of multiple ubiquitin hydrolases involved in pathways of intracellular protein ubiquitin-dependent modification and turnover and decreases the rate of ubiquitin-dependent degradation (57). The presence of Ubal prevented the down-regulation of p27kip1 (Fig. 5B), suggesting that ubiquitination of p27kip1 is a mandatory step for its down-regulation by TCR- and CD28-mediated signals.

To examine whether CD28 costimulation mediated degradation of p27kip1 in the proteasome complex, two different proteasome inhibitors (MG-132 and Pl-1) were used. Stimulation of T cells by CD3 and CD28 in the presence of either inhibitor resulted in the generation of multiple bands detected by the p27kip1-specific Ab (Fig. 5C). The electrophoretic mobility of these proteins was consistent with previously reported ubiquitinated forms of p27kip1 (54, 56). Importantly, the generation of these ubiquitinated forms was observed even in the presence of neutralizing anti-IL-2 Ab (Fig. 5C). These results show that signals mediated directly through CD28 costimulation are capable of inducing degradation of p27kip1 in the ubiquitin-proteasome pathway and strongly suggest that CD28 costimulation regulates cell cycle progression of T cells via a mechanism that is independent and upstream of IL-2 receptor-mediated signaling.

CD28 costimulation mediates IL-2-independent progression to the S phase of the cell cycle

p27kip1 has a unique role on the control of the cell cycle because it integrates extracellular and intracellular signals during the early G1 phase and regulates the activation of cdk-cyclin holoenzyme complexes, which determine the ability of the cell to progress through the G1 phase, pass the G1 restriction point, and enter the S phase. In light of the above results, the question arises whether CD28 costimulation can promote progression only into late G1 phase of the cell cycle characterized by synthesis of cyclin E and activation of cdk2 or whether it is sufficient to induce entry to the S phase and clonal expansion in the absence of IL-2. Although submitogenic anti-CD3 activation did not induce entry to the cell cycle (data not shown), addition of IL-2 resulted in increase of cells at the S phase of the cell cycle, which peaked at 72 h of culture (Fig. 6A) and temporally coincided with expression of cyclin A (Fig. 6B). CD28-mediated signals also increased the percentage of cells at the S phase of the cell cycle (Fig. 6A) and induced expression of cyclin A, which peaked at 48 h of culture (Fig. 6B). Anti-IL-2-neutralizing mAb abrogated IL-2-induced increase of cells in S phase of the cell cycle and expression of cyclin A (Fig. 6A and B). Anti-IL-2-neutralizing mAb induced a significant reduction in the percentage of cells at the S phase and levels of cyclin A expression induced by CD28 costimulation (Fig. 6A and B). Because these cells express cyclin E (Fig. 2A, top panel, lanes 15 and 16), they pass the G1 restriction point, and, therefore, CD28-costimulated cells that fail to enter S phase in the absence of IL-2 are blocked at the G1/S transition. Importantly, in the presence of anti-IL-2-eutalizing mAb, a significant fraction, equivalent to 37% of the CD28-costimulated cells entering the cycle in the absence of anti-IL-2 mAb, could progress into the S phase (Fig. 6A) and synthesize cyclin A (Fig. 6B), indicating that CD28 costimulation can mediate IL-2-independent T cell expansion.

FIGURE 5. Down-regulation of p27 by CD28 costimulation is mediated via ubiquitin-dependent degradation in the proteasome pathway. A, T cells were cultured as indicated, and after 12 and 24 h of culture T cells were isolated and used for RNA preparation and reverse transcription. Quantitation of p27kip1 mRNA was done by quantitative multiplex PCR using two sets of oligonucleotides specific for p27kip1 and G3PDH. B, T cells were cultured for the indicated time intervals with anti-CD3 + anti-CD28 in the presence or the absence of Ubal. Lysates were prepared, and equal amounts of protein were analyzed by 10% SDS-PAGE and immunoblotted with p27kip1-specific Ab used as a vehicle control for the reconstitution of the proteasome inhibitors. Viability of the cells at the end of the incubation period was determined by trypan blue exclusion. Lysates were prepared, and equal amounts of protein were analyzed by 10% SDS-PAGE and immunoblotted with p27kip1-specific mAb either alone or in the presence of two different proteasome inhibitors (MG-132, Pl-1). DMSO was used as a vehicle control for the reconstitution of the proteasome inhibitors. Viability of the cells at the end of the incubation period was determined by trypan blue exclusion. Lysates were prepared, and equal amounts of protein were analyzed by 10% SDS-PAGE and immunoblotted with p27kip1-specific mAb. For the conditions of TCR + CD28 culture, 50 μg of protein per sample were used, and, for the TCR + CD28 + anti-IL-2 mAb culture, 100 μg of protein per sample were used. Results are representative of three experiments.

CD28 costimulation mediates IL-2-independent progression to the S phase of the cell cycle

p27kip1 has a unique role on the control of the cell cycle because it integrates extracellular and intracellular signals during the early G1 phase and regulates the activation of cdk-cyclin holoenzyme complexes, which determine the ability of the cell to progress through the G1 phase, pass the G1 restriction point, and enter the S phase. In light of the above results, the question arises whether CD28 costimulation can promote progression only into late G1 phase of the cell cycle characterized by synthesis of cyclin E and activation of cdk2 or whether it is sufficient to induce entry to the S phase and clonal expansion in the absence of IL-2. Although submitogenic anti-CD3 activation did not induce entry to the cell cycle (data not shown), addition of IL-2 resulted in increase of cells at the S phase of the cell cycle, which peaked at 72 h of culture (Fig. 6A) and temporally coincided with expression of cyclin A (Fig. 6B). CD28-mediated signals also increased the percentage of cells at the S phase of the cell cycle (Fig. 6A) and induced expression of cyclin A, which peaked at 48 h of culture (Fig. 6B). Anti-IL-2-neutralizing mAb abrogated IL-2-induced increase of cells in S phase of the cell cycle and expression of cyclin A (Fig. 6A and B). Anti-IL-2-neutralizing mAb induced a significant reduction in the percentage of cells at the S phase and levels of cyclin A expression induced by CD28 costimulation (Fig. 6A and B). Because these cells express cyclin E (Fig. 2A, top panel, lanes 15 and 16), they pass the G1 restriction point, and, therefore, CD28-costimulated cells that fail to enter S phase in the absence of IL-2 are blocked at the G1/S transition. Importantly, in the presence of anti-IL-2-eutalizing mAb, a significant fraction, equivalent to 37% of the CD28-costimulated cells entering the cycle in the absence of anti-IL-2 mAb, could progress into the S phase (Fig. 6A) and synthesize cyclin A (Fig. 6B), indicating that CD28 costimulation can mediate IL-2-independent T cell expansion.

FIGURE 5. Down-regulation of p27 by CD28 costimulation is mediated via ubiquitin-dependent degradation in the proteasome pathway. A, T cells were cultured as indicated, and after 12 and 24 h of culture T cells were isolated and used for RNA preparation and reverse transcription. Quantitation of p27kip1 mRNA was done by quantitative multiplex PCR using two sets of oligonucleotides specific for p27kip1 and G3PDH. B, T cells were cultured for the indicated time intervals with anti-CD3 + anti-CD28 in the presence or the absence of Ubal. Lysates were prepared, and equal amounts of protein were analyzed by 10% SDS-PAGE and immunoblotted with p27kip1-specific Ab used as a vehicle control for the reconstitution of the proteasome inhibitors. Viability of the cells at the end of the incubation period was determined by trypan blue exclusion. Lysates were prepared, and equal amounts of protein were analyzed by 10% SDS-PAGE and immunoblotted with p27kip1-specific mAb. For the conditions of TCR + CD28 culture, 50 μg of protein per sample were used, and, for the TCR + CD28 + anti-IL-2 mAb culture, 100 μg of protein per sample were used. Results are representative of three experiments.

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down-regulation of p27kip1, resulting in massive T cell expansion. Our results provide a potential molecular mechanism explaining the progression is the down-regulation of p27kip1 cdk inhibitor. Our regulatory mechanism for the activation of cdks and cell cycle progression is the early down-regulation of p27kip1, which releases cyclin D2/cdk4-cdk6 and cyclin E/cdk2. This release is controlled at the posttranscriptional level and is mediated via its ubiquitination and degradation in the proteasome pathway. Although the signals that activate the proteasome-mediated degradation of p27kip1 have not been fully determined, several observations implicate Ras and the mitogen-activated protein kinase (MAPK) signaling in this process (64–67). Expression of a dominant negative Ras allele in NIH 3T3 cells abolished down-regulation of p27kip1 in response to epidermal growth factor (65). Conversely, expression of an activated allele of Ras decreased p27kip1 levels in rat fibroblasts growth-arrested in G1 (64). In that system, a chemical inhibitor of MAPK blocked degradation of p27kip1, hyperphosphorylation of Rb, and activation of cdk2. Ubiquitin-dependent degradation of p27kip1 requires its prior phosphorylation (56, 68). MAPK and cyclin E-cdk2 holoenzyme have been shown to phosphorylate p27kip1 in vitro (64, 69). Moreover, protein tyrosine kinase (PTK) activity is required for the induction of protein ubiquitination (70, 71). Importantlly, although p27kip1 does not undergo tyrosine phosphorylation, PTK activity is required for subsequent ubiquitination of p27kip1 (56). CD28-mediated co-stimulation has a direct effect on the activation of PTKs (72–75), Ras, and the MAPK pathway (76), thereby regulating the phosphorylation of various intracellular substrates. Therefore, these events may directly link CD28-mediated signals to p27kip1 degradation. Protein ubiquitination is a dynamic process that is controlled by the coordinate action of multiple ubiquitin-conjugating enzymes and deubiquitinating enzymes (77). Whether CD28 costimulation directly controls ubiquitination of p27kip1 by regulating the activity of ubiquitinating or deubiquitinating enzymes remains to be determined.

Regardless of the level and the mechanism of regulation, our results show that CD28 is capable of mediating down-regulation of p27kip1 in a direct, IL-2-independent, and a secondary IL-2-dependent manner. This is supported by the observations 1) that ubiquitination of p27kip1 is detected even in the presence of neutralizing anti-IL-2 mAb and 2) that neutralizing anti-IL-2 mAb only partially inhibits CD28-mediated down-regulation of p27kip1 expression whereas it efficiently prevents down-regulation of p27kip1 induced by IL-2 added in 100-fold higher concentration than that produced by CD28 costimulation. Therefore, CD28 costimulation regulates T cell cycle progression via two distinct mechanisms. First, it directly primes for entry to the cycle by down-regulating p27kip1 before IL-2 accumulation. CD28 mediates the initial drop of p27kip1, which releases cyclin D2/cdk4-cdk6 and cyclin E-cdk2 from the constraint of this inhibitor and, therefore, allows the activation of its enzymatic activity and progression to the late G1 phase. Second, since significant amounts of IL-2 accumulate as a consequence of CD28-mediated increased IL-2 secretion and since T cells are ready to uptake them because expression of IL-2 receptor-α, β-, and γ-chains has been induced, IL-2 receptor-mediated signals result in enhanced and prolonged activation of cyclin E-cdk2, which induces phosphorylation and further degradation of p27kip1, amplification of the activation signal, and clonal expansion. This hypothesis is supported by the observation...
that, although anti-IL-2 mAb does not affect CD28-mediated activation of cdk4 and cdk6, it partially inhibits CD28-mediated activation of cdk2. Most importantly, despite the partial inhibition of CD28-mediated anti-IL-2 mAb, a significant fraction of cells can enter S phase, indicating that signals directly mediated through CD28 can be sufficient for T cell clonal expansion. Therefore, CD28 costimulatory signals mediate entry of T cells into the S phase and clonal expansion in an IL-2-dependent and IL-2-independent manner.

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**References**

9. Guerder, S., J. Meyerhoff, and R. Flavell. 1994. We thank Dr. Lee Nadler for helpful discussions and critical reading of the manuscript.