Interleukin-7 Inactivates the Pro-apoptotic Protein Bad Promoting T Cell Survival*

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Interleukin-7 (IL-7) is a cytokine that is required for T cell development and survival. The anti-apoptotic function of IL-7 is partly through induction of Bcl-2 synthesis and cytosolic retention of Bax. Here we show that the Bcl-2 homology 3 domain-only protein, Bad, is involved in cell death following IL-7 withdrawal from D1 cells, an IL-7-dependent murine thymocyte cell line. IL-7 stimulation resulted in the inactivation of Bad by phosphorylation at Ser-112, -136, and -155. The phosphoinositide 3-kinase (PI3K)/Akt pathway has been implicated previously in Bad phosphorylation. In response to IL-7, the PI3K/Akt pathway induced phosphorylation at Ser-136 and -155, but Ser-112 was partly independent of the PI3K/Akt pathway, indicating an as yet unknown pathway in this response. Following IL-7 withdrawal, dephosphorylation led translocation from cytosol to mitochondria, and Bax deficiency rescues thymocyte apoptosis. Here we show that the inactivation of IL-7 is partly through induction of Bcl-2 synthesis and overexpression of a bax transgene in the thymus inhibits thymopoiesis (16). Bad was implicated as a death-promoting factor in IL-7 signaling based on our observation that IL-7 inactivates Bax by preventing its translocation from cytoplasm to mitochondria (17), and Bad deficiency rescues thymocyte development in IL-7Rα-deficient mice early in life (18). However, beyond 8 weeks of life, Bax deletion no longer protects thymocytes from IL-7Rα deficiency (18). This suggests that another death mediator in addition to Bax counters Bcl-2 later in life following the loss of an IL-7 signal.

Bad is a pro-apoptotic member of the Bcl-2 family and belongs to the group of “BH3 domain-only” proteins that bind to Bcl-2 and block its function. Growth factors such as IL-3 can induce the phosphorylation of Bad at Ser-112, -136, and -155 to promote cell survival (19, 20). Several kinases have been proposed to mediate Bad phosphorylation, including Akt, cAMP-dependent protein kinase, and ribosomal S6 kinase (19, 21–23). The phosphorylation of Bad promotes binding to 14-3-3 proteins, which retain Bad in the cytoplasm. In the absence of survival signals, Bad is dephosphorylated, translocates to mitochondria, and blocks the survival function of Bcl-2 and Bcl-XL in the outer mitochondrial membrane (19, 20). Bad is a potential apoptotic factor in T lymphocyte development because T cells from bad transgenic mice are highly sensitive to apoptotic stimuli (24). A constitutively active form, Bad^3SA/3SA (introduced as a transgenic), induces lower thymic weight and reduces thymic cellularity (25). Bad null mice develop B cell lymphoma and show an increased incidence of pre-T cell lymphoblastic lymphomas after γ irradiation (26), also consistent with a role in Bad for the death of lymphocytes.

IL-7 has been reported to activate PI3K through its p85 regulatory subunit, which is recruited to the Tyr-449 residue of the IL-7Rα chain in murine B cells (27). This pathway could activate Akt and therefore inactivate Bad following IL-7 stimulation. Although the knockout of p85 does not reduce T cell development (28), the knockout of the catalytic subunit of PI3Kγ shows somewhat reduced thymic cellularity (29), and there are additional isoforms of PI3K that could provide survival signals in T cells. We reported previously that a pharmacological inhibitor of PI3K, which blocks multiple isoforms, induces apoptosis in pro-T cells (18). In the present study, we evaluated the roles of PI3K and Bad in IL-7 signaling using D1 cells, a murine thymocyte cell line that depends on IL-7 for survival and proliferation. We observed that Bad was inactivated by IL-7, that it was capable of mediating apoptosis in these cells, and that its inactivation was partly through PI3K.

MATERIALS AND METHODS

Cell Culture—The IL-7-dependent thymocyte cell line D1 was generated from a p53 knock-out mouse (30). D1 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (HyClone), 1-glutamine (2 mM), 1% penicillin-streptomycin, β-mercaptoethanol (50 μM, 29160

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IL-7 Withdrawal Activates Bad

The Akt kinase assay was carried out following the manufacturer's protocol. Akt was immunoprecipitated with immobilized Akt1 monoclonal antibody from cell lysates. The Akt kinase activity was assayed by an in vitro kinase reaction containing ATP and GSK-3 fusion protein as substrate. The products of the reaction were analyzed by Western blotting with phospho-Akt-Ser-473 and Akt antibodies.

RESULTS

IL-7 Activates Akt—Akt is the key downstream target of PI3K and is activated by phospholipid binding and dual phosphorylation at Thr-308 and Ser-473. To evaluate the role of the PI3K/Akt pathway in IL-7 signaling, we first investigated whether Akt was activated by IL-7. D1 cells were deprived of IL-7 for 12 h and then stimulated with IL-7 for various times. Western blotting using anti-phospho-Akt-Ser-473 antibody revealed that Akt was activated by IL-7 stimulation. As shown in Fig. 1A, IL-7 induced Akt phosphorylation at Ser-473 within 5 min, and phosphorylation continually increased up to 2 h. We next examined the rate of dephosphorylation of Akt following IL-7 withdrawal. D1 cells were deprived of IL-7 for various times, and as shown in Fig. 1B, Akt phosphorylation sharply declined by 2 h after IL-7 withdrawal. The PI3K inhibitor LY294002 blocked IL-7-induced Akt activation in D1 cells (data not shown). Thus, IL-7 activates the PI3K-Akt pathway in D1 cells, suggesting this pathway could play a role in cell survival induced by IL-7.

IL-7 Induces Bad Phosphorylation—We then examined the Akt substrate, Bad, to determine whether it could account for the survival effect of IL-7 via the inactivation of this proapoptotic member of the Bcl-2 family. Bad is inactivated by phosphorylation at three serine sites, Ser-112, -136, and -155, whereas the phosphorylation at Ser-136 and -155 peaked an hour later (Fig. 2A), whereas the phosphorylation at Ser-136 and -155 peaked an hour later (Fig. 2C). We then analyzed the rate of decline in Bad phosphorylation after IL-7 withdrawal. The level of Bad phosphorylation at Ser-112 was decreased by 2 h and was nearly undetectable by 6 h of IL-7 withdrawal (Fig. 2B). The phosphorylation at Ser-136 and -155 showed a somewhat faster

In vitro Akt Kinase Assay—The Akt kinase assay was carried out following the manufacturer's protocol. Akt was immunoprecipitated with immobilized Akt1 monoclonal antibody from cell lysates. The Akt kinase activity was assayed by an in vitro kinase reaction containing ATP and GSK-3 fusion protein as substrate. The products of the reaction were analyzed by Western blotting with phospho-Akt-Ser-473 and Akt antibodies.

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Bad antibody. were stripped and reprobed with total IL-7, and subjected to immunoblotting with antibodies specific for Bad Ser-112 or -155. Pro-T cells were isolated from the thymuses of Rag-1-deficient mice, cultured for 8 h with (+) or without (−) IL-7, and subjected to immunoblotting with antibody specific for Bad Ser-112 phosphorylation (∼). The blots were stripped and reprobed with total Bad antibody.

We assessed whether the Bad phosphorylation in response to IL-7 that we observed in the D1 thymocyte cell line also occurred in pro-T cells. Thymocytes from Rag-1-deficient mice are mainly pro-T2 and -T3 cells and are dependent on IL-7 for survival. Thymocytes from Rag-1-deficient mice were cultured in suspension for 8 h with or without IL-7. We observed that IL-7 promoted Bad phosphorylation in these pro-T cells in primary culture (Fig. 2E), verifying that Bad is phosphorylated by IL-7 signaling and could therefore regulate T cell survival.

IL-7 induces Bad phospho-112lation Partially Depends on the PI3K/Akt Pathway—Akt is a principle kinase capable of phosphorylating Bad at Ser-136 in vitro and in vivo (21, 35). However, the kinases responsible for Ser-112 and -155 phosphorylation can differ between different cell types and stimuli. We assessed the effect of the PI3K inhibitor LY294002 on Bad phosphorylation induced by IL-7 in D1 cells. After overnight deprivation of IL-7, the D1 cells were stimulated with IL-7 for various times in the presence or absence of LY294002. We observed that LY294002 blocked IL-7-induced Bad phosphorylation at Ser-136 and -155 (Fig. 3B). LY294002 only inhibited Ser-112 phosphorylation at the early time point (1 h) but not at the late time point (8 h) (Fig. 3A), suggesting that an additional pathway is involved for Ser-112. As an additional approach to determine whether the PI3K/Akt pathway could induce Bad phosphorylation at Ser-112, we stably expressed a constitutively active form of murine Akt1 containing the myristoylation/palmitoylation motif from the tyrosine kinase Lck (32). The activity of this overexpressed Akt was verified by immunoprecipitation from the cell lysates and an in vitro kinase assay using the recombinant GSK fusion protein as a substrate (Fig. 4A). We compared the level of Bad phosphorylation at Ser-112 in D1 with that in active Akt-transfected D1. The status of Ser-112 phosphorylation did not increase in D1 cells expressing active Akt in the presence or absence of IL-7 (Fig. 4B), and thus active Akt could not replace IL-7 to induce Bad phosphorylation at Ser-112. Taken together, our findings suggest that IL-7-induced Bad phosphorylation at Ser-136 and -155 is PI3K/Akt-dependent, but Ser-112 phosphorylation is largely PI3K/Akt-independent.

IL-7 Withdrawal Triggers Bad Translocation to Mitochondria and Binding to Bcl-2—In healthy cells, phospho-Bad is largely PI3K/Akt-independent. Ser-112 phosphorylation did not alter D1 survival in the presence of IL-7 but accelerated the death of D1 after IL-7 removal (Fig. 6B). D1 cells were next transfected with the constitutively active Bad, Bad3SA, in which the three serine phosphorylation sites had been mutated to alanine (19). Fig. 6C shows that 66% of D1 cells bearing Bad3SA died 48 h after transfection even in the presence of IL-7, and more than 81% died after 72 h. These data indicate that activation of Bad could contribute to cell death following the loss of the IL-7 signal.

It was observed that IL-7-induced Bad phosphorylation, we measured Bad protein levels in cytoplasm versus mitochondria in D1 cells after IL-7 withdrawal. As shown in Fig. 5A, in the presence of IL-7, Bad was found mainly in the cytoplasmic fraction, whereas little was seen in the mitochondrial fraction. Withdrawal of IL-7 induced a rise in the Bad protein level in mitochondria by 2 h, and a large increase was observed by 6 h. Bcl-2 was constitutively present in the mitochondrial membrane fractions. We then investigated the effect of LY294002 on the intracellular location of Bad. LY294002 induced Bad translocation from cytoplasm to mitochondria (Fig. 5B), which could result from inhibition of phosphorylation of Bad Ser-136 and -155. Next, we evaluated Bad association with Bcl-2 after IL-7 withdrawal by immunoprecipitating Bcl-2 and immunoblotting for Bad. Indeed, we found that the binding of Bad to Bcl-2 decreased in the absence of IL-7 (Fig. 5C). Therefore, on the loss of the IL-7 signal in D1 cells, Bad translocates to mitochondria and binds to Bcl-2.

Bad Promotes the Death of D1 Cells—To determine whether Bad contributed to the death of D1 cells following IL-7 withdrawal, we first tested the effect of a cell-permeable Bad peptide on D1 cell survival. This peptide contains the BH3 domain of Bad and binds to Bcl-2 (36). D1 cells were treated with the Bad peptide or the control peptide in the presence of IL-7, and a large increase was observed by 6 h. Bcl-2 was constitu-
cells (30), we therefore evaluated whether overexpression of Bcl-2 could replace the IL-7 signal. Stably transfecting D1 cells with a Bcl-2 retroviral expression vector indefinitely protected D1 cells from death induced by IL-7 deprivation for the long term. Only 2% of D1 cells expressing Bcl-2 died compared with 88% of normal D1 cells when deprived of IL-7 for 72 h (Fig. 7A).

We then co-transfected Bcl-2 and Bad3SA into D1 cells, and as shown in Fig. 7B, Bcl-2 clearly rescued D1 cells from death induced by Bad3SA. Thus, IL-7 induces the synthesis of Bcl-2, which is sufficient to protect cells from activated Bad and IL-7 withdrawal.

**PI3K/Akt Pathway Prolongs D1 Cell Survival following IL-7 Withdrawal**—The PI3K/Akt pathway has been shown to promote the survival of a wide range of cell types (19). Having shown that IL-7 stimulation activates Akt and inactivates Bad, we verified the overall survival effect of blocking the PI3K/Akt pathway. D1 cells were treated with a PI3K inhibitor, LY294002, and cell survival was quantified by propidium iodide staining. The PI3K inhibitor did not kill D1 cells in the presence of IL-7 (Fig. 8A). As an alternative method for manipulating this pathway, we transfected DN-Akt or active Akt into D1 cells. Similar to the PI3K inhibitor, DN-Akt did not interfere with D1 cell survival in the presence of IL-7 (Fig. 8B). However, either LY294002 or DN-Akt accelerated the death of D1 cells following IL-7 deprivation (Fig. 8). The converse effect was seen with the active form of Akt, which slowed the cell death process following IL-7 deprivation but did not maintain D1 survival without IL-7 beyond 72 h (Fig. 8B). Thus, the PI3K/Akt pathway partly contributes to the IL-7 survival func-

**Fig. 3.** PI3K inhibitor blocks IL-7-induced Bad phosphorylation at Ser-136 and Ser-155. D1 cells (A) or Bad-transfected D1 cells (B) were deprived (−) of IL-7 for 12 h and then stimulated (+) with IL-7 (50 ng/ml) for different times points in the presence (+) or absence (−) of 30 μM LY294002. Immunoblotting was performed with antibodies specific for Bad phosphorylation at Ser-112, -136, -155, and total Bad. DMSO, dimethyl sulfoxide.

**Fig. 4.** IL-7-induced Bad phosphorylation at Ser-112 is Akt-independent. D1 cells were stably transfected with the constitutively active Akt and cultured in the presence (+) or absence (−) of IL-7 for 12 h. Akt kinase activity was analyzed by kinase assay from D1 or the transfected D1 cells (A). Bad phosphorylation at Ser-112 was examined by immunoblotting from different dilutions of the cell lysates (B).

**Fig. 5.** IL-7 stimulation retains Bad in cytosol and prevents Bad binding to Bcl-2. Bad intracellular location and Bcl-2 association were examined. A, D1 cells were deprived of IL-7 for various times, and cytoplasmic and mitochondrial protein fractions were prepared and immunoblotted with anti-Bad or -Bcl-2 antibodies. B, D1 cells were treated with IL-7 and 30 μM LY294002 for various times, and Bad protein levels from cytoplasmic or mitochondrial fractions were analyzed. C, lysates were prepared from D1 cells cultured with (+) or without (−) IL-7 for 8 h, immunoprecipitated (IP) with anti-Bcl-2, and blotted with anti-Bad or anti-Bcl-2. IgG was used as a control (c).
tion, consistent with its contribution to Bad inactivation, and there must also be additional IL-7 survival effects, such as the PI3K/Akt-independent pathway to phosphorylation of Bad at Ser-112 and the synthesis of Bcl-2.

**DISCUSSION**

IL-7 receptor signaling is required for the survival of pro-T cells and mature T lymphocytes. These anti-apoptotic effects of IL-7 have been partly attributed to induction of the synthesis of Bcl-2 (11) and suppression of the action of Bax (17). A third anti-apoptotic effect of IL-7 is described here, which is the inactivation of the BH3-only protein Bad. IL-7 stimulation induced Bad phosphorylation at three regulatory serine residues, blocking Bad translocation from cytoplasm to mitochondria. Introducing activated Bad induced the death of D1 cells, and this was countered by overexpressing Bcl-2. The PI3K/Akt

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**Fig. 6.** Bad is a death mediator of IL-7 deprivation in D1 cells. The sensitivity of D1 cells to Bad was determined. A, cells were cultured in 96-well plates with cell-permeable peptides corresponding to Bad or control (50 μM) for 48 h, and cell viability was determined by MTT assay. DMSO, dimethyl sulfoxide; B, cells were transfected with WT Bad or Bad3SA (C), cultured with or without IL-7 for various times, and viable cells were determined by propidium iodide staining.

**Fig. 7.** Bcl-2 protects from IL-7 withdrawal. D1 cells were stably transfected with bcl-2 and cultured without IL-7 for certain of time points (A). bcl-2 and bad3SA were co-transfected in to D1 cells (B). Cell viability was analyzed by propidium iodide staining following flow cytometric analysis.
pathway mediated phosphorylation at two of the three sites on Bad and contributed to, but could not replace, the survival signal from IL-7 stimulation.

In many cell types, the PI3K/Akt pathway is sufficient to promote survival, and the pharmacological blockade of PI3K activity can suppress the ability of trophic factors to support survival (32, 35). The survival function of the PI3K/Akt pathway is through inactivation of Bad by phosphorylation at Ser-136 (21). It has been shown that survival factors required phosphorylation of Ser-136 and -155 (but not Ser-112) to block Bad-mediated apoptosis (19, 21). We observed that the PI3K inhibitor did not promote apoptosis in D1 cells in the presence of IL-7 (Fig. 3B), although Bad phosphorylation at Ser-136 and -155 was abolished (Fig. 3B). Why does blocking Bad inactivation (by inhibiting PI3K) fail to kill D1 cells in the presence of IL-7 but accelerates death following withdrawal? One possibility is that Ser-112 phosphorylation is sufficient for Bad inactivation because the PI3K inhibitor did not block Ser-112 phosphorylation of Bad in D1 cells at later time points. However, we observed Bad translocation to mitochondria in the presence of the PI3K inhibitor LY294002 (Fig. 5B), which would primarily affect the other two serines, suggesting that phosphorylation of Ser-112 may not be enough to block Bad translocation. We favor a model in which IL-7 regulates the balance of Bad versus Bcl-2. In the presence of IL-7, Bcl-2 is synthesized (11), and Bad is inactivated. Following IL-7 withdrawal, Bcl-2 synthesis ceases, and Bad (which is newly activated) binds and inactivates the residual Bcl-2, accelerating death. Thus, blocking Bad inactivation (using PI3K inhibitors) does not kill D1 cells as long as IL-7 is present, because the level of Bcl-2 exceeds the level of active Bad. If the amount of active Bad is artificially increased by overexpression of the active Bad mutant, all of the Bcl-2 is inactivated (even in the presence of IL-7), and the cell dies. Conversely, eliminating active Bad by overexpressing active Akt would extend life after IL-7 withdrawal, because the amount of functional Bcl-2 would persist for a longer period of time, but the cell would eventually run out of Bcl-2 and die. A similar model has recently been proposed by Datta et al. (25), who suggest that Bad "raises the mitochondrial threshold of apoptosis."

Our present model based on the D1 cell line would not appear to explain our previous findings in the IL-7 response of pro-T2 and -T3 thymocytes (18). In that study, we find that blocking PI3K kills pro-T2 and -T3 thymocytes in the presence of IL-7, whereas in the D1 cell line, blocking PI3K accelerates death following IL-7 withdrawal. One possibility is that the Bad/Bcl-2 balance is more delicate in pro-T cells compared with D1 cells, i.e. more Bad and less Bcl-2. Another possibility is that pro-T cells are more sensitive to glucolysis-related survival, because the phosphorylation status of Bad could affect mitochondria-based glucokinase activity (38). It is also possible that inhibition of PI3K in pro-T cells could affect other pathways in addition to Bad, such as cell cycling or another apoptotic protein such as Bim. Induction of Bim synthesis occurred following cessation of PI3K/Akt signaling through a forkhead transcription factor (39). These other pathways could be more dominant in pro-T cells than D1 cells.

IL-7 was shown previously to activate PI3K in a murine B cell line, and the activation was dependent on the Tyr-449 of IL-7Rα, which is near the C terminus of the intracellular domain (27). Although we also observed that the PI3K/Akt is activated in the D1 thymocyte line by IL-7 (Fig. 1), other studies did not find a requirement for Tyr-449 or even the intracellular domain of IL-7Rα in this activation. Thus, mutation of Tyr-449 or deletion of the entire intracellular domain of IL-7Rα still retained Akt activation following IL-7 stimulation in D1 cells. These data suggest that the IL-7 signaling to activate the PI3K/Akt pathway comes from the γc chain in T cells. This would be consistent with the report showing that in human T cells stimulated with IL-7, Jak3 (which is associated with the γc) is associated with the p85 subunit of PI3K. IL-7-induced phosphorylation of p85, which could lead to the observed PI3K activation (40). Thus, IL-7 appears to activate the PI3K/Akt pathway differently between the T cell and B cell.

In a different cell line responding to IL-3, phosphorylation of Bad by Akt at Ser-112 and Ser-136 induces dissociation from Bcl-XL and translocation to the cytoplasm where it binds to 14-3-3 proteins and thus promotes cell survival (20, 35). In our system, the IL-7-induced Bad phosphorylation at Ser-112 at later time points did not appear to be mediated by Akt, because neither did LY294002 block nor active Akt promote Bad phosphorylation at Ser-112 (Figs. 3A and 4B). We have also distinguished the intracellular region of IL-7Rα that induces Bad phosphorylation from the region that induces the PI3K/Akt pathway, in which Box1 is required for the former but not the latter. cAMP-dependent protein kinase or ribosomal Ub kinase via protein kinase C-dependent pathways has been implicated in Bad phosphorylation at Ser-112 in some cell types (23, 41). However, we found that neither cAMP-dependent protein kinase nor protein kinase C inhibitors blocked Bad phosphorylation at Ser-112 in D1 cells (data not shown). The preceding observations suggest that there might be a novel kinase (neither Akt, nor cAMP-dependent protein kinase, nor protein kinase C, which is associated with the Box1 region of IL-7Rα) mediating Bad phosphorylation at Ser-112.

The Ser-155 residue of Bad undergoes phosphorylation via cAMP-dependent protein kinase in response to growth factors, including platelet-derived growth factor and insulin-like growth factor (19, 22). However, we found that this phosphorylation took place via the PI3K/Akt pathway in D1 cells responding to IL-7. The phosphorylation of the Ser-136 residue has been shown in many reports to require Akt, and we confirmed this in our system. Therefore, in T cells, IL-7 inactivation of Bad takes place only partially through the PI3K/Akt pathway.
pathway, distinguishing this mechanism from the action of other survival factors.

Bad-deficient mice show no dramatic increase in pro-T cells (26). This need not indicate that Bad inactivation is irrelevant to IL-7 signaling, because neither Bax-deficient nor Bcl-2 transgenic mice show striking increases in pro-T cells, whereas both dramatically restore pro-T cells in the absence of IL-7 (12, 13, 18). Thus, eliminating death pathways in pro-T cells reveals that, in addition to apoptosis, there must be other limits on their population size. On the other hand, Bad(3SA/3SA) knock-in mice (25) or Bcl-2 knock-out mice show a reduction in pro-T cells, indicating that these apoptotic mechanisms, once activated, can eliminate this cell type, thus supporting our model. As noted in the Bad(3SA/3SA) knock-in mouse study, we showed that overexpression of active Bad, Bad3SA, induced the death of D1 cells, an IL-7-dependent thymic cell line (Fig. 6C).

BH3-only proteins have been classified into two groups by Letal et al. (42), the “Bid-like” versus the “Bad-like.” The Bid-like proteins act on Bax and Bak; instead, they bind Bcl-2 and block its ability to sequester BH3-only proteins. We have shown previously that IL-7 with-
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