The Anti-apoptotic Effect of Notch-1 Requires p56\textsuperscript{Lck}-dependent, 
Akt/PKB-mediated Signaling in T Cells*

Received for publication, September 8, 2003, and in revised form, October 24, 2003
Published, JBC Papers in Press, October 28, 2003, DOI 10.1074/jbc.M309924200

Hadassah Sade, Sudhir Krishna, and Apurva Sarin‡

From the National Centre for Biological Sciences, University of Agricultural Sciences-Gandhi Krishi Vignan
Kendru Campus, New Bellary Road, Bangalore 560065, Karnataka, India

The Notch family of transmembrane receptors have been implicated in a variety of cellular decisions in different cell types. Here we investigate the mechanism underlying Notch-1-mediated anti-apoptotic function in T cells using model cell lines as the experimental system. Ectopic expression of the intracellular domain of Notch-1 (AcN1) increases expression of anti-apoptotic proteins of the inhibitors of apoptosis (IAP) family, the Bel-2 family, and the FLICE-like inhibitor protein (FLIP) and inhibits death triggered by multiple stimuli that activate intrinsic or extrinsic pathways of apoptosis in human and murine T cell lines. Numb inhibited the AcN1-dependent induction of anti-apoptotic proteins and anti-apoptotic function. Using pharmacological inhibitors and dominant-negative approaches, we demonstrate that p56\textsuperscript{Lck} is required for the Notch-1-mediated activation of Akt/PKB in the regulation of AcN1-mediated anti-apoptotic function and the expression of FLIP and IAP family proteins. Using a cell line deficient for the T cell-specific, Src family protein, the tyrosine kinase p56\textsuperscript{Lck} and by reconstitution approaches we demonstrate that p56\textsuperscript{Lck} is required for the Notch-1-mediated activation of Akt/PKB function. Furthermore, the Src tyrosine kinase inhibitor, PP2, abrogated ectopically expressed AcN1-mediated anti-apoptotic function and phosphorylation of p56\textsuperscript{Lck}. We present evidence that endogenous Notch-1 associates with p56\textsuperscript{Lck} and PI3K but that Akt/PKB does not co-immunoprecipitate with the Notch1-p56\textsuperscript{Lck}-PI3K complex. Finally, we demonstrate that the Notch1-p56\textsuperscript{Lck}-PI3K complex is present in primary T cells that have been activated in vitro and sustained in culture with the cytokine interleukin-2.

The Notch family of transmembrane receptors are important regulators of cell fate determination events in different cell types. Upon activation by ligands usually located on the surface of neighboring cells, Notch undergoes intramembrane proteolysis and release of an intracellular region resulting in signaling (1). The mechanism of Notch-1 signaling is incompletely understood even in well characterized genetic models like Droso-
sophila or the worm. There is recent evidence of the interaction of Notch together with T cell antigen receptor in determination of cell fate within the T cell lineage (2, 3). Furthermore, Notch-1 has been shown to protect against anokis (apoptosis induced by matrix withdrawal) or p53-mediated apoptosis in immortalized epithelial cells (4, 5), T cell receptor-induced apoptosis in mature cells (6), and dexamethasone-mediated apoptosis in thymocytes (7).

In mammalian cells apoptosis is initiated via extracellular receptor-mediated apoptotic signaling (8, 9) or death pathways that converge on the mitochondrion (10). Both pathways culminate in the activation of caspases, a family of proteases that mediate the orderly dismantling of cells. Death receptors of the tumor necrosis factor (TNF)\textsuperscript{1} receptor superfamily trigger apoptosis principally via the activation of procaspase 8/10 (8, 9), whereas mitochondrial signaling activates caspase-9. Caspase-induced apoptosis is regulated by endogenous antagonists like FLICE-like inhibitor protein (FLIP) that prevent processing and anti-apoptotic proteins of the inhibitors of apoptosis protein (IAP) family that block function (11). The release of intermediates from the mitochondrial intermembrane space that occurs as an initial step is blocked by anti-apoptotic members of the Bel-2 family (12).

Mature T cells develop from immature precursors through complex but ordered processes (13). There is considerable evidence that Notch signaling regulates the earliest stages of T cell commitment and promotes differentiation in the T cell lineage (14–16). During T cell development, negative selection ensures deletion of autoreactive thymocytes and allows maturation of CD4\textsuperscript{+} or CD8\textsuperscript{+} T cells (13). Notch involvement in Bel-2-dependent survival signaling in immature T cells was first reported in this model (7). Thymic maturation into CD4\textsuperscript{+} or CD8\textsuperscript{+} subsets of mature T cells is accompanied by the emigration of these cells to peripheral lymphoid organs. Mature T cells remain susceptible to varied apoptotic stimuli, many of which are regulated via pathways distinct from those in thymocytes (17–19). In this study we ask if Notch-1 regulates apoptosis in model T cell lines and have attempted to identify the molecular mechanism of Notch-1 dependent anti-apoptotic function.

We show that ectopic expression of the intracellular domain of Notch-1 (AcN1) confers protection against diverse stimuli that trigger intrinsic and extrinsic pathways of death in model T cell lines. This anti-apoptotic function, we suggest, can result from the AcN1-induced elevated expression of IAP-2, Bcl-xL.

† To whom correspondence should be addressed: National Centre for Biological Sciences, UAS-GKVK Campus, New Bellary Rd., Bangalore 560065, Karnataka, India. Tel.: 91-80-363-6420; Fax: 91-80-363-6462; E-mail: sarina@ncbs.res.in.

* This work was supported by grants from the Department of Science and Technology, Ministry of Health, Government of India; Life Sciences Research Board, India; and an International Senior Research Fellowship in Biomedical Sciences in India from the Wellcome Trust, UK (to A. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: TNF, tumor necrosis factor; FLIP, FLICE inhibitor protein; IAP, inhibitors of apoptosis; PI3K, phosphatidylinositol 3-kinase; GFP, green fluorescent protein; LC, loading control; PP2, 4-amino-5-(4-chlorophenyl)-7-([3R]-butyl)pyrazolo[3,4-d]pyrimidine; TRAIL, TNF-related apoptosis-inducing ligand; WBA, Western blot analysis.
and FLIP, which represent three major families of anti-apoptotic proteins. The protective effect of AcN1 was attenuated by the Notch antagonist Numb and disruption of Akt/PKB-dependent signaling. Furthermore, we show that loss of function of the T cell-specific, Src family, non-receptor tyrosine kinase p56\textsuperscript{Lck} compromised Akt/PKB phosphorylation and anti-apoptotic function. This allows us to propose a functional hierarchy of interactions between Notch-1, PI3K, and p56\textsuperscript{Lck} that might operate in T cells.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents—**Jurkat, a T lymphoblastoid cell line of human origin, and 2B4, a murine T cell hybridoma, were used in all experiments. The J.CaM1.6 mutant was obtained from ATCC (Manassas, VA). Activated T cells were generated by stimulation in vitro as described before and sustained in interleukin-2 (20). Activated T cells were used on day 5 after stimulation. All reagents were obtained from Calbiochem unless specified otherwise. The mitochondrial dye JC-1 was obtained from Molecular Probes (Eugene, OR). Recombinant TNF, TRAIL, and antibodies to Bid, Bcl-2, Bcl-x\textsubscript{L}, and FLIP were obtained from R & D Systems (Minneapolis, MN). Antibodies to Akt, BIM, IAP-1, IAP-2, Jagged, p85MAPK, p56\textsuperscript{Lck}, and GFP were from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies to phosphorylated p56\textsuperscript{Lck} or Akt were obtained from Cell Signaling Technology (Beverly, MA) and the monoclonal antibody to GFP was from Clontech, BD Biosciences. Antibodies to the various domains of Notch and their sources are as follows: Notch-1 intracellular domain referred to as NICT\textsuperscript{290} (clone C17.9C6) and to Notch-1 intracellular domain nucleotides 6658–7131 (clone bTan 20) were obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA); Notch-1 carboxyl terminus referred to as NICD\textsuperscript{sc} (M-20) and to the Notch-1 extracellular domain, residues 20–150, referred to as Notch-1 EC\textsuperscript{H11032} (H-131), were from Santa Cruz Biotechnology and to residues 1299–1492 in the extracellular domain of Notch-1, referred to as Notch-1 EC\textsuperscript{H11032} (clone 8G10), was from Upstate Biotechnology (Lake Placid, NY).

**Plasmids and Transfections—**The AcN encoding plasmid pcDNA3-ICTX (AcN1; Ref. 21) was a kind gift of J. Aster (Harvard Medical School, Boston). This construct was used to generate stable transfectants. AcN-IRES-GFP was a gift from Annapoorni Rangarajan (Whitehead Institute for Biomedical Research, Cambridge, MA). In addition, we generated the AcN1-GFP construct for which the AcN1 region (spanning amino acids 1759–2556) was amplified using the following specific primers: FF, 5'-GGAGAATTCATGCCAACCCGCCC-3' and RR, 5'-GGG-GATCCTCAACAGTCGGCTCG-3') cloned into EcoRI and BamHI sites of pEGFPN1. All three constructs, which principally comprise the intracellular domain of Notch-1, yielded the same results in biochemical and functional assays. The dominant-negative mutant of Akt, AH-Akt, was obtained from Julian Downward (Imperial Cancer Research Fund, London, UK). The Bcl-2 plasmid was a generous gift of Dr. S. Perwez Hussain (NCI, National Institutes of Health). The construct for Numb (22) was obtained from Weimin Zhong (Yale University). The mouse Lck clone NT-18 was obtained from ATCC (Manassas, VA). The open reading frame of Lck was PCR-amplified and cloned into the Scal and KpnI sites of the pCruz Myc expression vector (Santa Cruz Biotechnology). The sequences of the primers used for the PCR are 5'-AAAGTACTATGG-GCTTGTTGTCGGC-3' (FF) and 5'-GGGATACCTCAAGGCTGCTGCTG-3' (RP). The sequence of all constructs was verified by automated sequencing.

Stable cell lines expressing neo, AcN1 or Bcl-2 were generated by transfection with 2 \mu g of DNA of appropriate plasmids by electroporation. After 48 h, cells were cultured for 10–14 days in complete medium supplemented with 80 \mu g/ml G418 for selective growth of transfected cells. Selected cells were not subcloned further. A minimum of two separate sets of stable transfectants have been tested in these experiments. All experiments were performed as described previously (23).

**Electroporations—**Electroporations were performed as described previously (23). All flow cytometric analysis was performed on the FACSCalibur (BD Biosciences). Apoptotic nuclear damage was assessed in GFP-transfected cells as described before (23).

**Immunoprecipitations—**Immunoprecipitations were performed in transiently transfected populations. 10\textsuperscript{6} cells were lysed in 20 mM Tris (pH 7.5) containing 450 mM NaCl, 1 mM EDTA, 10 \mu g/ml apotinin and leupeptin, 100 \mu g/ml phenylmethylsulfonyl fluoride, and 1% Nonidet P-40 for 15 min at 4 °C. Cell debris was cleared by centrifugation at 14,000 rpm for 30 min, and the resulting supernatant was divided into 2 aliquots, one aliquot incubated overnight with Sepharose G beads (Amersham Biosciences, Uppsala, Sweden) preincubated for 1 h with specific antibody and the other aliquot with beads similarly incubated but with an isotype-matched antibody as a control. Lysates were immunoprecipitated overnight at 4 °C by gentle rocking, and then beads with bound proteins (immune complex) were separated by centrifugation at 14,000 rpm for 1 min. Immune complexes were washed in lysis buffer, resolved on SDS gels, and the proteins detected by Western blot analysis. In immunoprecipitations using p56\textsuperscript{Lck} or, a polyclonal antibody to Akt (raised in rabbit) from the same company was used as negative controls. In experiments where the polyclonal antibody (goat) to Notch intracellular domain was used, a polyclonal antibody (raised in goat) recognizing Bim and from the same company was used as the control group. In other experiments, purified rabbit and goat Ig have been used as negative controls with identical results. In experiments where GFP was immunoprecipitated with a monoclonal to GFP, purified monoclonal mouse Ig (Sigma) was used as a control.

**Western Blot Analysis of Proteins—**Cell lysates of 0.2–0.5 × 10\textsuperscript{6} cells were resolved by SDS-PAGE, and protocols recommended by the manufacturers were used for Western blot analysis of Bcl-x\textsubscript{L}, FLIP, and Notch. Blots were developed by chemiluminescence using Super Signal (Pierce). p85MAPK was used to establish parity of loading in 10–12% SDS gels, and Akt or spectrin was used as the loading control (LC) for 8–10% SDS gels. The term LC has been used to indicate these proteins in the various Western blots.

**RESULTS**

The murine T cell hybridoma 2B4 and the human lymphoblastoid cell line Jurkat have been used in all experiments. All experiments have been performed using both the stable and transient expression of AcN1 in these cell lines. Stable transfectants of Jurkat and 2B4 were derived as described under “Experimental Procedures.” For clarity of presentation, the data presented in the figures were derived from either transient or stable transfections in different experiments.

**AcN1 Blocks Apoptosis and Induces Expression of Anti-apoptotic Proteins—**The 2B4 T cell hybridoma has been used in many studies as a model system to examine the regulation of apoptosis in mature T cells (24, 25). Stable transfectants of 2B4 cells constitutively expressing the intracellular domain of Notch-1 (AcN1) (hereafter referred to as 2B4-AcN1S) has significantly increased levels of the anti-apoptotic proteins Bcl-x\textsubscript{L}, FLIP, and IAP-2 (Fig. 1A). Bcl-2 expression is relatively high in 2B4 cells and was not substantially increased in 2B4-AcN1S cells in comparison with cells transfected with the neomycin gene alone. To test whether elevated levels of these proteins correlated with functional outcomes, apoptotic damage was assessed in 2B4-AcN1S cells (closed symbols or filled bars) or 2B4-neos cells (open symbols or open bars) treated with dexamethasone, etoposide, or cultured on anti-CD3-coated plates to trigger Fas-ligand-Fas signaling (Fig. 1, B–D). In all cases 2B4-AcN1S-expressing cells were protected from apoptotic damage, albeit to varying extents. Similar results were obtained on transient transfection of AcN1 (data not shown).

**AcN1 Blocks Apoptosis in Jurkat Cells—**To confirm that the results described above were not a peculiarity of the 2B4 hybridoma, we ectopically expressed AcN1 in Jurkat, a human lymphoblastoid T cell line. This result in elevated levels of Bcl-x\textsubscript{L} and IAP-2 proteins in Jurkat cells stably transfected with AcN1 (J-AcN1S) as compared with Jurkat cells transfected with the control plasmid (J-neoS) (Fig. 2A). Endogenous FLIP expression is higher in Jurkat than in 2B4 cells; however, the increase in protein expression induced by AcN1 was significant in both cell lines. Bcl-2 on the other hand was not detectable in untransfected Jurkat cells, and its expression could not be induced by AcN1. We established that our assay could detect
modulated mitochondrially regulated apoptotic signaling. Since many apoptotic signaling pathways have been characterized in the Jurkat cell line, we used the cell line to test whether AcN1 regulated apoptosis triggered by extrinsic pathways of death as well. J-neoS and J-AcN1S cells were assessed for susceptibility to apoptosis induced by the death receptor ligands TNF or TRAIL. Apoptosis was triggered by treatment with TNF and cycloheximide (the combination referred to as TNF) in all analysis. J-AcN1S cells were protected from TNF-induced apoptotic nuclear damage (Fig. 2B, solid bars), loss of mitochondrial transmembrane potential, which is a measure of mitochondrial integrity (Fig. 2D, dark hatched bars) or cell lysis (data not shown). AcN1 also protected cells from different concentrations of TRAIL-induced apoptosis at a level equivalent to that achieved by overexpressing Bcl-2 in these cells (Fig. 2C).

Furthermore, on transient transfection with AcN1-GFP, Jurkat cells were protected from apoptosis triggered by etoposide (Fig. 2D) or the PKC inhibitor, staurosporine, at 20 ng/ml staurosporine but not at the higher concentration of staurosporine (Fig. 2E). In Fig. 2, D and E, J-AcN1-GFP-transfected cells are represented by filled symbols and cells transfected with GFP alone by open symbols. Thus, ectopic expression of AcN1 resulted in increased expression of anti-apoptotic proteins and protection from diverse apoptotic stimuli. We then attempted to identify the mechanism by which AcN1 could initiate these events.

AcN1 Activates PI3K-mediated Signaling—Previous experiments have shown that Notch-1 acts through the PI3K pathway to trigger survival in epithelial cells (4). If a similar mechanism operated in T cells, modifications in intermediates of this pathway would, in principle, be detected upon AcN1 ectopic expression. We used pharmacological inhibitors of PI3K and a dominant-negative inhibitor of the serine-threonine kinase Akt (AH-Akt) to test the functional interactions between Notch signaling and the PI3K pathway in T cells. We detected increased phosphorylation Akt-Thr308 with no change in levels of total Akt in either 2B4 or Jurkat cell lines transiently transfected with AcN1 (Fig. 3A). In functional assays of apoptosis, LY294002 a pharmacological inhibitor of PI3K (26) reversed the anti-apoptotic function of AcN1 in 2B4 cells (Fig. 3B). TNF-induced apoptosis is substantially lower in J-AcN1S cells (Fig. 3C, hatched bars) as compared with TNF-induced apopto-

Fig. 1. AcN1 increases anti-apoptotic protein expression and blocks apoptosis in 2B4 cells. A, 2B4-neo cells or 2B4-AcN1S cells were analyzed by immunoblotting for the expression of different proteins shown in the figure. p38MAPK was used to establish parity of loading/LC. B–D, 2B4-neo (open squares or open bars) and 2B4-AcN1S (filled squares or dark bars) cells were cultured overnight with indicated concentrations of dexamethasone (B), etoposide (C), or plate-bound anti-CD3 (clone 2C11, 5 μg/ml) (D). Cells were scored for apoptotic nuclear morphology as described previously (23).

Fig. 2. AcN1 up-regulates anti-apoptotic proteins and blocks apoptosis in Jurkat cells. A, J-neo, J-AcN1S, or J-Bcl-2S cells were analyzed for the expression of proteins by WBA. p38MAPK was the LC. B, J-neo or J-AcN1S cells were cultured untreated or with 50 ng/ml TNF + 100 ng/ml cycloheximide for 18 h. Apoptotic nuclear damage in the presence (black bars) or absence (white bars) of TNF, and the loss of mitochondrial transmembrane potential in the presence (dark hatched bars) or absence (light, hatched bars) of TNF was measured as described under “Experimental Procedures.” C, J-neo (open squares) or J-AcN1S (filled circles) or J-Bcl-2S (filled triangles) cells were cultured with indicated concentrations of TRAIL for 12–15 h and analyzed for apoptotic nuclear damage. D and E, Jurkat cells transiently transfected with 1.8 μg neo-GFP (open squares) or 5 μg of AcN1GFP (filled circles) or J-AcN1S cells or 2B4-AcN1S cells were cultured for 14 h to allow expression of transfected genes. Cells were then cultured as such or with the indicated concentrations of etoposide (D) or staurosporine (STS, E). After an additional 12 h of culture with the apoptotic stimuli, GFP-positive cells in the two transfection groups were scored for apoptotic nuclear damage.

Bel-2 protein by detecting the protein in Jurkat cells that stably express bel-2 (Fig. 2A, third lane).

The experiments with the 2B4 cell line indicated that AcN1
LY294002 reversed the protective effect of AcN1 but not Bcl-2 inhibition of TRAIL-induced death (Fig. 3D, filled symbols). We verified that LY294002 was not toxic to cells (Fig. 3C and D, open symbols). In subsequent experiments we used a dominant-negative form of Akt (AH-Akt) to test the outcome of more specific disruption of Akt signaling on AcN1-inhibition of apoptosis. Apoptosis induced by TRAIL is substantially reduced in cells that express AcN1 (Fig. 3E, AcN1) when compared with those expressing neomycin (Fig. 3E, neo). In this assay, the co-expression of AH-Akt attenuated the anti-apoptotic effect of AcN1 (Fig. 3E, AcN1+AH-Akt), indicating that disrupting Akt signaling interfered with the anti-apoptotic function of AcN1.

Ectopic expression of AH-Akt in 2B4 cells also blocked AcN1-mediated induction of IAP-2 and FLIP but did not affect the up-regulation of Bcl-xL (Fig. 3F). J-AcN1S cells treated with 20 \( \mu \text{M} \) LY294002 for 6–8 h were assessed for the expression of anti-apoptotic proteins. LY294002 blocked Akt phosphorylation and expression of IAP-2 (Fig. 3G). Again, Bcl-xL expression was not modified by LY294002. Taken together, these experiments using genetic and pharmacological approaches suggest that AcN1 triggered activation of a PI3K signaling pathway that culminated in the expression of the anti-apoptotic proteins FLIP and IAP. However, Bcl-xL expression appeared to be regulated independently of Akt signaling.

Numb Blocks AcN1-induced Signaling in T Cells—We used the Notch antagonist Numb to confirm that the observations in the preceding set of experiments were specifically initiated by Notch signaling. As shown in Fig. 4A, co-transfection of Numb disrupted AcN1 inhibition of dexamethasone or etoposide in the 2B4 cell line. Ectopic expression of Numb alone enhanced Fas-mediated apoptosis (triggered by cross-linking the TcR-CD3 complex) in the 2B4 cell line (Fig. 4B) and TRAIL-induced apoptosis in Jurkat cells (Fig. 4C). Furthermore, Numb blocked AcN1-mediated induction of Bcl-xL, IAP-2, and FLIP in both cell lines (Fig. 4, D and E) and substantially reduced the phosphorylation of Akt (Fig. 4F). Using the GFP-tagged construct of AcN1 (AcN1-GFP) we could determine that AcN1 is principally localized to the nucleus, and this distribution was not disrupted in cells that co-expressed Numb (Fig. 4G).

p56\(^{\text{lck}}\) Is a Putative Adapter in the AcN1-mediated Activation of Akt—The experiments thus far suggest that AcN1 signals via the PI3K pathway to activate Akt culminating in cell survival. What is the link between Notch-1 and Akt activation? p56\(^{\text{lck}}\) is a member of the Src-like family of non-receptor tyrosine kinases associated with T cell receptor signaling. p56\(^{\text{lck}}\) is activated when CD4 or CD8 co-receptors are cross-linked and phosphorylates and activates other intracellular substrates such as Akt via PI3K. Ectopic expression of AcN1 resulted in enhanced phosphorylation of p56\(^{\text{lck}}\) in Jurkat and 2B4 cells (Fig. 5A). To explore the functional implications of this phosphorylation, if any, we used the isogenic Jurkat cell line that was defective for p56\(^{\text{lck}}\) (J.CaM1.6). In J.CaM1.6 cells AcN1 did not induce phosphorylation of Akt or expression of IAP-2 or FLIP (Fig. 5B). TNF-dependent induction of IAP-2 and increased phosphorylation of Akt indicated that the J.CaM1.6 cell line was not defective in the modulation of these proteins if signaled appropriately (Fig. 5C). The cell line was refractory to AcN1-mediated inhibition of etoposide-induced apoptosis (Fig. 5D). However, reconstitution with p56\(^{\text{lck}}\) restored the ability of AcN1 to block etoposide-induced apoptotic nuclear damage in these cells (Fig. 5E). In another approach, treatment with the general Src kinase inhibitor, PP2, abrogated the anti-apoptotic effect of AcN1 in Jurkat cells (Fig. 5F). Furthermore, when tested after overnight treatment, at concentrations that abrogated the anti-apoptotic function of AcN1, PP2 also blocked AcN1-induced phosphorylation of p56\(^{\text{lck}}\) (Fig. 5F, inset).
FIG. 5. Role of p56\(\text{lck}\) in Notch-1 function. A, lysates of Jurkat (first and second lanes) and 2B4 cells (third and fourth lanes) transiently overexpressing neo (first and third lanes) or AcN1GFP (second and fourth lanes) were probed for levels of phospho-p56\(\text{lck}\) by immunoblotting. Equal loading was confirmed by re-probing with p56\(\text{lck}\). B, J.CaM1.6 cells were transfected with GFP (first lane) or AcN1GFP (second lane) and assessed for the expression of proteins indicated in the figure. C, J.CaM1.6 cells were cultured as such (first lane) or with 100 ng/ml TNF (second lane) for 12 h and lysates of 4 \(\times\) 10^5 cells were analyzed for expression of proteins shown in the figure. D, J.CaM1.6 cells were transfected with 1.8 \(\mu\)g of GFP or 5 \(\mu\)g of AcN1GFP. 12 h after transfection, cells were cultured in culture with (filled bars) or without (open bars) TRAIL (5 ng/ml) for 12 h. Apoptotic nuclear damage in GFP-positive cells was assessed by counting the number of cells with characteristic nuclear morphology. E, whole cell lysates were prepared of Jurkat (first lane) or 2B4 (second lane) cells transfected with GFP or AcN1GFP and cultured for 14 h. Lysates were analyzed for expression of different proteins shown in the figures. F, WBA of phosphorylated Akt in Jurkat cells transfected with 5 \(\mu\)g AcN1-GFP (first lane), 5 \(\mu\)g of AcN1GFP + 2.5 \(\mu\)g of Numb (second lane). The membrane was re-probed for Akt (LC). G, nuclear localization of AcN1-GFP in 2B4 cells transfected with AcN1 in the absence (upper panel) or presence of Numb (lower panel) by fluorescence microscopy. The panels on the left are the same field of cells stained with Hoechst 33342.

FIG. 4. Numb regulates AcN1 function. A, 2B4 cells transfected with GFP or 5 \(\mu\)g of AcN1GFP or 5 \(\mu\)g of AcN1GFP + 2.5 \(\mu\)g of Numb were cultured for 14 h to allow expression of transfected genes. Cells were then transfected in culture with (open bars) or treated with 25 nM dexamethasone (Dex) (black bars) or 2.5 \(\mu\)g/ml etoposide (Etop) (gray bars). 12 h after the addition of the apoptotic stimuli, GFP-positive cells were scored for apoptotic nuclear damage as described under “Experimental Procedures.” B, 2B4 cells were transfected with GFP or GFP + 2.5 \(\mu\)g Numb. 10 h after transfection, cells were transfected in culture on wells that had plate bound anti-CD3 (10 \(\mu\)g/ml, filled bars) or on uncoated wells (open bars). Cells were harvested after 14 h and assessed for apoptotic nuclear damage in the GFP-positive cells in all groups. C, Jurkat cells were transfected with GFP or GFP + 2.5 \(\mu\)g Numb and cultured for 10 h. Cells from all transfection groups were then cultured in culture with (filled bars) or without (open bars) TRAIL (5 ng/ml). Apoptotic nuclear damage in GFP-positive cells was assessed 8–10 h after the addition of TRAIL. D and E, whole cell lysates were prepared of Jurkat (D) or 2B4 (E) cells transfected with neo, 5 \(\mu\)g of AcN1GFP, or 5 \(\mu\)g of AcN1GFP + 2.5 \(\mu\)g of Numb and cultured for 14 h. Lysates were analyzed for expression of different proteins shown in the figures. F, WBA of phosphorylated Akt in Jurkat cells transfected with 5 \(\mu\)g AcN1-GFP (first lane), 5 \(\mu\)g of AcN1GFP + 2.5 \(\mu\)g of Numb (second lane). The membrane was re-probed for Akt (LC). G, nuclear localization of AcN1-GFP in 2B4 cells transfected with AcN1 in the absence (upper panel) or presence of Numb (lower panel) by fluorescence microscopy. The panels on the left are the same field of cells stained with Hoechst 33342.
Notch-1 Inhibits Apoptosis in T Cells

---

**Fig. 6. Endogenous Notch-1 associates with PI3K and p56
 in T cells.** A, Jurkat cell lysates prepared 8 h after transfection with AcN1-GFP were immunoprecipitated (IP) with mouse Ig (isotype control, first lane) or mouse anti-GFP (second lane) and analyzed by Western blot and sequential strip and re-probe for GFP, NICDSC, and PI3K. B, Jurkat (second and fourth lanes) and J CaM1.6 (first and third lanes) cells were transfected with 5 μg of AcN1-GFP and cultured for 8 h were immunoprecipitated (IP) with beads coated with goat Ig (isotype control, first lane) or NICDSC, the Notch-1 antibody raised in goat (second lane). Immunoprecipitates were analyzed by WBA for expression of the proteins indicated in the panel on the right of the blots. WCL indicates the whole cell lysate. C, untransfected Jurkat cell lysates were immunoprecipitated (IP) using an antibody to p56
 as described under “Experimental Procedures.” Immunoprecipitates were analyzed for the presence of NICDSC and PI3K by Western blot analysis. E and F, whole cell lysates of Jurkat cells transiently transfected with GFP (first lane) or 5 μg of AcN1GFP (second lane) were analyzed for the expression of full-length Notch-1 protein using the bTan20 antibody (E). Spectrin was used to assess porosity of loading (LC). In the same experiment, cells were also probed for the expression of Jagged (F). G, Jurkat cells transfected with GFP or AcN1-GFP were immunoprecipitated (IP) with the antibody to p56
. The immunoprecipitates were resolved on 8% SDS gels and probed for the expression of Notch-1: Notch-1 ECUB and Notch-1 EC SC. Expectedly, PI3K and p56
 were selected for survival rather than death they must also be equipped with proteins such as FLIP and members of the Bcl-2 family that serve to buffer them from various apoptotic stimuli.

---

Notch-1 Associates with PI3K and p56
 in AcN1-transfected and -activated T Cells—The transfected construct AcN1 is principally nuclear-localized (Fig. 4G), although PI3K is a membrane-localized signaling complex, as is p56
. We hypothesized that if indeed there is a direct interaction of the PI3K-p56
 complex with Notch, then the association must involve Notch-1 localized outside the nucleus, most likely full-length, endogenous Notch-1. The subsequent experiments were designed to test this possibility. When an antibody to GFP was used to immunoprecipitate AcN1-GFP from transfected cells, it failed to immunoprecipitate PI3K (Fig. 6A). The immunoprecipitated GFP-tagged AcN1 protein (>116 kDa) can be detected by the antibody (clone C17.9C6) raised to the Notch-1 intracellular region (NICDHB). The AcN1-GFP protein is, however, not recognized by another antibody (NICDSC) to the intracellular domain. The NICDSC clone did recognize endogenous protein (third lane, WCL) thereby providing a means to distinguish the transfected gene product (>116 kDa) from the endogenous protein (>85 kDa).

Immunoprecipitating endogenous p56
 from AcN1-GFP-transfected Jurkat cells (Fig. 6B, second and fourth lanes) revealed a complex that contains the p85 regulatory subunit of PI3K but not AcN1-GFP or Akt (Fig. 6B, fourth lane), although these are detected in the whole cell lysate (second lane). The NICDSC antibody, which does not recognize AcN1-GFP, revealed that endogenous Notch-1 is in association with PI3K and p56
 (Fig. 6B, fourth lane). Expectedly, there was no signal for PI3K or Notch-1 on immunoprecipitating of p56
 in J.CaM1.6 cells, as these cells do not express p56
 (Fig. 6B, first and third lanes). In reverse immunoprecipitations we confirmed that immunoprecipitating endogenous Notch-1 using NICDSC brings down a complex that contains p56
 and PI3K but not Akt from AcN1-GFP-transfected Jurkat cells (Fig. 6C). Thus, from these experiments we concluded that endogenous Notch-1 associates with PI3K and p56
. The complex does not stably associate with Akt or AcN1-GFP.

Does p56
 normally exist in association with endogenous Notch-1 and PI3K? Indeed, in untransfected Jurkat cells, p56
 associates with PI3K and Notch-1, although expectedly the levels of Notch-1 are relatively low in these cells (Fig. 6D). Since the phosphorylation of both Akt and p56
 is consistently enhanced on AcN1 transfection, and cells are protected from apoptosis, we hypothesized that AcN1 may stabilize the Notch-1-p56
-PI3K interaction by driving expression of full-length endogenous Notch-1. A similar feedback mechanism has also been reported in other systems (28, 29). As already shown, the levels of p56
, PI3K, or Akt are not enhanced in AcN1 transfected cells, but the possibility that AcN1 triggers an increase in expression of endogenous Notch-1 was confirmed in subsequent experiments. Jurkat cells stably transfected with AcN1GFP express increased levels of a >250-kDa Notch-1 species that is detected using an antibody that recognizes an epitope in the Notch-1 extracellular domain (Fig. 6E). Furthermore, the Notch-1 ligand, Jagged, was also elevated in these cells (Fig. 6F), which suggests the possibility of increased ligand-mediated Notch signaling via the endogenous receptor in ACN1-transfected cells. We then tested whether the extracellular domain of Notch-1 is also present in the complex with p56
 and PI3K. As shown in Fig. 6G, in experiments that replicate the protocol used for B and D, p56
 immunoprecipitates a complex that contains the Notch-1 extracellular domain detected using two antibodies raised to the extracellular region of Notch-1: Notch-1 ECUB and Notch-1 ECSC. Expectedly, PI3K and NICD are also present in this complex.

Finally we asked whether Notch-1, PI3K, and p56
 associate in non-transformed T cells. We tested for this association in polyclonally activated T cells sustained in culture with interleukin-2. The cytokine is important for T cell proliferation and survival and some of these effects are dependent on signaling through PI3K. In activated T cells we observed that endogenous Notch-1 (NICDSC) immunoprecipitated p56
 and PI3K (Fig. 6H) from untransfected, in vitro activated splenic T cells sustained in culture with the cytokine interleukin-2.

**DISCUSSION**

Mature T cells are known to express anti-apoptotic proteins to protect themselves from diverse apoptotic stimuli that they encounter in the course of their existence. Thus, when T cells are selected for survival rather than death they must also be equipped with proteins such as FLIP and members of the Bcl-2 family that serve to buffer them from various apoptotic stimuli.
Notch-1 Inhibits Apoptosis in T Cells

We show that Notch-1 functions as part of cytokine signaling relays to regulate cell survival in specific spatio-temporal contexts in T cell compartments. Following an antigen response, effector T cells are eliminated by active mechanisms (40). Linked to the loss of the effector pool of T cells is generation of the memory subset (41). While initial events resulting in the generation of memory may be stochastic, long term survival of memory T cells is likely dependent on instructional signals delivered by cytokines (42). Anti-apoptotic proteins like Bel-2 and Bel-x, are critical for immune memory, and their expression is also linked to cytokine signaling in T cells (43). Our experiments reveal a functional relationship between Notch-1, p56Lck, and PI3K-dependent activation of Akt, molecules that are key intermediates in T cell survival. Elucidation of the mechanism by which Notch and PI3K influence signaling events that link T cell stimulation to the regulation of apoptotic responses and long term survival of antigen-stimulated T cells would yield further insights into molecular mechanisms underlying T cell homeostasis.

Acknowledgments—We are grateful to Satyajit Rath, National Institute of Immunology, New Delhi and Veronica Rodrigues, DBS, TIFR for discussion and comments. The monoclonal antibodies-bTan 20 and C17.9C6 developed by Spyros Artavanis-Tsakonas were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD, National Institutes of Health, and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA. The costs of publication of this article were defrayed in part by the page charge payments. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

REFERENCES

1. Artavanis-Tsakonas, S., Rand, M. D., and Lake, R. J. (1999) Science 284, 770–776
2. Robey, E., Chang, D., Itano, A., Cado, D., Alexander, H., Lans, D., Weinmaster, G., and Salmon, P. (1996) Cell 87, 485–492
3. Washburn, T., Schweighoffer, E., Gridley, T., Chang, D., Fowlkes, B. J., Cado, D., and Robey, E. (1997) Cell 88, 833–843
4. Rangarajan, A., Syal, R., Selvarajah, S., Chakraborti, O., Sarin, A., and Krishna, S. (2001) Virology 286, 25–30
5. Nair, P., Somasundaram, K., and Krishna, S. (2003) J. Virol. 77, 7106–7112
6. Jhe, B. M., Bielek, W., Pear, W. S., and Osborne, B. A. (1999) J. Immunol. 162, 635–638
7. Deftos, M. L., He, Y. W., Ojala, E. W., and Bevan, M. J. (1998) Immunol. Rev. 166, 239–252
8. Ashkenazi, A., and Dixit, V. M. (1998) Science 281, 1305–1308
9. Wallach, D., Varfolomeev, E. E., Malinin, N. L., Golstein, Y. V., Kovalevsky, A. B., and Boldin, M. P. (1999) Annu. Rev. Immunol. 17, 331–367
10. Green, D. R., and Reed, J. C. (1998) Science 281, 1309–1312
11. Datta, S. R., Brunet, A., and Greenberg, M. E. (1999) Cell 99, 403–414
12. Gottlieb, E., Vander Heiden, M. G., and Thompson, C. B. (2000) Mol. Cell. Biol. 20, 5680–5689
13. Goldberg, A. W., and Bevan, M. J. (1999) Nature 402, 255–262
14. Itoh, J., Punt, J. A., and Pear, W. S. (2002) Curr. Opin. Immunol. 14, 192–199
15. Radtke, F., Wilson, A., Ernst, B., and MacDonald, H. R. (2002) Immuno. Rev. 187, 65–74
16. Schmitt, T. M., and Záitze-Pfücker, J. C. (2002) Immunity 17, 749–756
17. Boehme, S. A., and Lenardo, M. J. (1996) J. Immunol. 156, 4075–4078
18. Kuida, K., Haydar, T. F., Kuan, C. Y., Gu, Y., Taya, C., Karasuyama, H., Su, M. S., Rakic, P., and Flavell, R. A. (1998) Cell 94, 325–337
19. Yoshida, H., Kong, Y. Y., Yoshida, R., Elia, A. J., Hakem, A., Hakem, R., Penninger, J. M., and Mak, T. W. (1998) Cell 94, 739–750
20. Sane, A., Wu, M. L., and Henkart, P. A. (1996) J. Exp. Med. 184, 2445–2450
21. Aster, J. C., Robertson, E. S., Hasserjian, R. P., Turner, J. R., Kieff, E., and Sklar, J. (1997) J. Biol. Chem. 272, 11336–11343
22. Zhang, W., Fedor, J. N., Jiang, M. M., Jan, L. Y., and Jan, Y. N. (1996) Neuron 17, 45–53
23. Sade, H., and Sarin, A. (2003) Eur. J. Immunol. 33, 913–919
24. Yang, Y., Liu, Z. H., Ware, C. F., and Ashwell, J. D. (1997) Blood 89, 550–557
25. Sarin, A., Adams, D. H., and Henkart, P. A. (1993) J. Exp. Med. 178, 1693–1700
26. Vlahos, C. J., Matter, W. F., Hui, K. Y., and Brown, R. F. (1994) J. Biol. Chem. 269, 5241–5248
27. Yano, H., Nakamishi, S., Kimura, K., Hanai, N., Saito, Y., Fukui, Y., Nonomura, Y., and Matsuda, Y. (1993) J. Biol. Chem. 268, 25854–25856
28. Luo, B., Aster, J. C., Hasserjian, R. P., Kuo, F., and Sklar, J. (1997) Mol. Cell. Biol. 17, 6057–6067
29. Carmena, A., Boff, E., Halmon, M. S., Gisellbrecht, S., Jimenez, F., Baylies, M. K., and Michelson, A. M. (2002) Dev. Biol. 244, 226–242
30. Datta, S. R., Brunet, A., and Greenberg, M. E. (1999) Genes Dev. 13, 2995–2997
31. Pages, F., Raguneau, M., Rottapel, R., Truneh, A., Nunes, J., Imbert, J., and Olive, D. (1994) Nature 368, 327–329
32. Delsolmenne, M., Tan, C., Gray, V., Rue, L., Woodgett, J., and Dhafer, S. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11211–11216
33. Perez, O. D., Kinosita, S., Itoh, Y., Payan, D. G., Kitamura, T., Nolant, G. P., and Lorenz, J. B. (2002) Immunity 16, 51–65
34. Cepionis, P. J., Botelho, F., Richards, C. D., and McKay, D. M. (2000) J. Biol. Chem. 275, 29121–29127
35. Sohn, S.J., Forbush, K. A., Pan, X. C., and Perlmuter, R. M. (2001) J. Immunol. 166, 2209–2217
36. Chen, W. S., Xu, P. Z., Gottlob, K., Chen, M. L., Sokol, K., Shiyanova, T., Roninson, I., Weng, W., Suzuki, R., Tohe, K., Kadowaki, T., and Hay, N. (2001) Genes Dev. 15, 2203–2208
Notch-1 Inhibits Apoptosis in T Cells

37. Seddon, B., Legname, G., Tomlinson, P., and Zamoyska, R. (2000) Science 290, 127–131
38. Cuevas, B., Lu, Y., Watt, S., Kumar, R., Zhang, J., Siminovitch, K. A., and Mills, G. B. (1999) J. Biol. Chem. 274, 27583–27589
39. Raab, M., Cai, Y. C., Bunnell, S. C., Heyeck, S. D., Berg, L. J., and Rudd, C. E. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8891–8895
40. Van Parijs, L., and Abbas, A. K. (1998) Science 280, 243–248
41. Sprent, J., and Surh, C. D. (2002) Annu. Rev. Immunol. 20, 551–579
42. Lodolce, J. P., Boone, D. L., Chai, S., Swain, R. E., Dassopoulos, T., Trettin, S., and Ma, A. (1998) Immunity 9, 669–676
43. Plas, D. R., Rathmell, J. C., and Thompson, C. B. (2002) Nat. Immunol. 3, 515–521