Akt inhibits the orphan nuclear receptor Nur77 and T cell apoptosis*

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Running title: Inhibition of Nur77 by Akt

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SUMMARY

Akt is a common mediator of cell survival in a variety of circumstances. Although some candidate Akt targets have been described, the function of Akt is not fully understood especially because of the cell type- and context-dependent apoptosis regulation. In this paper, we demonstrate that one of the mechanisms by which Akt antagonizes apoptosis involves the inhibition of Nur77, a transcription factor implicated in T cell receptor-mediated apoptosis. It has been suggested that Akt phosphorylates Nur77 directly, but it remains unknown whether Akt suppresses biological functions of Nur77. We found that Akt inhibited the DNA binding activity of Nur77 and stimulated its association with 14-3-3 in a phosphorylation-site dependent manner. Moreover, we found that expression of Akt suppressed Nur77-induced apoptosis in fibroblasts, and it suppressed activation-induced cell death of T cell hybridomas. The inhibition of Nur77 by Akt suggests a mechanism that explains how T cell receptor activation can promote survival in some instances even when Nur77 is induced. Collectively, these results may suggest that Akt is a negative regulator of Nur77 in T cell apoptosis.
INTRODUCTION

The regulation of survival versus apoptosis is a central issue during T cell development and activation. The survival/death regulation of thymocytes through T cell receptor (TCR)\(^1\) activation plays a key role in establishing a functional T cell repertoire. In the thymus, immature T cells that moderately recognize self peptide-MHC complexes survive and are induced to differentiate (positive selection), whereas self-reactive T cells which recognize self peptide-MHC complexes with high affinity/avidity are induced towards cell death (negative selection), thus eliminating potentially toxic T cells (1, 2). Although both forms of thymic selection, as well as activation-induced cell death (AICD) of mature T cells, are mediated by TCR, the intracellular signaling mechanisms by which TCR regulates T cell survival and apoptosis are not fully understood, and have been subject to intense investigation.

Amongst the molecules implicated in T cell apoptosis is Nur77 (also known as NGFI-B or TR3), a member of the orphan nuclear receptor superfamily. Nur77 was originally identified as an immediate-early gene transiently induced by serum, growth factors and NGF (3, 4). It has also been shown that Nur77 and Nor-1, a related member of the Nur77 family, are induced during TCR-mediated apoptosis (5, 6). Expression of a dominant negative Nur77 blocks activation-induced cell death in T cell hybridomas as well as negative selection in transgenic mice (7, 8). Conversely, transgenic mice that express wildtype Nur77 or Nor-1 exhibit massive apoptosis, and reduction in thymocyte numbers and the proportion of double-positive (DP) thymocytes (7, 9). Therefore it seems likely that Nur77 plays an important role in T cell apoptosis. Nur77 transcription activity correlates well with its apoptotic function in T cells (10), although this might not be the case in other cell types (11).

When Nur77 is induced by growth factors or TCR stimulation, it often becomes transcriptionally inactive due to posttranslational modification (12). This is consistent with the observation that expression of Nur77 does not necessarily correlate with induction of apoptosis. In particular, we found that TCR activation resulted in Nur77 expression even
under a condition mimicking positive selection, in which TCR promotes survival (this study). This and other studies suggest the existence of a mechanism to inhibit Nur77’s proapoptotic function (13). It has been reported that phosphorylation of Nur77 on Ser350 negatively regulates its function. Ser350 resides within the domain required for specific binding of Nur77 to DNA, and its phosphorylation results in reduction of both the DNA binding and transcriptional activities of Nur77 in PC12 cells (12, 14). In vivo phosphorylation of Ser350 indeed takes place in T cells as well as in other cells (15-17). Therefore, it appeared likely that a kinase involved in survival signaling would phosphorylate Nur77 and thus antagonize the proapoptotic function of Nur77.

The phosphatidylinositide 3-kinase (PI3-K)-Akt pathway is activated in response to TCR activation, and is implicated in mediating survival signals in T cells. For instance, thymocytes derived from mice deficient in p110γ, a catalytic subunit of PI3-K, exhibited enhanced apoptosis (18), whereas those derived from pten−/− mice, in which the PI3-K-Akt pathway is constitutively active, were more resistant to TCR-mediated cell death and resulted in a defect in thymic negative selection (19, 20). Moreover, both DP thymocytes and mature T cells derived from transgenic mice expressing gag-akt, a constitutively active Akt, showed enhanced viability in culture and resistance to various apoptosis-inducing stimuli such as γ-irradiation, dexamethasone and Fas ligand, further supporting the survival promoting function of the PI3-K-Akt pathway in T cells (21). Recently, it has been shown that Akt phosphorylates Nur77 directly in vitro and in vivo (22). However, it remains to be determined whether Akt inhibits proapoptotic functions of Nur77, and how Akt-mediated phosphorylation inhibits Nur77. In this study, we show that phosphorylation of Nur77 by Akt results in reduction of its DNA binding activity and stimulation of its association with 14-3-3 in a phosphorylation-site dependent manner. We also show that Akt suppresses Nur77-mediated cell death and thus propose an antagonistic interaction between apoptosis and survival signalings downstream of TCR.

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EXPERIMENTAL PROCEDURES

Cell culture, transfection and MHC knockout mouse

DO11.10 mouse T cell hybridomas were cultured at 37°C in RPMI1640 medium containing 10% fetal bovine serum (FBS) and penicillin-streptomycin supplemented with non-essential amino acids, sodium pyruvate, 10 mM Hepes buffer and β-mercaptoethanol (Gibco-BRL). 293T, Rat1a and PLAT-E cells were cultured in Dulbecco’s modified Eagle medium (DMEM) containing 10% FBS at 37°C. For electroporation, 1x 10^6 cells were mixed with 15 µg of plasmid DNA and electroporated with 240 V pulse at 960 µF. Cell transfection was performed using FuGENE reagent (Roche Diagnostics) according to manufacturer's instruction. T cells were stimulated with plate-bound anti-TCR-β monoclonal antibody (H57-597; Pharmingen), or 10 µg/ml of anti-CD3ε monoclonal antibody (145-2C11; Pharmingen) and 1 µg/ml of anti-CD28 antibody. Cells were also treated with 10 nM of PMA and 1 µM of calcium ionophore (CaI) A23187 for 7 h to induce activation-induced cell death.

MHC-deficient mice had been generated by crossing the I-Aβ-/- line of MHC class II-deficient mice with β2-microglobulin-deficient mice (23). These mice fail to develop mature T cells, and most of the thymocytes derived from MHC knockout mice are immature, CD8/CD4 double positive cells. A previous report suggested that engagement of T cells with anti-TCR-β monoclonal antibody mimicked positive selection and differentiation, as indicated by increase in cell surface expression of TCR-α chain and CD5, and decrease in RAG-1 and CD4/CD8 expression (24). On the other hand, stimulation with anti-CD3ε antibody resulted in induction of apoptosis, mimicking negative selection (25).

Plasmid construction, recombinant proteins and antibodies

Nur77 plasmid and Nur77-responsive luciferase reporter plasmid (NBRE-luc) are kind gifts of Drs. Y. Katagiri, G. Guroff and J. Milbrandt. Site-directed mutagenesis was performed to generate Ser350 to Ala mutant of Nur77 by using QuickChange Site-Directed Mutagenesis...
Kit (Stratagene). Wildtype, constitutively active or dominant-negative Akt constructs were generously provided from Dr. D. Alessi. Each of Akt mutant was inserted into an expression vector pCS2+ (26). Bacterially expressed recombinant proteins were produced and purified using standard GST fusion protein protocol (Amersham Pharmacia Biotech). We purchased anti-Nur77, anti-TCR-β (H57-597), anti-CD3ε (145-2C11), anti-CD28 (37.51) from Pharmingen and anti-Myc (Santa Cruz), anti-Flag (Sigma), anti-His-tag (Novagen), anti-Akt and anti-phospho-Ser473 Akt (Cell Signaling). Anti-phospho Ser350 Nur77 antibody was raised by immunizing rabbits with a KLH-conjugated phosphopeptide corresponding to amino acid sequence of rat Nur77 343-353 (GRRGRLPpSKPK). Antiserum was purified as a bound fraction of phosphopeptide-conjugated column.

**Immunoprecipitation and immunoblotting**

Cells were washed twice with phosphate buffered saline (PBS) and lysed with a cell lysis buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, 1 mM dithiothreitol and protease inhibitors, PMSF, leupeptin, aprotinin and pepstatin. Proteins were immunoprecipitated from cell lysates after incubating with 2 μg of appropriate antibodies and protein A-Sepharose beads (Amersham Pharmacia Biotech) for 2 h at 4°C. Cell lysates were subjected to SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore). Immunoreactive bands were detected by an ECL system (Amersham Pharmacia Biotech).

**Electrophoretic mobility shift assay (EMSA)**

Cells were lysed in a buffer containing 50 mM Heps-KOH pH 7.8, 420 mM KCl, 0.1 mM EDTA, 5 mM MgCl₂, 20% glycerol and protease inhibitors. Oligonucleotide probes were end-labelled with T4 polynucleotide kinase. Cell lysates and labelled probes were incubated for 1 h in a buffer containing 10 mM Heps-KOH pH 7.8, 50 mM KCl, 1 mM EDTA, 5 mM MgCl₂, 5 mM dithiothreitol, 10% glycerol and protease inhibitors and subjected to polyacrylamide gel electrophoresis. The electrophoretically shifted bands including the

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complex of Nur77 and DNA were detected after autoradiography.

**Retroviral infection and cell sorting**

Human CD8 sequence lacking cytoplasmic domain was subcloned into pMX-IRES retroviral vector to generate pMX-IRES-CD8. Akt mutants were inserted into this vector to generate pMX-Akt-IRES-CD8. Recombinant retroviruses were obtained by transfection of retroviral vector plasmids into ecotropic virus packaging cells, PLAT-E as described elsewhere (27). Conditioned media from these cells were collected and added to T cell cultures and incubated for 16 h in the presence of 5 µg/ml of polybrene (Sigma). Infected cells (CD8-positive) or non-infected cells (CD8-negative) were separated by use of microbeads-conjugated anti-CD8 antibody and auto magnetic activated cell sorting (MACS) separation units (Miltenyi Biotec) according to the manufacturer’s instruction.

**Flow cytometric analysis**

Cells were stained with FITC-conjugated anti-CD8 antibody (Pharmingen) or 1 ng/ml of 7-amino-actinomycin D (7-AAD; Molecular Probes) in a FACS staining buffer (PBS with 0.2% bovine serum albumin and 0.1% NaN$_3$). Labelled cells were analysed on FACScan flow cytomter (Becton Dickinson).

**Luciferase assay**

After stimulation, the luciferase activity in the cell lysates was measured according to the manufacturer’s instruction (Promega). Relative luciferase activity was normalized with co-expressed β-galactosidase activity.

**Fetal thymus organ cultures (FTOC)**

Fetal thymus organ cultures (FTOC) was performed essentially as described previously (28). Briefly, fetal thymi were dissected from C57BL/6 mouse embryos (E15) and incubated for 8 days at 37°C in RPMI1640 medium containing 10% fetal bovine serum. Then cells were

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harvested and dissociated to analyse cell surface expression of CD8 and CD4 using FACScan flow cytometer (Becton Dickinson).
RESULTS

Nur77 induction and Akt activation in thymocytes-

A major question in T cell development is how TCR activation in immature CD4+ CD8+ double positive (DP) thymocytes is translated into either survival (positive selection) or apoptotic (negative selection) signals (1, 2). One possible mechanism to discriminate these outcomes might be that TCR activation in negative selection, but not that in positive selection, induces expression of proapoptotic proteins such as Nur77. Therefore we compared expression levels of Nur77 under conditions mimicking either positive or negative selection. Immature DP thymocytes isolated from the MHC knockout mice were treated with anti-TCR-β antibody or with anti-CD3ε/CD28 antibodies to mimic positive or negative selections, respectively ((24) and see details in Experimental Procedures). Consistent with previous reports, CD3ε (plus CD28) engagement in thymocytes derived from MHC knockout mice resulted in sustained expression of Nur77 protein (Fig. 1). Importantly, the stimulation of thymocytes with anti-TCR-β antibody also induced expression of Nur77 protein, although the induction was transient and not robust (Fig. 1). This result suggests that Nur77 is induced to a higher level in the context of negative selection (apoptosis) compared with positive selection (survival), and also suggests that a threshold level should exist for the apoptosis-inducing function of Nur77. One mechanism for determination of such a threshold might be a survival signal that would counteract this proapoptotic function of Nur77.

Although it has been shown that Akt is activated in response to TCR activation in mature T cells and contributes to their survival, it was unknown whether this is also the case in immature DP thymocytes. We found that Akt became phosphorylated in response to the crosslinking of both anti-TCR-β and anti-CD3ε/CD28 to a similar extent (Fig. 1). We then asked whether the PI3-K-Akt pathway might serve as a survival promoter to counteract the proapoptotic function of Nur77 in thymocytes.

PI3-K activity is necessary for thymocyte survival-

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To ask whether the PI3-K(/Akt) pathway is indeed essential for survival of immature thymocytes, we examined the effect of LY294002, a PI3-K inhibitor, on fetal thymus organ cultures (FTOC). FTOC taken from E15 mouse embryos were incubated for 2 days in the presence or absence of LY294002, and then the numbers of double negative (DN, CD4-CD8-), single positive (SP, CD4+CD8- or CD4-CD8+) and DP cells were determined by FACS analysis. Treatment of FTOC with LY294002 markedly reduced all types of immature thymocytes in a dose dependent manner (Fig. 2). A low dose (5 µM) of LY294002 resulted in reduction of DP cells primarily (Fig. 2), suggesting that DP cells are most sensitive to PI3-K inhibition. This result is consistent with the recent finding that PI3Kγ knockout mice results in moderate reduction of immature thymocytes (18).

Akt suppresses transcriptional activation of Nur77 induced by TCR stimulation-

Because the PI3-K(-Akt) pathway and Nur77 appear to have opposite effects on death/survival regulation of T cells, and because it has recently been reported that Akt is capable of phosphorylating Nur77 directly (22), we asked whether Akt has an inhibitory effect on the functions of Nur77 in T lymphocytes. We first examined the transcriptional activity of endogenous Nur77 in T lymphocytes, as monitored by a luciferase gene reporter construct under the control of Nur77 binding elements (NBREs). We obtained DO11.10 T cell hybridomas stably expressing either wildtype, a constitutively active or a kinase-negative form of Akt, by infecting retrovirus encoding both Akt and human CD8, an expression marker, linked with a bicistronic internal ribosome entry site (IRES) sequence (see Fig. 3A). After infection, Akt-expressing cells were separated by cell surface expression of human CD8. The profile of human CD8 expression levels was not affected by the types of Akt (Fig. 3B). In non-infected cells or cells expressing only human CD8, the transcriptional activity of Nur77 increased 12 to 14-fold after TCR stimulation for 4 h, and was accompanied by an increase in the level of endogenous Nur77 protein (Fig. 3C). This increase in the transcriptional activity was blocked by expression of N-terminally truncated dominant-negative Nur77 (Fig. 3C), confirming that this reporter assay indeed reflects the
transcriptional activity of Nur77. Expression of either wildtype or a constitutively active Akt, but not a kinase-negative Akt, inhibited activation of Nur77 transcriptional activity in response to TCR stimulation (Fig. 3C). Importantly, the level of Nur77 protein induced by TCR stimulation was not altered by expression of any of the Akt constructs (Fig. 3C).

Induction of NBRE-driven luciferase by ectopic expression of Nur77 was also suppressed when active Akt was coexpressed, whereas the level of Nur77 protein was not suppressed (Fig. 3D). Therefore it is likely that Akt suppresses the transcriptional activity of Nur77 in T lymphocytes through posttranslational modification, presumably through phosphorylation.

**Akt suppresses DNA binding activity of Nur77**

How does Akt-mediated phosphorylation inhibit the transcriptional activity of Nur77? The most likely possibility may be that phosphorylation on Ser350 reduces DNA binding activity as previously reported using recombinant Nur77 protein (29). We therefore examined whether the DNA binding activity of endogenous Nur77 induced by T cell activation could be indeed inhibited by expression of an active Akt. A shifted band (Nur77-DNA complex) was detected in electrophoretic mobility shift assay (EMSA) when control cells or cells expressing a kinase-negative Akt were stimulated with phorbol ester (PMA) plus calcium ionophore (CaI) (Fig. 4). The identity of the shifted band (Nur77-DNA complex) was confirmed by supershift analysis with anti-Nur77 antibody (data not shown) and a chase experiment with cold probes (Fig. 4). Expression of an active Akt resulted in reduction of the Nur77-DNA complex (Fig. 4), although the protein levels of Nur77 were essentially unchanged between the control cell extract and the Akt-expressing cell extract used in these experiments (Fig. 3C), suggesting that Akt inhibits the DNA binding activity of Nur77.

**Association of Nur77 with 14-3-3 in a phosphorylation-dependent manner**

Another mechanism by which Akt-mediated phosphorylation inhibits Nur77's function might be phosphorylation-dependent binding of 14-3-3 to Nur77. There are a large number
of proteins whose functions are regulated by phosphorylation-dependent binding of 14-3-3. For example, some of the targets of Akt, such as Bad and Forkhead family of transcription factors, bind to 14-3-3 and are functionally modulated (30, 31). Interestingly, the sequence around Ser350 of Nur77 corresponds to the consensus sequence for 14-3-3 binding, R-x-x-phosphoS/T-x-P, when it is phosphorylated, and is highly conserved among the Nur77 family (Nor-1 and Nurr1) and between species (Fig. 5A). Therefore, it is an intriguing possibility that Nur77 forms a complex with 14-3-3 upon phosphorylation of this site. We observed Akt phosphorylation of Nur77 on S350 in vitro (Fig. 5B) and in vivo (Fig. 5C) by use of a phosphorylation-site mutant (S350A) and phospho-S350-specific antibody, which confirmed the recent finding of Pekarsky, Y. et al. (22). We then tested whether recombinant (Histagged) Nur77 binds to recombinant (GST-tagged) 14-3-3 in vitro when it is phosphorylated by Akt using a GST-pull down assay. We found that the wildtype phosphorylated His-Nur77 could be bound to GST-14-3-3 (Fig. 5D). Neither S350A mutant or non-phosphorylated Nur77 were able to bind to GST-14-3-3 (Fig. 5D). We also tested their in vivo association. Myc-tagged Nur77 and Flag-tagged 14-3-3 were cotransfected into 293T cells with or without various Akts, and coprecipitation of the Flag-tagged 14-3-3 with Myc-tagged Nur77 was observed. 14-3-3 was found to associate with wildtype Nur77, but not S350A Nur77, and only when active Akt was co-expressed (Fig. 5E). These data strongly suggest that 14-3-3 binds to Nur77 phosphorylated on Ser350 both in vitro and in vivo. As it has been shown that some of 14-3-3 binding proteins were excluded from nucleus after complex formation via a mechanism utilizing a nuclear export signal of 14-3-3 protein, we examined subcellular localization of green fluorescent protein (GFP)-fused Nur77 in DO11.10 cells. However, Nur77 protein remained in the nucleus even after cells were stimulated with PMA/CaI or when active Akt was co-expressed (data not shown).

Akt inhibits activation-induced cell death in T cell hybridomas-

Nur77 is thought to play a key role in activation-induced cell death (AICD) of mature T cells as well as in negative selection of immature DP thymocytes. We examined whether the
activation of Akt is capable of suppressing AICD in the T cell hybridoma DO11.10. AICD was induced by treating cells with PMA/CaI for 7 h, and dead cells were detected by use of a membrane-impermeable dye, 7-amino-actinomycin D (7-AAD). Cells expressing only human CD8 or a kinase-negative Akt underwent AICD to a similar extent as the control (non-infected) cells (Fig. 6A, B). In contrast, expression of wildtype or a constitutively active Akt markedly inhibited the cell death induced by PMA/CaI treatment (Fig. 6B), suggesting that Akt mediates survival signals to prevent AICD. This result further supports the idea that Akt might counteract the effect of Nur77 in death/survival regulation of T cells.

Akt inhibits Nur77-induced cell death in a Ser350-dependent manner-

Finally, we asked whether Akt directly inhibits Nur77’s proapoptotic function. To address this, we attempted to examine the proapoptotic function of Nur77 in T cell hybridomas, but expression of Nur77 per se appeared insufficient for inducing apoptosis in these cells (data not shown). Therefore, we utilized Rat1 fibroblasts, in which expression of Nur77 was sufficient to induce apoptosis. Expression of active Akt suppressed apoptosis induced by Nur77 almost to the levels seen in the absence of Nur77 (Fig. 7). In contrast, active Akt was unable to inhibit apoptosis induced by the mutant Nur77 containing the Ser350-to-Ala substitution (Fig. 7). These results suggest that Akt inhibits Nur77-induced apoptosis directly through phosphorylation of Ser350.
DISCUSSION

Akt, a downstream target of PI3-K, is emerging as a common mediator of cell survival in a variety of models. Recent data suggest that Akt antagonizes the proapoptotic functions of Bad, Caspase-9, the Forkhead family members and glycogen synthase kinase-3 (GSK-3), or activates the NF-κB pathway, which may participate in the survival-promoting effects of Akt (32, 33). However, the function of Akt in T cells was still largely unknown. Our studies demonstrate that one of the mechanisms by which Akt antagonizes apoptosis involves the inhibition of Nur77. We show here that expression of an active Akt suppresses activation-induced cell death of T cell hybridoma DO11.10, in which Nur77 plays a pivotal role. Moreover, Akt also suppresses apoptosis induced by expression of Nur77 in a phosphorylation site-dependent manner, suggesting that Akt directly inhibits the proapoptotic function of Nur77. This role in inhibition may account for the observation that Nur77 is expressed in the absence of induction of apoptosis under conditions in which TCR activation promotes survival in thymocytes, as well as in other cells.

The fate of a developing thymocyte is determined by the interaction between TCR and its ligands (MHC/peptide complexes). Studies suggest that both the qualitative and quantitative aspects of TCR activation guide a T cell to either survive and mature or undergo apoptosis. In the quantitative model, strong activation signals result in cell death (negative selection) while moderate signals result in survival (positive selection) (34). Although the signaling requirement for regulating strong versus weak signals from the TCR remains largely unknown, the Nur77 family proteins appear to play a qualitatively distinct role, that is, an important role only in apoptosis (negative selection), not in survival (positive selection). In our study, the levels of Nur77 expressed in response to TCR activation under conditions mimicking either positive or negative selection were low and high, respectively (Fig. 1). Therefore, we would predict the existence of a threshold determining whether Nur77 induces apoptosis or not. We propose that a survival signal via the PI3-K-Akt pathway might play a part in this threshold based on several reasons; First, the proapoptotic
function of Nur77 can be antagonized by Akt-mediated phosphorylation (this study).
Second, the inhibition of PI3-K induces robust apoptosis of DP thymocytes (this study and
(18)) and third, the activation of the PI3-K-Akt pathway inhibits negative selection (20, 21)
or activation-induced cell death (this study).

The phosphorylation of Ser350 has been shown to inhibit the transcriptional activity of
Nur77 (12). Consistently, expression of an active Akt suppressed the transcriptional activity
of both exogenous and endogenous Nur77 induced by TCR activation (this study). In this
study we have suggested possible mechanisms by which Akt inhibits the transcriptional
activity of Nur77. One mechanism would be that Akt inhibits the DNA binding activity of
Nur77, as we observed reduced DNA binding activity of Nur77 upon expression of Akt in T
cell hybridomas (this study), and because a previous study has shown that the
phosphorylation of the recombinant DNA binding domain of Nur77 results in reduction of
the DNA binding activity in vitro (29). Another mechanism, although not mutually exclusive,
might be the 14-3-3 binding of Nur77 induced by the phosphorylation of Ser350. The
binding of 14-3-3 often leads to inactivation of the protein or sequestration of the protein
from its functional targets (30, 31). Therefore, the binding of Nur77 with 14-3-3 might result
in inactivation of its transcriptional activity or sequestration from its targets or functional
compartments such as nucleus (or mitochondria, see below). This sequestration model is
supported by the previous observations that phosphorylated form of Nur77 is predominantly
cytoplasmic (22, 35).

It is not clear how Akt suppresses the proapoptotic function of Nur77 in T cells and in
fibroblasts. We show that expression of Akt suppresses both the transcriptional and
proapoptotic activities of Nur77, but this does not necessarily mean that Akt inhibits Nur77-
induced apoptosis through inhibition of its transcriptional activity. A recent study has shown
that expression of Nur77 induces apoptosis in prostate cancer LNCaP cells through targeting
to mitochondria even in the absence of the DNA binding domain (11), which is apparently
controversial to other reports in which the transcriptional activity correlates well with the
apoptosis-inducing activity of Nur77 (7, 8, 10). In our hands, Nur77 was localized diffusely

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in the nucleus, not in mitochondria, when Nur77 was ectopically expressed with either a Myc-tag or a GFP-tag in T cell hybridomas and Rat-1 fibroblasts (data not shown), although this does not exclude the possibility that a trace amount of mitochondrially-targeted Nur77 is responsible for the induction of apoptosis. In that case, the 14-3-3 binding of Nur77 induced by Akt-mediated phosphorylation might play an essential role to inactivate the proapoptotic function of Nur77.

In summary, we have shown that Akt antagonizes the proapoptotic function of Nur77 through its direct phosphorylation. This finding reveals a novel nexus between apoptosis and survival signalings, which might play a critical role in determination of cell death/survival decision of T cells and other cells.

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1The abbreviations used are: TCR, T cell receptor; MHC, major histocompatibility complex; PI3-K, phosphatidylinositol 3-kinase; AICD, activation-induced cell death; 7-AAD, 7-amino-actinomycin D; FTOC, fetal thymus organ cultures; DP, double positive; SP, single positive; DN, double negative; IRES, bicistronic internal ribosome entry site; GST, glutathione S transferase; PMA, Phorbol 12-myristate 13-acetate; CaI, calcium ionophore; EMSA, electrophoretic mobility shift assay; DMEM, Dulbecco’s modified Eagle medium; PBS, phosphate buffered saline; FBS, fetal bovine serum.
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FIGURE LEGENDS

Fig. 1.
*Induction of Nur77 expression after T cell stimulation.* Double-positive thymocytes prepared from MHC knockout mice were stimulated with plate-bound anti-CD3ε plus anti-CD28 antibodies or anti-TCR-β antibody for indicated times to mimic negative or positive selection, respectively. The level of Nur77 protein was detected by western blotting with anti-Nur77 antibody. Phosphorylation state at Ser473 and protein level of Akt were also examined by immunoblotting with anti-phospho Akt and anti-Akt antibodies.

Fig. 2.
*Effects of PI3-K inhibitor on survival of immature thymocytes in mouse fetal thymus organ culture (FTOC).* Fetal thymi were dissected from mouse embryos (E15) and incubated at 37°C in RPMI1640 medium containing 10% fetal bovine serum for 6 days. Then the thymi were treated with a PI3-K inhibitor, LY294002, at indicated concentrations and were further incubated for 2 days. Thymocytes were harvested and dissociated to analyse cell surface expression of CD8 and CD4 by FACScan flow cytometer. The percentage of each subpopulation is indicated.

Fig. 3.
*Effects of Akt on the transcriptional activity of Nur77.* (A) Schematic structure of retroviral vectors harboring both Akt and human CD8 linked with a bicistronic internal ribosome entry site (IRES) sequence to ensure simultaneous expression of both genes. (B) CD8 expression on the cell surface was confirmed after retrovirus infection of DO11.10 cells. After the magnetic-activated cell sorting (MACS), the CD8-negative cells (dashed line) and CD8-positive cells (solid line) were detected by FACScan using FITC-conjugated anti-CD8 antibody. (C) Transcriptional activity of Nur77 after TCR stimulation. Nur77 responsive reporter plasmid (NBRE-luc) was electroporated into DO11.10 cells that stably expressed Nur77.
each Akt mutant or dominant-negative Nur77. After cells were stimulated with plate-bound anti-CD3ε monoclonal antibody for 4 h, the luciferase activity in the cell lysates was measured. The level of Nur77 protein in each subpopulation after TCR stimulation was examined by immunoblotting with anti-Nur77 antibody. (D) Rat1a cells were transfected with Nur77 expression plasmid and reporter plasmid together with or without Akt, then the luciferase activity and expression level of Nur77 protein were measured after 18 h.

Fig. 4.

Effects of Akt on the DNA binding activity of Nur77. Either control or Akt-expressing DO11.10 cells were stimulated with 10 nM of PMA plus 1 µM of calcium ionophore (CaI) A23187 for 7 h to induce Nur77 expression. Cell lysate were analysed for electrophoretic mobility shift assay (EMSA) with a probe specific for Nur77 binding. Shifted bands of Nur77-DNA complexes were confirmed after chasing with 10-fold excess of unlabelled cold probe but not mutated cold probe.

Fig. 5.

Association of Nur77 protein with 14-3-3 in a phosphorylation-dependent manner. (A) Schematic structure of Nur77. Boxed region indicates amino acid sequences of the Nur77 family that correspond to the consensus sequence for 14-3-3 binding (R-x-x-S/T-x-P). The consensus sequence for Akt phosphorylation is underlined. (B) In vitro phosphorylation of Nur77 by Akt. Bacterially expressed GST-fused recombinant Nur77 proteins (WT or Ser350 to Ala mutant) were incubated with or without active Akt immunoprecipitates in a kinase reaction buffer, and subjected to immunoblotting with anti-phospho Ser350 Nur77 antibody. (C) In vivo phosphorylation of Nur77. Myc-tagged Nur77 (wildtype or S350A) and each Akt were coexpressed in 293T cells. Anti-Myc immunoprecipitates (anti-Myc) were blotted with either anti-phospho Ser350 Nur77 antibody or with anti-Myc antibody. Total cell extracts were also subjected to immunoblotting with anti-Akt antibody. An active Akt which lacks preckstrin homology domain migrates faster than full-length Akt. (D) In vitro binding of
Nur77 with 14-3-3ζ protein. Bacterially expressed His-tagged Nur77 proteins (wildtype or S350A) were preincubated with or without active Akt immunoprecipitate in a kinase reaction buffer. Nur77 proteins were subsequently mixed with either GST or GST-14-3-3ζ protein and glutathione-Sepharose for 1 h at 4°C allowing the complexes bind to the resin. The Nur77 proteins bound to GST or GST-14-3-3 were detected by immunoblotting with anti-His antibody. (E) In vivo binding of Nur77 with 14-3-3ζ protein. 293T cells were transfected with or without Myc-tagged Nur77 (WT or S350A), Flag-tagged 14-3-3ζ and each Akt. Complex formation was analysed by immunoprecipitation with anti-Myc antibody followed by immunoblotting with anti-Flag antibody to detect bound 14-3-3 proteins.

Fig. 6.

Suppression of activation-induced cell death by Akt. (A) Analysis of T cell hybridomas by flow cytometry. DO11.10 cells that stably express each mutant of Akt were stimulated with PMA plus CaI for 7 h. Cells were stained with a cell-impermeable dye, 7-amino-actinomycin D (7-AAD) and profiled by flow cytometry. The bars represent the range counted as apoptotic cells. (B) Statistical representation of the percentage of cell death after PMA/CaI stimulation. The results represent the average of three independent experiments.

Fig. 7.

Suppression of Nur77-induced cell death. Rat1a fibroblasts were transfected with Nur77 (wildtype or Ser350A) with or without active Akt. Dead cells were scored 24 h after transfection, by apoptotic nuclear morphology visualized with coexpressed GFP-fused human histone H2B. Essentially the same results were obtained in three independent experiments.
Fig. 1

- **Nur77**

- **Phospho-Akt**

- **Akt**

|        | anti-TCRβ | anti-CD3ε + anti-CD28 |
|--------|-----------|-----------------------|
| 0      | 3         | 3                     |
| 3      | 6         | 6                     |
| hr     |           |                       |

Fig. 2

The figure shows the cell number per thymus lobe in response to different concentrations of LY294002 (μM). The y-axis represents the cell number, and the x-axis shows the concentration of LY294002 in μM. The graph is divided into five bars, each representing a different concentration level of LY294002. The bars are color-coded to indicate different cell types: CD8- CD4+, CD8+ CD4+, CD8+ CD4-, and CD8- CD4-. The percentage of each cell type is indicated at the top of each bar.

- At 0 μM, 10% CD8- CD4+ and 52% CD8+ CD4+.
- At 2 μM, 14% CD8+ CD4+ and 50% CD8- CD4+.
- At 5 μM, 18% CD8+ CD4-.
- At 10 μM, 27% CD8+ CD4-.

The graph illustrates the change in cell distribution with increasing concentrations of LY294002.
**Fig. 3**

A) Diagrams showing the structure of LTR-IRES-hCD8-LTR and LTR-Akt-IRES-hCD8-LTR constructs.

B) Flow cytometry analysis showing FITC-CD8 expression in CD8-alone, CD8+Akt wt, CD8+Akt active, CD8+Akt KA, and CD8+Nur77 DN conditions. The graphs display the counts of FITC-CD8 fluorescence intensity.

C) Bar graph showing the luciferase activity (NBRE-Luc) with control and anti-CD3ε Ab conditions. The graph includes Vector, Akt wt, Akt active, Akt KA, and Nur77 DN conditions with and without CD8-negative and CD8-positive environments.

D) Western blot analysis of Nur77 with and without Akt active conditions.
Fig. 5

A

Transcriptional activation DNA binding Dimerization
N [ ] C

rNur77 VRTDSLKGGCGRLPSKPKQP
hNur77 VRTDSLKGGCRGRLPSKPKQP
mNur77 VRTDSLKGRGGRLPSKPKQP
hNurr1 VRTDSLKGRGGRLPSKPKSP
rNurr1 VRTDSLKGRGGRLPSKPKSP
hNor-1 VRTDSLKGRGGRLPSKPKSP
rNor-1 VRTDSLKGRGGRLPSKPKSP

B

GST-Nur77 - Akt

WT SA WT SA

anti-P S350 Nur77

CBB

anti-Myc

anti-Nur77

total anti-Akt

C

Myc-Nur77 WT S350A

IP IB

anti-phospho S350 Nur77

anti-Myc

anti-Nur77

total anti-Akt

D

His-Nur77 WT S350A

GST GST-14-3-3

His-Nur77

E

Akt - - WT Active KN - Active KN

Myc-Nur77 WT WT WT WT SA SA SA

Flag-14-3-3 - + + + + + +

IP IB

anti-Myc anti-Flag

total anti-Flag

Fig. 5
Fig. 6

A

B

- Control
- + PMA/Cal

Apoptotic cells (%)

CD8 negative
CD8 alone
CD8 + Akt wt
CD8 + Akt active
CD8 + Akt KA
