Cutting Edge: Protective Effects of Notch-1 on TCR-Induced Apoptosis

Birgit M. Jehn, Wolfgang Bielke, Warren S. Pear and Barbara A. Osborne

*J Immunol* 1999; 162:635-638;
http://www.jimmunol.org/content/162/2/635

**References**
This article cites 13 articles, 3 of which you can access for free at:
http://www.jimmunol.org/content/162/2/635.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Cutting Edge: Protective Effects of Notch-1 on TCR-Induced Apoptosis

Birgit M. Jehn, Wolfgang Bielke, Warren S. Pear, and Barbara A. Osborne

The Notch receptor protein was originally identified in Drosophila and is known to mediate cell to cell communication and influence cell fate decisions. Members of this family have been isolated from invertebrates as well as vertebrates. We isolated mouse Notch-1 in a yeast two-hybrid screen with Nur77, which is a protein that has been shown previously to be required for apoptosis in T cell lines. The data presented below indicate that Notch-1 expression provides significant protection to T cell lines from TCR-mediated apoptosis. These data demonstrate a new antiapoptotic role for Notch-1, providing evidence that, in addition to regulating cell fate decisions, Notch-1 can play a critical role in controlling levels of cell death in T cells. The Journal of Immunology, 1999, 162: 635–638.

Notch-dependent signal transduction involves a ligand-dependent proteolytic cleavage of Notch, resulting in the release and translation of its intracellular domain to the nucleus, where it is able to regulate gene transcription (reviewed in Ref. 5).

Activated forms of Notch or Notch-like proteins are often associated with tumorigenic phenotypes in mammals. Translocation-associated notch homologue-1 (TAN-1)3, the human homologue of mouse Notch-1, is rearranged in some human T cell lymphomas (T cell acute lymphoblastic leukemia) bearing a t(7;9)(q34;q34.39) chromosomeal translocation. This rearrangement results in a constitutively active, oncogenic protein lacking the extracellular domain (6). Int-3/Notch-4, another Notch-related protein, is found to be truncated by the insertion of mouse mammary tumor virus, resulting in the formation of breast tumors in mice exposed to this virus; insertion of this truncated form of Notch-4 is sufficient to cause mammary gland tumors (7).

Here, we report the novel observations that the nuclear receptor protein Nur77 and the transmembrane receptor Notch-1 interact physically and that Notch-1 protects from Nur77-dependent cell death in T cell lines. The data presented provide strong evidence that Notch-1 is a key player in nuclear hormone receptor-mediated cell death processes in vertebrates and suggest a new role for Notch-1 as an antiapoptotic protein.

Materials and Methods

Yeast two-hybrid screen

A transcriptionally inert but nuclear form of Nur77 (amino acids 268–536) was inserted into the bait vector pEG202 and transformed into the yeast strain EGY48/pSH18–34 (8, 9). Interactor clones were isolated by screening 6 × 106 colonies of a mouse T cell lymphoma cDNA library (Clontech, Palo Alto, CA). cDNA fragments of 55 interactor clones were analyzed. Retransformation of different bait and prey constructs into control yeast strains was performed as described previously (8, 9).

In vitro protein binding assay

A full-length Nur77 cDNA fragment was fused to a glutathione S-transferase (GST) tag (Pharmacia, Uppsala, Sweden). Protein purification from bacteria and coupling to GST-Sepharose beads was performed as described previously (10). COS-1 cells were transiently transfected, and extracts were prepared (10). GST-Nur77 protein coupled to beads was incubated with either buffer alone or the indicated extracts. Samples were washed six times and subjected to SDS-PAGE analysis.

Cell death induction and analysis

DO11.10 cells and retrovirally infected derivatives were plated at a density of 1.5 × 105 cells/ml and stimulated to die apoptotically as indicated. At
Retroviral infection

Retroviral infections were performed as described previously (11). The retroviral constructs pGD-ICT and pGD-ECT were transiently transfected into Bosc 23 cells. DO11.10 cells were infected with retroviral particles for 24 h. G418 selection (1 μg/ml) was started at 24 h postinfection and was maintained throughout propagation of the infected cells. Apoptosis was induced as described previously.

Transient transfections and luciferase (Luc) reporter assays

A triple repeat of the Nur77-specific DNA response element (NurRE) was subcloned 5′ of a thymidine kinase-Luc reporter gene. Transient cotransfections of 1.2 × 10⁵ COS-1 cells/well in 6-well plates using SuperFect reagent (Qiagen, Chatsworth, CA) and a total amount of 4.5 μg of plasmid DNA per well (1.5 μg each plasmid DNA). Cellular extracts were prepared and samples were analyzed at 24 h posttransfection for Luc activity according to the manufacturer’s instructions (Promega, Madison, WI). Transfections were normalized for the level of total cellular protein (Bradford Assay; Bio-Rad, Hercules, CA), and Luc activity was calculated per microgram of protein. Mean values and SDs are shown for at least four independent experiments (each done in duplicate).

Results and Discussion

Yeast two-hybrid system reveals that Notch-1 interacts with Nur77

Using the yeast two-hybrid system (8, 9), a transcriptionally inert form of Nur77 that was able to enter the nucleus was used as bait to screen a mouse T cell lymphoma cDNA library. Among 55 independently isolated interactor clones, 48 clones encoded the mouse Notch-1 protein. The majority of the clones that were isolated contained a region encoding the Notch-1 ankyrin-like repeats (12, 13), suggesting that the observed interaction with Nur77 occurs via this protein motif. To confirm the specificity of the interaction between Nur77 and Notch-1, various control experiments were conducted. Retransformation of either different or unrelated bait constructs as well as the reintroduction of both bait and prey constructs alone into different control yeast strains verified the specificity of the observed original interaction (Fig. 1A) and indicated that Nur77 and Notch-1 form a protein complex in yeast.

To test whether Nur77 can also interact with Notch-1 in cellular extracts, we confirmed a direct protein interaction between Nur77 and Notch-1 using an in vitro protein binding assay (Fig. 1B). A bacterially expressed and purified GST-Nur77 fusion protein was coupled to GST-Sepharose beads and mixed with whole cell extracts derived from either untransfected or Flag-tagged Notch-1 transfected COS-1 cells. In addition, GST-Nur77 was incubated with Flag-Notch-1-containing extracts and purified Nur77 protein derived from a baculovirus insect cell expression system (PharMingen, San Diego, CA) (lane 3), or whole cell extracts derived from untransfected COS-1 cells (lane 4). Whole cell extracts derived from either Flag-tagged Notch-1-transfected COS-1 cells (lane 5) or untransfected COS-1 cells (lane 6) were loaded in the absence of GST-Nur77. M represents the biotinylated low range protein standard (Bio-Rad).

FIGURE 1. Notch-Nur77 protein interactions. A, Summary of protein interactions in yeast. Activation of a LacZ reporter gene is only detected when pGAL4 is fused to Notch-1 and cotransfected with a pLexA-Nur77 plasmid indicating the specificity of the interaction. B, Western blot analysis (15) using the FlagM2 mAb (10 μg/ml) (Kodak, Rochester, NY). A GST-Nur77 fusion protein coupled to GST-Sepharose beads was incubated with either NETN buffer alone (150 mM NaCl, 1 mM EDTA, 20 mM Tris, and 0.5% Nonidet p-40) (lane 1), whole cell extracts derived from COS-1 cells that had been transiently transfected with a Flag-tagged Notch-1 construct (lane 2), COS-1/Flag-tagged Notch-1 whole cell extracts plus 1 μg of purified Nur77 protein derived from a baculovirus insect cell expression system (PharMingen, San Diego, CA) (lane 3), or whole cell extracts derived from untransfected COS-1 cells (lane 4). Whole cell extracts derived from either Flag-tagged Notch-1-transfected COS-1 cells (lane 5) or untransfected COS-1 cells (lane 6) were loaded in the absence of GST-Nur77.

Retrovirally overexpressed forms of TAN-1 protect from Nur77-dependent cell death

To investigate Notch-1 function in lymphoid cells, we retrovirally infected different forms of the TAN-1 protein (Fig. 2). The human homologue of Notch-1 (6), in the T hybridoma cell line DO11.10. DO11.10 cells are a commonly used model of T cell death. DO11.10 cells do not express endogenous Notch-1. The cytoplasmic construct extracellular domain-containing form of TAN-1 (ECT⁺) contains parts of the extracellular domain and the complete transmembrane and intracellular domains of TAN-1, resulting in a 120-kDa protein (11) (Fig. 2). The nuclear construct intracellular domain of TAN-1 (ICT) encodes a 110-kDa protein containing only the intracellular domain (11). Retroviral expression of both constructs was confirmed by Western blot analysis using the bTAN18 mAb (data not shown). To exclude any nonspecific, long terminal repeat-derived effects on the parental cell line DO11.10, the empty retroviral vector MSCV2.1 was introduced into these cells.

FIGURE 2. TAN-1 constructs. The presented TAN-1 constructs were used for retroviral infection of DO11.10 cells. Infected cells were selected with G418 (1 mg/ml) and grown in G418-containing medium through further propagation. TM, transmembrane domain; ANK, ankyrin-repeat domain.
We subsequently compared apoptotic cell death in TAN-1-expressing cells vs control cells. Apoptosis induced via TCR cross-linking or treatment with PMA/CaI or dexamethasone leads to similar levels of cell death in both the parental cell line DO11.10 and the MSCV2.1-infected control line (Fig. 3A). In contrast, DO11.10 cells expressing ECT \(^{+}\) or ICT are protected from the Nur77-dependent apoptosis induced via TCR cross-linking or PMA/CaI treatment (Fig. 3B). Protection in the range of \(\sim 45-60\%\) was observed with the different constructs compared with uninfected or MSCV2.1-infected DO11.10 cells.

**FIGURE 3.** Effects of retrovirally infected forms of TAN-1 on apoptosis in DO11.10 cells. DO11.10 cells were cultured in RDG medium RPMI 1640 plus DMEM mixed 1:1) containing 10% horse serum. For cell death induction and analysis, cells were plated at a density of 1–2 \(\times\) \(10^5\) cells/ml and stimulated either with a combination of 10 nM of PMA and 500 nM of CaI A23187 or with 20 \(\mu\)M of dexamethasone. For TCR cross-linking, DO11.10 cells were plated in a cell culture flask coated with the mAb F23.1, which specifically interacts with the V\(\beta\)8 chain present on DO11.10 cells. A, FACScan analysis of DO11.10 cells or empty retroviral vector-infected MSCV2.1 cells that had been induced to die apoptotically either with the TCR-specific Ab F23.1, with a combination of 10 nM of PMA and 500 nM of CaI, or with 20 \(\mu\)M dexamethasone. YO-PRO-3 iodide-stained cells were analyzed at 16–18 h poststimulation by FACScan. B, Western blots of the 0 and 4 h timepoints; C, Summary of the data at all timepoints.

**FIGURE 4.** TAN-1 protein is found in the nucleus and impairs Nur77-dependent transcription. DO11.10 cells were stimulated with PMA (10 nM) and CaI (500 nM) and assayed at 0, 2, 4, and 8 h poststimulation. At each timepoint, nuclear extracts were prepared and run on SDS-PAGE. A total of 15 \(\mu\)g of nuclear protein was loaded per lane. The proteins were transferred and probed with the bTAN18 mAb. ECT\(^{+}\) and TANFull were not detectable in nuclear extracts at 0 and 2 h but were detectable by 4 h, indicating that these forms of the protein are initially cytoplasmic and are subsequently translocated into the nucleus. All other forms of TAN were immediately detectable in the nucleus. A, Western blots of the 0 and 4 h timepoints; B, Summary of the data at all timepoints. C, Cotransfected forms of TAN-1 impair Nur77-dependent promoter activity. A total of 1.2 \(\times\) \(10^5\) COS-1 cells were cotransfected using a total amount of 4.5 \(\mu\)g of plasmid DNA. Cotransfection of a NurRE-thymidine kinase (TK)-Luc construct and a Nur77 expression plasmid leads to a strong induction of Luc activity (black). Additional cotransfection of either an ICT expression plasmid (light gray) or a ECT\(^{+}\) expression plasmid (dark gray) results in an impairment of Nur77-dependent promoter activity.
To investigate whether the protection conferred by Notch-1 was specific for Nur77 signaling pathways, we subsequently examined the effect of Notch-1 on glucocorticoid-induced death, an event that does not require Nur77 (1). Cell death initiated with dexamethasone remains unaffected and is equivalent in TAN-1-expressing cells and MSCV2.1-infected cells (Fig. 3C). Therefore, Notch-1-mediated protection from cell death is dependent upon the presence of Nur77 and most likely on the physical interaction with this nuclear hormone receptor protein.

To determine where Notch-1 is found in T cells, subcellular localization of ICT and ECT proteins was determined in DO11.10 cells signaled to undergo apoptosis after combinatorial PMA/Ca2+ treatment. In Fig. 4A, the results of Western blots of nuclear extracts demonstrate that ICT is found exclusively in the nucleus, whereas ECT is first expressed in the cytosol and, after 2 h, translocates to the nucleus. Data are summarized in Fig. 4B.

To demonstrate that TAN-1 directly influences Nur77 function, we cotransfected a Nur77 Luc reporter construct with an expression construct of Nur77. As shown in Fig. 4C, Nur77 expression drives the Luc reporter, and this induction was dramatically impaired in the presence of cotransfected TAN-1 protein, indicating that TAN-1 directly modulates the functional activity of Nur77. These data support the conclusion that Notch expression alters Nur77 activity.

The proposed complex formation between Nur77 and Notch-1 and the observed suppression of cell death in T cells might have unique implications in the context of neoplasia and tumor formation. In this regard, it is interesting to note that during normal thymic development, Notch-1 expression is observed in early CD4+/CD8− T cells and is significantly reduced in CD4+/CD8+ T cells undergoing negative selection (14). Recently, it was observed that Nur77 is expressed in both CD4+/CD8− double-negative and CD4+/CD8+ double-positive thymocytes (K. Fortner, K. Newell, and B.A.O., unpublished observations), suggesting that Notch-1 might be involved in protecting double-negative thymocytes from cell death while the decrease in Notch-1 levels in double-positive thymocytes renders them sensitive to TCR-mediated death. Therefore, it is possible that after the development of double-negative thymocytes, Nur77 and Notch-1 are not expressed simultaneously in a normal context. However, in those situations in which Nur77 and Notch-1 are inappropriately coexpressed, we propose that this interaction provides protection from the normal cell death pathway operating during thymic development. These observations have important implications for our understanding of tumorigenesis in lymphoid cells. Peer et al. (11) demonstrated recently that expression of different TAN-1 alleles in hemopoietic cell types correlates with tumor formation. In a bone marrow reconstitution assay with TAN-1-expressing cells, T cell leukemias were induced (11). In all cases, the T cell neoplasms were of an immature phenotype and phenotypically resembled tumors caused by endogenous murine TAN-1. In this context, it appears that TAN-1 is oncogenic for T cells (11).

In conclusion, the data presented in this paper demonstrate an interaction between Nur77 and Notch-1. The functional consequences of Notch-1 expression are twofold. First, TAN-1 can inhibit Nur77-dependent cell death. Second, the expression of TAN-1 protein leads to reduced activity of the Nur77 reporter gene, indicating that TAN-1 represses Nur77 function. These data support a model whereby Nur77-dependent apoptosis is inhibited by Notch-1 through direct protein binding and subsequent inhibition of Nur77 transcriptional activity.

Acknowledgments

We thank R. Brent and coworkers for sharing the yeast strain EGY48 and various plasmid constructs for the yeast two-hybrid screen. E. Robey for providing the Flag-tagged Notch-1 construct. J. Aster for providing retroviral TAN constructs, and S. Artavanis-Tsakonas for sharing the bTAN18 mAb. B.M.J. thanks Stefan Meuer for his advice and support.

References