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Biochemical and Biophysical Research Communications 334 (2005) 1102–1106
BBRC
www.elsevier.com/locate/ybbrc

Nur77 nuclear import and its NBRE-binding activity in thymic lymphoma cells are regulated by different mechanisms sensitive to FK506 or HA1004

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Received 30 June 2005
Available online 14 July 2005

Abstract

Thymic lymphoma cells restore their sensitivity to ionomycin-induced apoptosis when treated with FK506 or HA1004. In apoptosis-resistant cells, ionomycin-induced Nur77 strongly binds DNA during the first 2 h of response, in contrast to lymphoma cells treated with ionomycin together with FK506 or HA1004, which undergo massive apoptosis. We show that Nur77 could discriminate between calcium signals sensitive to FK506 and those sensitive to HA1004, as the inhibitors differentially regulate the kinetics of Nur77 nuclear import, and FK506, unlike HA1004, inhibits Nur77 DNA-binding activity. In the presence of HA1004, NBRE binding by Nur77 protein increases with time (6 h vs 2 h), whereas the final outcome of both inhibitors is apoptosis of thymic lymphoma cells.

Keywords: Nur77; Apoptosis; Thymic lymphoma; FK506; HA1004; NBRE; DNA-binding; Nuclear import; Ionomycin; Calcium signals; Nuclear receptors

Studies on the nuclear receptor Nur77 (TR3, NGFI-B) usually refer to its control via DNA binding, of the regulation of the gene expressions involved in biological processes such as proliferation [1], differentiation [2], steroid hormones synthesis [3,4], cell cycle arrest [5], or survival promotion [6,7]. The function of nuclear orphan receptor Nur77 may be regulated at multiple levels: its expression [8,9], subcellular distribution [5,10–12], and ability to bind DNA [13,14]. It has been demonstrated that in several cell lines, e.g., prostate cancer [15], gastric cancer [5,11], rat cerebellar granule neurons [12], colon cancer [10], and peripheral blood lymphocytes [16], the induction of apoptosis depends on successful Nur77 migration from the nucleus to mitochondria. On the mitochondrial membrane, Nur77 interacts with Bcl-2, which leads to conformational changes within Bcl-2 and the switching of this protein from a protector to a killer. This event enables the release of cytochrome *c* [16], which is a widely known prerequisite to caspase cascade activation.

In contrast, Nur77 is believed to exert its effect in T cells by activating the transcription of proapoptotic genes or/and silencing the transcription of antiapoptotic ones [17,18]. However, it remains unclear whether potential downstream genes are direct transcriptional targets of Nur77 [19]. In T cells the expression of Nur77 is induced by elevated intracellular calcium level (mimicked by ionomycin treatment) as a result of TCR activation and is required in activation-induced T-cell death [20–22].

Impaired induction of apoptosis is one of the main features liberating tumor cells from host surveillance mechanisms as well as from anticancer therapies. The latter aims at restoration of tumor susceptibility to cell

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0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.07.004

death by pharmacological treatment. In agreement with this, we found that thymic lymphomas resistant to Nur77-mediated apoptosis [23] treated with FK506 [24], an inhibitor of calcineurin (calcium-dependent phosphatase), known as immunosuppresant or HA1004 [25], an inhibitor of serine/threonine protein kinases, restored their sensitivity to ionomycin-induced apoptosis. In order to examine the underlying mechanism, we studied endogenous Nur77 expression in subcellular fractions and its DNA-binding activity in lymphoma cells resistant and with restored sensitivity to ionomycin-induced apoptosis. The obtained results show, that in thymic lymphoma cells Nur77 discriminates between two signaling pathways sensitive to FK506 and HA1004, which differentially regulate the kinetics of Nur77 protein nuclear import as well as its DNA-binding activity.

Materials and methods

Cells. VIII/d cells derived from primary thymic lymphoma of anti-HY-TCR transgenic mice were cultured at 37 °C and in 5% CO_2 in 6-well tissue culture plates (Corning Costar) in Iscove's modified Dulbecco's medium (IMDM from Gibco) supplemented with 20 μM β-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS (Boehringer Mannheim).

Treatment of cells. Stimulations were performed in 24-well plates (Costar) (2×10^6 cells per well). Cells were cultured (37 °C, 5% CO_2) for 2, 6 (for lysate preparation), or 20 h (for apoptosis detection) in the presence of 1.5 μg/ml ionomycin (Sigma), 40 nM FK506 (generous gift from Dr. M. Wasik, University of Pennsylvania, USA), or 4 μg/ml HA1004 (Sigma). Cells treated with medium alone were used as a control.

Detection of apoptosis. The detection of apoptosis was based on DNA content evaluation with the use of propidium iodide and flow cytometry [26]. Briefly, after 20-h of treatment, the cells were washed twice with phosphate-buffered saline (PBS) containing 2.5% FCS, fixed with 70% ethanol for 30 min at 4 °C, and then, after washing with PBS, the cells were stained with propidium iodide (Sigma, 50 μg/ml in PBS Ca^-, Mg^-) overnight at 4 °C. The cell suspensions were analyzed with a FACSCalibur flow cytometer (Becton Dickinson). DNA content was evaluated on the basis of FL-2 histograms using WinMDI 2.8 software. Apoptosis was quantified as the percentage of cells with hypodiploidal DNA content.

As a control, the MTS Assay (Promega) was performed according to the manufacturer's directions. Briefly, cells were treated in 96-well flat-bottomed tissue culture plates (Corning Costar) (2×10^5 cells per well) for 20 h, then MTS was added to the wells, and incubation was continued for another 3 h. Absorbance at 490 nm was measured on a Victor^2 (Wallac) 96-well plate reader. The number of metabolically active cells was estimated on the basis of a standard curve previously calibrated for VIII/d cells.

Nuclear extract preparation. Nuclear extracts were prepared as described previously [27] with minor modifications. Cells were washed in cold PBS, suspended in three volumes of buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, 0.1 mM sodium orthovanadate, and 0.2% Nonidet P-40), incubated on ice for 15 min, and centrifuged at 10,000 rpm at 4 °C for 10 min. Supernatants were used as cytoplasmic lysates for Western blotting. The pellets were washed once with buffer A and resuspended in three volumes of buffer B (20 mM Hepes, pH 7.9, 10 nM KCl, 1 mM EDTA, 1 mM EGTA, 420 mM NaCl, 20% glycerol, 1 mM DTT, and 1 mM PMSF), and nuclear proteins were extracted by rocking for 30 min at 4 °C. After centrifugation (10 min, 10,000 rpm) the supernatants were stored at −70 °C and used as nuclear extracts for EMSA or nuclear fraction lysates for Western blotting. Protein concentrations were determined using BCA (Sigma).

Electromobility shift assay. Double-stranded consensus oligonucleotides, NGFI-B Response Element (NBRE) specific for monomeric Nur77 (from Bionovo), and CREB (Promega) were 5′end-labeled with [γ-^{32}P]ATP (NEN) by T4 Polynucleotide Kinase (Fermentas), according to the manufacturer's directions. Nuclear extracts (5 μg) were incubated with binding buffer (1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris–HCl, pH 7.5, and 4% glycerol), 0.1 μg of poly(dI–dC) (Amersham Biosciences), and labeled oligonucleotide (NBRE, 5′- TCGAGTTTTAAAAGGTCATGCTC AATTTG-3′; CRE, 5′-AGAGATTGCCTGACGTCAGAGAGCT AG-3′) at room temperature (in a total volume of 10 μl). DNA–protein complexes were resolved on 4% native polyacrylamide gel, in 0.5× TBE buffer. The gels were incubated overnight at −20 °C with a storage phosphor screen (Molecular Dynamics) and scanned with a Typhoon 8600 multi-imager (Molecular Dynamics). The specificity of complex formation was confirmed by performing the binding reactions in the presence of either unlabeled NBRE oligonucleotides or anti-Nur77 antibody.

Western blotting. Lysates of both fractions, prepared as described above, were mixed with concentrated sample buffer, boiled for 5 min, and subjected to SDS–PAGE on 10% polyacrylamide gel. The proteins were transferred to Immobilon P membrane (Millipore) using a semi-dry transfer apparatus (Bio-Rad Laboratories). The membrane was blocked with 1% casein (Sigma) in TBS and then incubated with 1 μg/ml of rabbit anti-Nur77 antibody (M-210), anti-histone H1 (FL-219), or goat anti-actin antibody (C-11) (Santa Cruz Biotechnology), followed by secondary horseradish peroxidase-linked antibodies (Dako) diluted 1:3000. The bound antibodies were visualized using the ECL Western blotting detection system (Amersham).

Results

Differential kinetics of Nur77 protein expression and its subcellular localization in the presence of FK506 and HA1004

We investigated endogenous Nur77 expression (Western blotting) in nuclear and cytoplasmic cell fractions as well as its DNA-binding ability in VIII/d cells of a thymic lymphoma line resistant to and with restored susceptibility to ionomycin-induced apoptosis after treatment with FK506 or HA1004. Apoptosis was checked in all experiments in simultaneously performed MTS assay (data not shown), as well as was quantified as the percentage of cells with hypodiploidal DNA content. In a series of experiments, the percentage of apoptotic lymphoma cells in the presence of ionomycin ranged between 15% and 36% (compared to 75–85% of normal thymocytes [25] and data not shown), and in the presence of ionomycin and FK506 or HA1004 between 53% and 81% and 50% and 77%, respectively. In the presence of inhibitors without ionomycin, the percentage of apoptotic cells did not exceed that of untreated controls ([24,25] and data not shown). Fig. 1B presents results of a representative experiment. Western