Short communication

THE MITOCHONDRIAL LOCALIZATION OF RelB AND NFATx IN IMMATURE T CELLS

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Abstract: In order to exert their activity, transcription factors must be transported to the nucleus. Certain transcription factors have also been found on mitochondria. Here, the localization of RelB and NFATx in the mitochondrial fractions of normal thymocytes and thymic lymphoma cells is shown for the first time. CREB was only found in the nucleus, while p50 (NFκB) was found in both the nucleus and the cytoplasm, but outside the mitochondria. The translocation of transcription factors to the mitochondria is differentially regulated. Unlike RelB, which is always present in the mitochondrial fraction, NFATx appeared on the mitochondria in cells treated with ionomycin together with an immunosuppressant and inhibitor of calcineurin (FK506). This data reveals that the mitochondrial localization of some transcription factors is precisely controlled by a calcium signal sensitive to FK506 in T cells.

Key words: Mitochondrial translocation, Transcription factors, NFATx, RelB, FK506, Thymocytes, Thymic lymphoma

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INTRODUCTION

Signal transduction within a cell proceeds via post-translational modifications of proteins (mainly phosphorylation) and protein trafficking. The strict control of the localization of transcription factors is crucial for their proper function. According to the general model, an activated transcription factor migrates from the cytoplasm to the nucleus, where it regulates gene expression to initiate the appropriate genetic program. An increasing number of reports reveal that "nuclear" transcription factors can also undergo translocation to the mitochondria under certain conditions. Some of these proteins, such as CREB [1], estrogen receptor [2], AP-1 [3] and NFκB [4], were found to bind mitochondrial DNA, while the localization of Nur77 [5], GR [6], p53 [7] and Elk-1 [8] to the mitochondrial membrane was found to be linked to the induction of apoptosis or decreased cell viability.

In T cells, the increase in the intracellular calcium level upon T-cell receptor (TCR) engagement, mimicked by ionomycin treatment, activates calcineurin [9] and subsequently multiple transcription factors, including Nur77, NFκB and NFAT. RelB, a member of the NFκB family [10], and NFATx [11] were shown to be involved in thymocyte survival and differentiation, while Nur77 is indispensable for the negative selection of autoreactive cells [12]. FK506, an inhibitor of the calcineurin and p38/JNK pathways [13], impairs the positive selection of thymocytes, but does not affect calcium-induced apoptosis [14]. This compound also restores thymic lymphoma cells' sensitivity to calcium-dependent apoptosis [15]. We recently showed that in mouse thymocytes, Nur77 localizes to the mitochondria in reaction to an apoptosis-inducing calcium signal [16], and that its translocation is unaffected by FK506. In this study, we attempted to determine the effect of ionomycin and FK506 on the subcellular distribution of NFATx, two NFκB subunits (p50 and RelB), and CREB in mouse thymocytes and thymic lymphoma cells.

MATERIALS AND METHODS

Cell culture and treatment

VIII/d cells derived from the primary thymic lymphoma of anti-HY-TCR transgenic mice [17] were cultured at 37ºC in 5% CO_2 in Iscove's modified Dulbecco's medium (Gibco) supplemented with 20 μM β-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma), and 10% FCS (Boehringer Mannheim). The VIII/d cells and thymocytes obtained from C57BL/6BoyIiw mice (2-3 months old) were treated in 24-well tissue culture plates (Corning Costar) for 6 h with 1 μg/ml of ionomycin (Sigma) or 40 nM of FK506 (donated by Dr. M. Wasik, University of Pennsylvania, USA).

Confocal microscopy
After the treatment, the cells were fixed in 4% paraformaldehyde (Sigma) in phosphate-buffered saline (PBS) for 30 min at 37ºC and washed three times with PBS. The cells were then permeabilized with 0.15% Triton X-100 (Sigma) in PBS containing 10% horse serum (Sigma) for 1 h at room temperature. The washed cells were incubated overnight with the polyclonal antibodies (Santa Cruz Biotechnology) anti-NFATx, anti-p50, anti-Rel B, or anti-CREB (1:100) and anti-Hsp60 (1:20), followed by staining for 1 h with the secondary antibodies anti-rabbit IgG conjugated with Alexa Fluor 488 (Molecular Probes; 1:200) and anti-goat IgG conjugated with Cy3 (Jackson Immuno Research; 1:400). After triple washing with PBS, the cell suspensions were placed onto microscope slides and directly examined under a confocal microscope (Bio-Rad MRC-1024ES). In each experiment, samples without primary antibody staining were examined as a control.

Subcellular fractionation
To obtain mitochondrial, nuclear and cytosolic fractions, we used a Mitochondria Isolation Kit (Pierce). Briefly, the cells were washed twice with cold PBS and lysed. After centrifugation at 800 x g for 10 min, the nuclear pellet was washed with lysis buffer, centrifuged at 1000 x g for 10 min, suspended in SDS sample buffer, and sonicated. The postnuclear supernatant was centrifuged at 10,000 x g for 15 min. The pellet containing the mitochondria was dissolved in the sample buffer, and the clear supernatant was used as the cytosolic fraction.

Western blotting
Cell fractions were boiled and subjected to SDS-PAGE on a 10% gel (20 μg/well for thymocytes and 50 μg/well for lymphoma cells). After transfer, the PVDF membrane (Millipore) was blocked with 1% casein in TBS, and then incubated with 1 μg/ml of the rabbit polyclonal anti-RelB, anti-p50, anti-CREB, or monoclonal anti-NFATx antibodies (Santa Cruz Biotechnology), followed by secondary horseradish peroxidase-labeled antibodies (DAKO). Anti-Hsp60, anti-histone H1, and anti-actin antibodies (Santa Cruz Biotechnology) were used as the controls for the protein content and purity of the fractions. The bound antibodies were visualized using the ECL blotting detection system (Pierce).

RESULTS

The mitochondrial localization of RelB and NFATx in thymocytes, as detected by confocal microscopy
We examined the subcellular localization of the chosen transcription factors in the intact thymocytes via confocal microscopy. In the untreated cells, we observed colocalization of RelB with Hsp60, a specific marker for the mitochondria. This was unaffected by the treatments. The colocalization of NFATx with Hsp60 was only detected after combined ionomycin/FK506 treatment, while the immunosuppressant alone caused cytosolic accumulation of
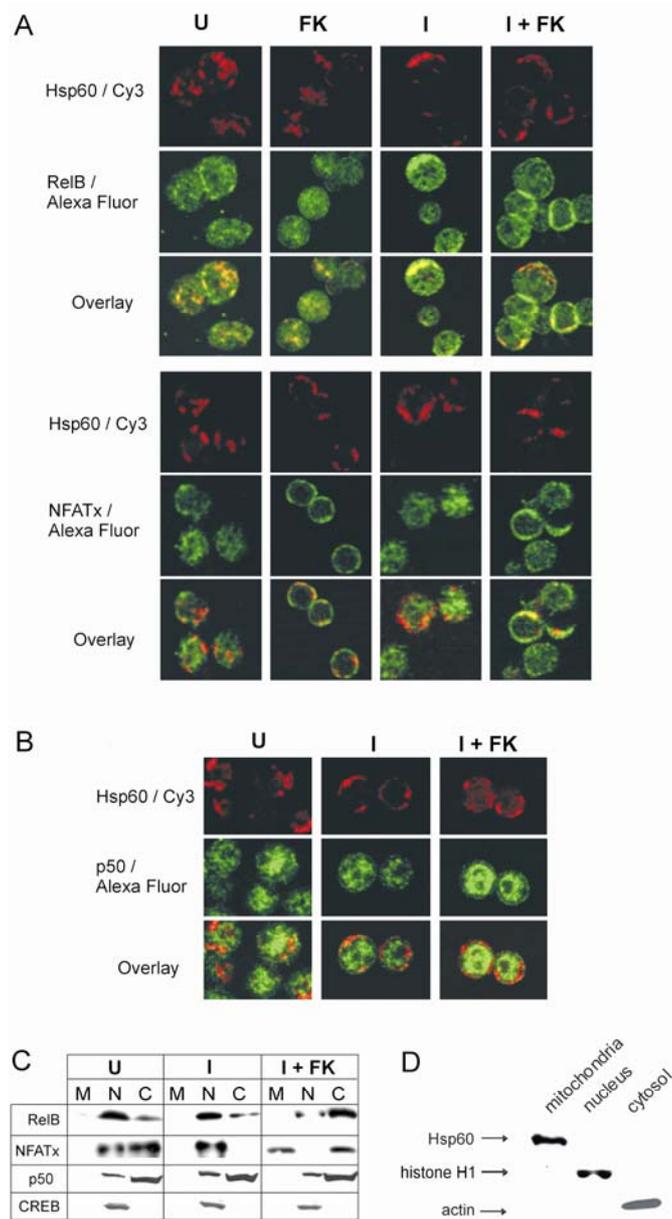
Fig. 1. The subcellular distribution of transcription factors in thymocytes after 6 h of treatment. The panels correspond to the particular treatment: U – untreated, FK – FK506, I – ionomycin, I + FK – ionomycin with FK506. The mitochondrial localization of RelB and NFATx (A), and non-mitochondrial localization of p50 (B) was detected by confocal microscopy. Transcription factors are stained with Alexa Fluor (green), Hsp60 is stained with Cy3 (red), colocalization is shown in yellow. C – Immunoblotting of the three fractions: M – mitochondrial, N – nuclear, and C – cytosolic. D – An exemplary analysis of purity of the fractions with anti-Hsp60, anti-actin, and anti-histone H1 antibodies. Representative results are shown.
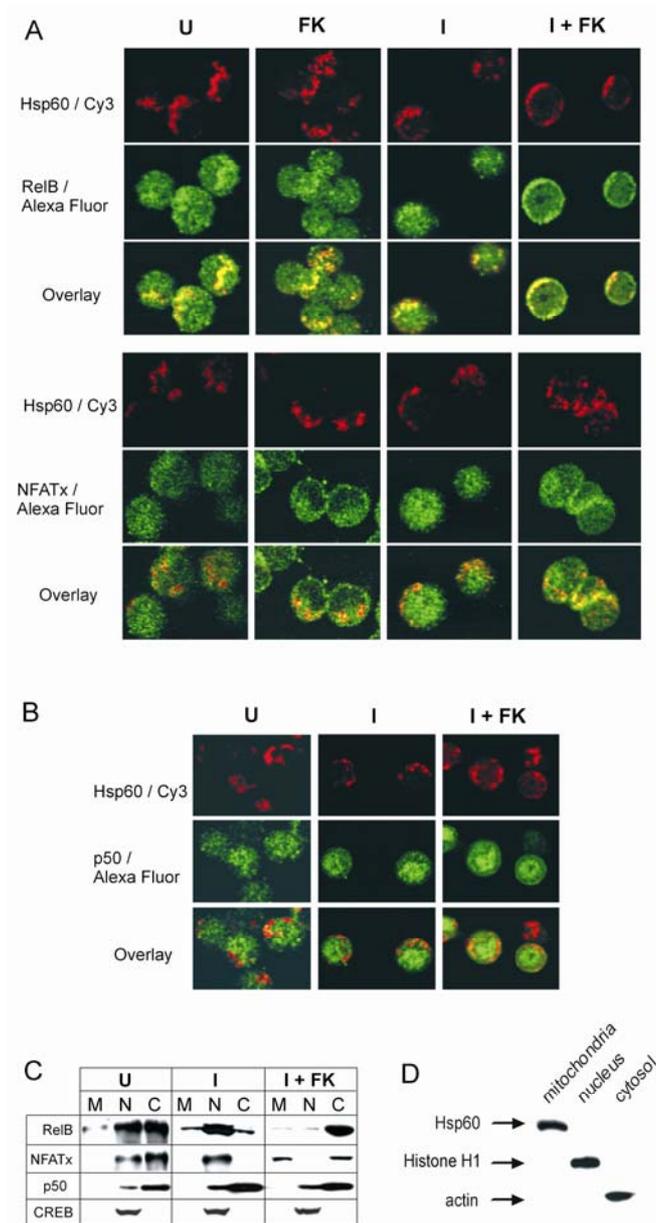
Fig. 2. The subcellular distribution of transcription factors in thymic lymphoma VIII/d after 6 h of treatment. The panels correspond to the particular treatment: U – untreated, FK – FK506, I – ionomycin, I + FK – ionomycin with FK506. The mitochondrial localization of RelB and NFATx (A), and non-mitochondrial localization of p50 (B) was detected by confocal microscopy. C – Immunoblotting of the three subcellular fractions: M – mitochondrial, N – nuclear, and C – cytosolic. D – As a control, each fraction was analyzed with anti-Hsp60, anti-actin, and anti-histone H1 antibodies. Representative results are shown.

this protein. No significant influence of FK506 alone on RelB subcellular localization could be detected (Fig. 1A). The distribution of p50 and CREB was non-mitochondrial regardless of the conditions (Fig. 1B and [16]). A similar pattern of localization of the studied proteins could be observed after 2 and 12 h of treatment (data not shown). To the best of our knowledge, this is the first time that a mitochondrial localization has been shown for NFATx and RelB in T cells.

The localization of transcription factors in thymocytes, as detected by Western blotting
To verify the RelB and NFATx mitochondrial localization, we performed studies of the distribution of these proteins in subcellular fractions from thymocytes treated for 6 h with ionomycin alone or ionomycin with FK506 (Fig. 1C). The mitochondrial localization of RelB in the untreated thymocytes was confirmed. However, Western blot analysis of the RelB distribution did not allow the detection of this protein in the mitochondrial fractions of the treated cells (Fig. 1C). This could be due to the low number of mitochondria in thymocytes, which resulted in a very low protein concentration in the mitochondrial fractions (data not shown), meaning the content of RelB in the mitochondrial fractions could have been too low to be visualized in our blots. The relatively higher sensitivity of confocal microscopy should also be taken into account.
In untreated cells, NFATx was detected in both the nucleus and cytosol. Ionomycin treatment resulted in the nuclear retention of this transcription factor. FK506 blocked the nuclear import of NFATx and, importantly, induced its association with the mitochondria. CREB was present solely in the nuclei, and the NFκB subunit p50 was detected in both the nuclear and cytosolic fractions, but not in the mitochondrial fraction, regardless of the treatment (Fig. 1C). The purity of each fraction was analyzed by Western blotting with the use of anti-Hsp60, anti-histone H1, and anti-actin antibodies (Fig. 1D).

The mitochondrial localization of RelB and NFATx in thymic lymphoma cells
We performed analogous studies on the chosen transcription factors' subcellular localization in mouse thymic lymphoma cells with confocal microscopy (Fig. 2A, B) and Western blotting (Fig. 2C). In general, the proteins displayed similar distribution patterns in the normal and transformed thymocytes. It is noteworthy that RelB was detected in the mitochondrial fraction regardless of the conditions. As a control, each fraction was analyzed with anti-Hsp60, anti-actin, and anti-histone H1 antibodies (Fig. 2D).

DISCUSSION

Our findings allow the addition of NFATx and RelB to the increasing group of "nuclear" transcription factors known to undergo mitochondrial translocation. The localization of NFATx was specifically controlled by a calcium signal sensitive to FK506, while RelB localization remained mitochondrial in normal and tumour-transformed thymocytes.

Our results on NFATx subcellular distribution are consistent with those obtained by other researchers. In untreated cells, NFATx cycles between the nucleus and the cytoplasm, but it resides in the nucleus too transiently to bind DNA. To efficiently arrest this protein in the nucleus, the activation of the calcium-dependent phosphatase calcineurin is required [9]. FK506, as an inhibitor of calcineurin, prevents the nuclear import of NFATx without affecting its nuclear export. We showed here for the first time that the presence of FK506 also leads to mitochondrial localization of this protein. The mechanism of NFATx addressing to the mitochondria remains to be elucidated.

Not all the transcription factors were directed to the mitochondria, as we observed no colocalization of Hsp60 with CREB or with p50. This further indicates the specificity of NFATx mitochondrial import under the given conditions. It also excludes the possibility that the detected NFATx and RelB colocalizations with a mitochondrial marker were artifacts of the experimental procedure. It is worth mentioning that the distributions of all the examined proteins were comparable in normal and transformed thymocytes, and could therefore be general for immature T cells.

Our results also show that the localization of RelB and p50, members of the same protein family (NFκB), is distinctly regulated. We showed that RelB is present on the mitochondria in untreated thymic lymphoma cells and freshly prepared mouse thymocytes, and that a pool of this protein remains mitochondrial regardless of the treatment. Bottero *et al.* [18] found p65, another member of the NFκB family, in the intermembrane mitochondrial space in quiescent Jurkat cells, which are derived from peripheral mature human T cells. p65 and p50 were found in the mitochondria of resting liver cells [4], contrary to the p50 nucleo-cytosolic distribution in our model. CREB was detected in the mitochondria of the rat brain [1], while we observed a strictly nuclear localization for this protein in immature T cells. This data reveals the complexity of this phenomenon and suggests that the mitochondrial localization of transcription factors is under strict control and depends on the cell type and development stage.

There is no correlation between NFATx translocation and apotosis or oncogenic transformation of thymocytes, and the role of the observed mitochondrial localizations is not known. However, these observations are interesting, since the subcellular distribution of proteins is important for their function. Do transcription factors deliver any information to the mitochondria as elements of signaling pathways or serve as adaptor proteins at the surface of the mitochondrial membrane? Is it simply an additional way of anchoring proteins and sequestering them far away from the nucleus (see NFATx after ionomycin with FK506)? Alternatively, mitochondrial NFATx or RelB could be involved in other calcium-regulated and/or energy-dependent biological processes, such as proliferation or differentiation. Explaining this phenomenon and its role requires further study.

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