Published in final edited form as:

Biochem Biophys Res Commun. 2010 November 26; 402(4): 778–783. doi:10.1016/j.bbrc.2010.10.112.

Mitochondrial translocation of signal transducer and activator of transcription 5 (STAT5) in leukemic T cells and cytokine-stimulated cells

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Abstract

Signal transducers and activators of transcription (STATs) were first identified as key signaling molecules in response to cytokines. Constitutive STAT activation also has been widely implicated in oncogenesis. We analyzed STAT5-associated proteins in a leukemic T cell line LSTRA, which exhibits constitutive tyrosine phosphorylation and activation of STAT5. A cellular protein was found to specifically interact with STAT5 in LSTRA cells by co-immunoprecipitation. Sequencing analysis and subsequent immunoblotting confirmed the identity of this STAT5-associated protein as the E2 component of mitochondrial pyruvate dehydrogenase complex (PDC-E2). Consistent with this interaction, both subcellular fractionation and immunofluorescence microscopy revealed mitochondrial localization of STAT5 in LSTRA cells. Mitochondrial localization of tyrosine-phosphorylated STAT5 also occurred in cytokine-stimulated cells. A time course experiment further demonstrated the transient kinetics of STAT5 mitochondrial translocation after cytokine stimulation. In contrast, cytokine-induced STAT1 and STAT3 activation did not result in their translocation into mitochondria. Furthermore, we showed that mitochondrial STAT5 bound to the D-loop regulatory region of mitochondrial DNA *in vitro*. It suggests a potential role of STAT5 in regulating mitochondrial genome. Proliferative metabolism toward aerobic glycolysis is well known in cancer cells as the Warburg effect and is also observed in cytokine-stimulated cells. Our novel findings of cytokine-induced STAT5 translocation into mitochondria and its link to oncogenesis provide important insights into the underlying mechanisms of this characteristic metabolic shift.

Keywords

STAT; PDC-E2; mitochondria; oncogenesis; cytokine; metabolism

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Introduction

Mitochondria are the powerhouses of the cell and serve as integrators of important regulatory signals that modulate numerous physiological processes [1]. Coordinated regulation of mitochondrial and nuclear functions is critical to maintain cellular metabolism and survival [2]. Defective mitochondrial-nuclear communication leads to mitochondrial dysfunction and, subsequently, the development of a wide variety of human disease [3]. Many cancer cells exhibit a characteristic metabolic shift toward aerobic glycolysis known as the Warburg effect [4]. During oxidative phosphorylation, pyruvate, a glucose metabolite, moves into mitochondria and is first converted to acetyl-CoA by pyruvate dehydrogenase complex (PDC). Through citric acid cycle and electron transport chain (ETC), maximum amounts of ATP are produced in the mitochondrion. Under aerobic glycolysis, however, pyruvate is directed away from mitochondria and metabolized to lactate in the cytoplasm. Although inefficient in generating ATP, aerobic glycolysis may facilitate the creation of biomass essential in making a new cell. Consistent with this hypothesis, growth signals, such as cytokines, have been shown to promote aerobic glycolysis [5]. Proliferative metabolism can also be controlled by signaling pathways involving both oncogenes and tumor suppressor genes [4]. The molecular mechanisms underlying this important metabolic shift, however, are still poorly defined.

Signal transducer and activator of transcription (STAT) proteins are latent cytoplasmic transcription factors essential for cellular response to cytokines [6,7]. Upon stimulation, tyrosine-phosphorylated STAT proteins dimerize, translocate to the nucleus, and regulate specific gene expression to modulate cellular functions. Increasing evidence suggests that STAT signaling may be involved in regulating cellular metabolism. In response to interferon- β (IFN- β), tyrosine-phosphorylated STAT3 is implicated in modulating mitochondrial ETC activity and oxidative phosphorylation [8]. Independent of tyrosine phosphorylation, however, STAT3 was reported to be constitutively present in mitochondria, associate with ETC complex I/II, and regulate mitochondrial respiration [9]. On the other hand, a recent study failed to detect significant amount of STAT3 in the mitochondria of porcine and murine heart and liver tissues [10]. It remains largely unknown whether STAT3 or other STAT family members translocate into mitochondria in a cytokine-dependent manner.

STAT3 and STAT5 are constantly activated in a large number of human cancers [11]. Constitutively active STAT3 and STAT5 mutants also function as oncogenes. The current paradigm is that STAT3 and STAT5 regulate the expression of nuclear target genes involved in various oncogenic processes, such as cell cycle progression, resistance to apoptosis, and angiogenesis. Gough *et al.* reported the role of mitochondrial STAT3 in Ras-mediated oncogenesis and a shift to cytoplasmic glycolysis in Ras-transformed cells [12]. It suggests that STAT proteins may exert additional functions in the mitochondrion to alter cellular metabolism that favors oncogenic transformation. In this report, we specifically examined the role of STAT5 in the context of both leukemia cells and cytokine response.

Materials and methods

Cell lines and culture conditions

Maintenance of the human CTLL-20 [13], the mouse BaF3 [14], the mouse NIH3T3 fibroblasts, the human Jurkat T cell line J77, the mouse T cell hybridoma BYDP, and the mouse T lymphoma cell line LSTRA [15] has been described elsewhere. For interleukin-2 (IL-2) stimulation experiments, CTLL-20 cells were deprived of IL-2 for 4 h and then stimulated with 30 units/ml of recombinant human IL-2 for various lengths of time as indicated in the figure legends. For IL-3 stimulation experiments, BaF3 cells were deprived

of IL-3 for 16 h and then stimulated with 10 ng/ml of recombinant mouse IL-3 for 15 min. For IFN- γ stimulation experiments, confluent culture of NIH3T3 fibroblasts were made quiescent in medium with 0.5% serum for 2 days and then stimulated with 10 ng/ml of recombinant mouse IFN- γ for 15 min. Recombinant human IL-2, mouse IL-3 and mouse IFN- γ were purchased from R&D Systems Inc. (Minneapolis, MN).

Subcellular fractionation

Cells were washed twice in phosphate-buffered saline (PBS), and then homogenized by passing through a 30-gauge needle. Cytosolic fraction and mitochondria-enriched heavy membrane fraction (mitochondrial fraction) were obtained through differential centrifugation as described previously [16,17]. The purity of each fraction was verified by immunoblotting of specific markers.

Immunoprecipitation, silver stain, and immunoblotting

Whole cell lysates and mitochondrial lysates were prepared by solubilizing whole cell pellets and mitochondrial fractions in RIPA buffer, respectively, followed by immunoprecipitation and immunoblotting as described before [15]. Antibodies specific for STAT5, STAT3, STAT1, PDC-E2 (the E2 component of PDC), VDAC1 (voltage-dependent anion-selective channel protein 1), and Eps15 (epidermal growth factor receptor substrate 15) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phosphotyrosine monoclonal antibody 4G10 was from Upstate Biotechnology, Inc. (Lake Placid, NY). Dilutions of different antibodies for immunoprecipitation, immunoblotting, and subsequent detection by the enhanced chemiluminescence (ECL) system were performed as recommended by the manufacturers. Silver staining was performed as described elsewhere [18]. Molecular weight markers in kDa are labeled in the figures where applicable.

Protein sequencing

STAT5 immunoprecipitates were resolved by 7% SDS-PAGE and transferred to PVDF membrane. Proteins were visualized by Ponceau S staining, cut from the membrane, and then subjected to amino-terminal sequencing using the Applied Biosystems Protein Sequencer.

Immunofluorescence microscopy

Exponentially growing LSTRA cells were adhered to 10-well slides at a concentration of 1.5×10^4 cells/well. Adhered cells were fixed with 4% paraformaldehyde in PBS for 15 min at 37°C and stained with 300 nM of MitoTracker Deep Red 633 for 20 min at room temperature. Cells were subsequently permeabilized with 0.2% Triton X-100 in PBS for 5 min, washed, and blocked with 4% bovine serum albumin in PBS for 30 min at room temperature. Permeabilized cells were stained with anti-STAT5 antibody for 1 h at 37°C followed by staining with Alexa Fluor 488-conjugated antibody for 30 min at 37°C. Nuclei were visualized by using DAPI as a counterstain. Anti-STAT5 antibody was purchased from BD Biosciences (San Jose, CA). MitoTracker, secondary antibody, and DAPI were purchased from Invitrogen (Carlsbad, CA). Antibodies were diluted following manufacturers' instructions before staining. Stained cells were viewed with appropriate filters using a fluorescence microscope with a Nikon Metamorph digital imaging system.

Electrophoretic mobility shift assay (EMSA)

Proteins were extracted from the mitochondrial fraction using high salt buffer as described previously [19]. The STAT5-binding consensus oligonucleotides (TTTCTAGGAATT) and anti-STAT5 supershifting antibody were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Mouse mitochondrial D-loop oligonucleotides

(TTCTAGTAGTTCCTCCAAAATATGACTTA) were custom synthesized and annealed. EMSA and antibody supershift assays were performed as previously described [19].

Results and discussion

Identification of PDC-E2 as a STAT5-binding partner

LSTRA is a mouse T cell leukemia that overexpresses active Lck kinase and exhibits constitutive STAT5 activation [15]. To further define the molecular details, we performed co-immunoprecipitation analysis to identify potential STAT5-binding partner(s). STAT5 and co-immunoprecipitated proteins were resolved by SDS-PAGE and visualized by silver staining. As a negative control, STAT5 co-immunoprecipitation experiments were also conducted in BYDP cells, a mouse T cell hybridoma with very low levels of STAT5 [15]. As shown in Fig. 1A, a predominant band with an apparent molecular weight of 60 kDa was observed exclusively in the STAT5 immunoprecipitates of LSTRA lysates.

In addition to STAT5, STAT3 is also constitutively activated in LSTRA cells [15]. However, the 60-kDa protein was not detected in the STAT3 immunoprecipitates of LSTRA lysates (Fig. 1B, compare *lanes 1* and *3*). As another negative control, rabbit control antibody did not bring down the 60-kDa protein (Fig. 1B, *lane 2*). These results demonstrate a specific interaction between STAT5 and a novel 60-kDa protein in a leukemic T cell line.

To determine the identity of this 60-kDa protein, we extracted the unknown protein from membrane after electrotransfer and subjected it to N-terminal amino acid analysis. Sequencing result (SLPPHQKVPLPS) identified the protein as PDC-E2 [20]. The interaction between STAT5 and PDC-E2 was verified by Western blot analysis using PDC-E2-specific antibody. As shown in Fig. 1C, PDC-E2 was co-precipitated with STAT5 in LSTRA lysates, but not in BYDP lysates.

Mitochondrial localization of STAT5 in LSTRA leukemia

PDC-E2 is localized predominantly in the mitochondrion [20]. To determine whether STAT5 might translocate to mitochondria, we performed subcellular fractionation of LSTRA cells. There was significant amount of STAT5 in the mitochondrial fraction of LSTRA cells (Fig. 2A, *lane 3*). Consistent with the co-immunoprecipitation observed in whole cell lysate (Fig. 2A, *lane 1*), PDC-E2 also co-precipitated with STAT5 in the mitochondrial fraction (Fig. 2A, *lane 3*).

We conducted immunofluorescence microscopy to further confirm subcellular localization of STAT5 in LSTRA cells (Fig. 2B). Consistent with constitutive STAT5 activation, a substantial amount of STAT5 could be detected in the nuclei of LSTRA cells. More importantly, outside the nuclei, there was clear co-localization of STAT5 and mitochondria visualized by the MitoTracker dye. Therefore, both biochemical analysis and immunofluorescence microscopy confirmed the presence of STAT5 in LSTRA mitochondria.

We showed previously that STAT5 was constitutively tyrosine-phosphorylated in LSTRA cells, but not in Jurkat T cells without cytokine stimulation [15]. To determine whether STAT5 tyrosine phosphorylation correlated with its mitochondrial localization, we compared mitochondrial fractions prepared from both LSTRA and Jurkat T cells. STAT5 was only present in the mitochondrial fraction of LSTRA cells and not Jurkat T cells (Fig. 2C, *middle panel*). Consistent with STAT5 activation in LSTRA, mitochondrial STAT5 exhibited high levels of tyrosine phosphorylation (Fig. 2C, *top panel*). Immunoblotting of VDAC1, a mitochondrial marker, confirmed normalized loading of mitochondrial proteins

(Fig. 2C, *bottom panel*). This result strongly suggests that mitochondrial localization of STAT5 is regulated and not constitutive.

Cytokine-induced STAT5 translocation into mitochondria

We showed previously that IL-2 activated STAT5 in an IL-2-dependent cell line CTLL-20 [21]. To determine whether IL-2-induced STAT5 activation led to mitochondrial translocation, we performed subcellular fractionation experiments in CTLL-20 cells. As shown in Fig. 3A (*upper panels*), STAT5 was only detected in the mitochondrial fraction after IL-2 stimulation (compare *lanes 3 and 4*). The stoichiometry of tyrosine-phosphorylated STAT5 was also significantly higher in the mitochondrial fraction (Fig. 3A, compare *lanes 2 and 4, upper panels*). Both cytosolic and mitochondrial fractions were analyzed by immunoblotting for Eps15, a cytosolic marker, and VDAC1. As shown in Fig. 3A (*lower panels*), there was no cross-contamination between the two fractions. Contamination from the nuclear fraction was also excluded by immunoblotting with Sp1, a nuclear marker (not shown).

In CTLL-20 cells, IL-2 induced rapid STAT5 tyrosine phosphorylation that peaked at 5 min and returned to basal level after 1 h [21]. It raises the possibility that IL-2-induced STAT5 mitochondrial translocation may exhibit similar transient kinetics. To test this hypothesis, we isolated mitochondrial fractions from IL-2-deprived and IL-2-stimulated CTLL-20 cells. Mitochondrial STAT5 was detectable within 5 min, peaked at 15 min, and diminished after 1 h (Fig. 3B, *middle panel*). The levels of tyrosine phosphorylation correlated with the amount of STAT5 within mitochondria (Fig. 3B, *top panel*). Anti-VDAC1 immunoblotting confirmed equal loading of mitochondrial proteins (Fig. 3B, *bottom panel*).

To determine if other cytokines have similar effects on STAT5 translocation into mitochondria, we expanded our studies to an IL-3-dependent BaF3 cell line. We showed previously that STAT5 was strongly activated in IL-3-stimulated BaF3 cells [22]. Similar to CTLL-20 cells, mitochondrial STAT5 was only detected in IL-3-stimulated, but not IL-3-deprived BaF3 cells (Fig. 4A, *lower panel*). Anti-phosphotyrosine immunoblotting further confirmed enrichment of phosphorylated STAT5 in the mitochondrial fraction (Fig. 4A, compare *lanes 2 and 4*). These results demonstrate the ability of STAT5 moving into mitochondria in two independent cell lines responding to two distinct cytokines.

Cytokine-induced transient mitochondrial localization has never been reported for STAT3 or other STAT family members. The mechanism underlying STAT3 mitochondrial translocation is not elucidated in the previous report [9]. Like STAT3, STAT5 also lacks discernible mitochondrion-targeting sequences. Unlike STAT3, however, our findings suggest the role of tyrosine phosphorylation in triggering STAT5 mitochondrial translocation. The maintenance of mitochondrial proteins is tightly regulated by both protein import/export machineries and selective proteolysis within the mitochondrion [23,24]. It remains to be determined whether mitochondrial STAT5 is degraded and/or transported back to the cytoplasm during the late phase of cytokine stimulation.

Specificity of cytokine-induced STAT mitochondrial translocation

Tyrosine-phosphorylated STAT3 did not interact with PDC-E2 in LSTRA cells (Fig. 1B). To determine if cytokine could induce STAT3 mitochondrial translocation, we repeated the subcellular fractionation experiment in CTLL-20 cells stimulated with IL-2 that also activates STAT3. As shown in Fig. 3C, STAT3 could not be detected in the mitochondrial fraction of IL-2-stimulated cells (*lane 4*) even though STAT3 is tyrosine-phosphorylated (*lane 2*).

We also examined IFN- γ -induced STAT1 activation in NIH3T3 fibroblasts [15]. While IFN- γ induced tyrosine phosphorylation of cytosolic STAT1 (Fig. 4B, *lane 2*), mitochondrial STAT1 could not be detected in IFN- γ -stimulated cells (Fig. 4B, *lane 4*). Because cytokines induce nuclear translocation of tyrosine-phosphorylated STAT proteins, the absence of tyrosine-phosphorylated STAT1 (Fig. 4B, *lane 4*) and STAT3 (Fig. 3C, *lane 4*) in mitochondria independently verified the purity of our mitochondrial preparation.

Our data support the notion that cytokine-induced STAT mitochondrial translocation is not a general property of all STAT family members. The absence of STAT3 in our mitochondrial preparation is consistent with the stoichiometrical analysis reported by Phillips *et al.* [10]. On the other hand, Wegrzyn *et al.* concluded that STAT3 constitutively localized in the mitochondrion independent of tyrosine phosphorylation [9]. We cannot exclude the possibility that cell type specificity may exist for mitochondrial localization of STAT proteins. Moreover, engagement of IFN- β receptors induces STAT3 tyrosine phosphorylation and active STAT3 upregulates ETC complex I function to stimulate mitochondrial respiration [8]. The absence of tyrosine-phosphorylated STAT3 in the mitochondrion of cytokine-stimulated cells (Fig. 3C) suggests that active STAT3 may indirectly regulate oxidative phosphorylation, such as through nuclear gene regulation.

In vitro DNA-binding activity of mitochondrial STAT5

There is extensive crosstalk between nuclear and mitochondrial gene regulation [25]. STAT signaling has been implicated in the regulation of metabolic genes through transcriptional profiling analyses [26,27]. It is not known whether STAT proteins directly participate in modulating the expression of mitochondrial genome encoding 13 ETC proteins. Numerous mitochondrial proteins associate with mitochondrial DNA to form an organized nucleoprotein particle called nucleoid [28]. PDC-E2 is a component of mitochondrial nucleoids in higher eukaryotes and interacts with mitochondrial transcription factor in co-immunoprecipitation [29]. The specific association between STAT5 and PDC-E2, therefore, raises the possibility that STAT5 may bind to mitochondrial DNA and regulate its expression.

Transcription of mitochondrial genes initiates from the control region (D-Loop) that contains a potential STAT5-binding site [30]. To determine whether mitochondrial STAT5 could bind DNA, mitochondrial extracts prepared from BaF3 cells were analyzed by EMSA. IL-3 strongly induced binding of mitochondrial STAT5 to both consensus STAT5 site (Fig. 4C, *lane 2*) and the putative STAT5-binding site in mitochondrial DNA (Fig. 4C, *lane 5*). Interestingly, anti-STAT5 antibody supershifted consensus STAT5 site-binding activity (Fig. 4C, *lane 3*) and reduced mitochondrial DNA-binding activity (Fig. 4C, *lane 6*). Similar results were observed in IL-2-stimulated CTLL-20 cells (not shown). It suggests that tyrosine-phosphorylated STAT5 may bind to mitochondrial DNA in a different conformation.

Previous studies suggest that mitochondrial STAT3 exerts its functions without affecting mitochondrial gene expression [9,12]. It was not addressed in those reports, however, if mitochondrial STAT3 acquired the ability to regulate mitochondrial DNA expression in cells stimulated with growth factors or cytokines. The stoichiometry of STAT5 and PDC-E2 in the mitochondrion remains to be determined in cell culture and animal tissues. As a transcriptional regulator, mitochondrial STAT5 at a low concentration may be sufficient to exert its function. The alternative, but not mutually exclusive, hypothesis is that STAT5 may regulate PDC-E2 activity via direct interaction. PDC proteins are the key enzymes in mitochondria that convert pyruvate into acetyl-CoA to be used in the citric acid cycle and subsequent oxidative phosphorylation. Therefore, altered PDC activity may also contribute to the metabolic shift observed in cancer cells and cytokine-stimulated cells.

Conclusions

In summary, we reported here a novel interaction between a transcription factor STAT5 and a metabolic enzyme PDC-E2 in the mitochondrion. It is distinct from the previously reported mitochondrial STAT3 because STAT5 translocation into mitochondria can be regulated by cytokines and involves tyrosine phosphorylation. Furthermore, mitochondrial STAT5 exhibits unique DNA-binding activity. The presence of mitochondrial STAT5 in tumor cells and cytokine-stimulated cells also coincides with their metabolic shift toward aerobic glycolysis. All together, our finding opens a new avenue in studying how STAT signaling integrates with mitochondrial biogenesis to modulate cellular metabolism both in cells responding to growth stimuli and in cancer cells.

Abbreviations used

STAT	signal transducer and activator of transcription
PDC-E2	E2 component of pyruvate dehydrogenase complex
ETC	electron transport chain
IFN	interferon
IL	interleukin
PBS	phosphate-buffered saline
VDAC1	voltage-dependent anion-selective channel protein 1
Eps15	epidermal growth factor receptor substrate 15
EMSA	electrophoretic mobility shift assay

Acknowledgments

We thank members in Dr. Bala Chandran's lab for their assistance in immunofluorescence microscopy and Dr. Kwang-Poo Chang for his comments on the manuscript. We would also like to give our special thanks to Dr. Steven J. Burakoff (Mount Sinai Medical Center, New York, NY) for reagents and technical support. This work was supported in part by National Institutes of Health Grant CA107210 and RFUMS H.M. Bligh Cancer Research Fund (to C.L.Yu).

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Fig. 1. Identification of PDC-E2 as a novel STAT5-interacting protein in LSTRA cells. (A) Whole cell lysates prepared from BYDP and LSTRA cells were first normalized for total proteins, and then immunoprecipitated (IP) using anti-STAT5 antibody. Proteins in the immunoprecipitates were subjected to SDS-PAGE and visualized by silver staining. (B) Normalized LSTRA whole cell lysates were immunoprecipitated with anti-STAT5, anti-STAT3 or control antibody, followed by SDS-PAGE, and visualized by silver staining. (C) BYDP and LSTRA whole cell lysates were normalized for total proteins, immunoprecipitated using anti-STAT5 antibody, followed by immunoblotting (IB) using antibodies specific for STAT5 and PDC-E2. The *arrowheads* mark the positions of STAT5, STAT3, PDC-E2, and immunoglobulin heavy chain (IgH) in the silver stain.

Fig. 2.

Mitochondrial localization of STAT5 in LSTRA cells. (A) LSTRA cells were either lysed directly to make whole cell lysate (WCL) or subjected to subcellular fractionation. Proteins in the whole cell lysates, cytosolic (Cyto), and mitochondrial (Mito) fractions were immunoprecipitated using anti-STAT5 antibody and then analyzed by SDS-PAGE with silver staining. (B) LSTRA cells were stained for mitochondria (*red*) and STAT5 (*green*). The two-color merge was overlaid with DAPI staining (*blue*) to visualize the nuclei. The *arrowheads* indicate STAT5 localized in mitochondria (*yellow*). (C) Mitochondrial fractions were prepared from equal numbers of Jurkat T cells and LSTRA cells. A small fraction of solubilized proteins were analyzed by anti-VDAC1 immunoblotting. Proteins in the remaining lysates were immunoprecipitated with anti-STAT5 antibody, followed by immunoblotting using antibodies specific for phosphotyrosine (P-Tyr) and STAT5.

Fig. 3.

IL-2-induced mitochondrial translocation of STAT5 but not STAT3. (A) Equal number of IL-2-deprived CTLL-20 cells were either left unstimulated (–) or stimulated with IL-2 for 15 min (+). A small fraction of cytosolic and mitochondrial proteins were analyzed by immunoblotting using antibodies specific for Eps15 and VDAC1. Proteins in the remaining lysates were immunoprecipitated with anti-STAT5 antibody, followed by anti-phosphotyrosine and anti-STAT5 immunoblotting. (B) Equal numbers of IL-2-deprived CTLL-20 cells were either left unstimulated (0) or stimulated with IL-2 for 5 min (5') to 60 min (60'). Aliquots of normalized mitochondrial lysates were analyzed by anti-VDAC1 immunoblotting. Proteins in the remaining lysates were immunoprecipitated with anti-STAT5 antibody, followed by anti-phosphotyrosine and anti-STAT5 immunoblotting. (C) Proteins in fractionated CTLL-20 were prepared as described in (A), subjected to anti-STAT3 immunoprecipitation, and followed by anti-phosphotyrosine and anti-STAT3 immunoblotting. The *arrowhead* on the left indicates the correct position of STAT3.

Fig. 4.

In vitro DNA-binding activity of mitochondrial STAT5 upon IL-3 stimulation. (A) Equal numbers of IL-3-deprived (–) and IL-3-stimulated (+) BaF3 cells were fractionated to prepare cytosolic and mitochondrial proteins. STAT5 immunoprecipitates were subjected to anti-phosphotyrosine and anti-STAT5 immunoblotting as described above. (B) Equal numbers of serum-starved NIH3T3 fibroblasts were either left unstimulated (–) or stimulated (+) with IFN- γ . Proteins in the cytosolic and mitochondrial fractions were immunoprecipitated with anti-STAT1 antibody, followed by immunoblotting with anti-phosphotyrosine and anti-STAT1 antibodies. (C) Mitochondrial proteins were extracted from IL-3-deprived (–) and IL-3-stimulated (+) BaF3 cells. Normalized mitochondrial extracts were incubated with radiolabeled consensus STAT5-binding oligonucleotides (lanes 1–3) or mitochondrial D-loop oligonucleotides containing a potential STAT5-binding site (lanes 4–6), and then subjected to gel shift experiments. Supershift assays were performed in the presence of anti-STAT5 antibody (lanes 3 and 6). The arrowheads on the right indicate the positions of DNA-protein complexes. The bracket on the right denotes the position of supershifted DNA-protein complex.