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Short Communication

Prolonged Activation of Transcription Regulating Factors in *Trypanosoma brucei brucei* Nuclear Proteins by Interferon-γ Stimulation

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Summary. In African trypanosomiasis the host-derived cytokine interferon-γ (IFN-γ) has been identified as a potent growth promoter of the causative agent *Trypanosoma brucei brucei (T.b.b.)*. The mechanism of growth promotion involves activation of tyrosine protein kinases (TPKs). In the present study, it is shown that in contrast to the situation in multicellular eukaryotic organisms, IFN-γ-stimulated TPKs in *T.b.b.* do not activate transcription factors (TFs) of the signal transducers and activators of transcription (STAT) family, but they activate the TF AP-1, and transcription regulating factors, which bind to E74 (ETS-like proteins) and to hSIE (STAT-like proteins), respectively. Prolonged activation of the transcription regulating factors was determined by electrophoretic mobility shift assay (EMSA) of IFN-γ stimulated *T.b.b*. In addition, c-fos, a component of the transcription factor AP-1, was detected immunocytochemically and by SDS-PAGE with subsequent Western blotting after IFN-γ stimulation of the trypanosomes. The findings support the reported growth enhancing properties of IFN-γ on trypanosomes and for the first time identify transcription regulating factors, which may be selectively involved in IFN-γ- dependent responses of protozoa.

Key words: IFN-γ, transcription factor, *Trypanosoma brucei.*

Abbreviations: AP-1 - activated protein-1, EMSA - electrophoretic mobility shift assay, ETS - external transcribed spacer, hSIE - high-affinity serum-inducible element, IFN-γ - interferon gamma, NF-κB - nuclear factor protein-κB, NO - nitric oxide, PARP - procyclic acidic repetitive protein, PBS - phosphate buffered saline, STAT - signal transducers and activators of transcription, *T.b.b.* - *Trypanosoma brucei brucei*, TFs - transcription factors, TLTF - T lymphocyte triggering factor, TPKs - tyrosine protein kinases, VSG - variant surface glycoprotein.

INTRODUCTION

Trypanosoma brucei is an extracellular hemoflagellate protozoan parasite creating major health problems in sub-Saharan Africa. It is the causative agent of human African trypanosomiasis or sleeping sickness, and 'nagana' disease in animals. The parasites multiply freely in the blood of their infected hosts and are capable of evading the host immune system by switching their outer body surface protein (Turner and Barry 1989). *Trypanosoma brucei* infection induces a massive polyclonal activation of B-lymphocytes, generalized immunosuppression with multiple changes in the cells of the lym-

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phoid tissues, and induction of suppressive T-cells and macrophages (Askonas *et al.* 1979). In addition, early induction of various other immune mediators such as nitric oxide (NO) (Sternberg and McGuigan 1992) and IFN-γ (Olsson *et al*. 1991) are also associated with the infection.

IFN-γ is an important cytokine for the host response against different infections, especially viral infections. However, recent studies demonstrated that in *Trypanosoma brucei* infection this cytokine plays an important role for the benefit of the trypanosomes. It was shown that IFN-γ could stimulate the growth and proliferation of *Trypanosoma brucei brucei* (*T.b.b.*) *in vitro* and *in vivo* (Bakhiet *et al*. 1996). A parasite-released protein called T lymphocyte triggering factor (TLTF) induces $CD8^+$ T lymphocytes to produce IFN-γ (Olsson *et al*. 1991, 1993; Bakhiet *et al*. 1996). The gene for this protein was cloned and the recombinant version was shown to induce IFN-γ secretion by $CD8^+$ but not $CD4^+$ T lymphocytes (Vaidya *et al*. 1997). The mechanism of IFN-γ activation of *T.b.b* was studied step-wise. A direct effect of IFN-γ on *T.b.b.* was the rapid and strong tyrosine phosphorylation detected in *T.b.b.* lysates by Western blotting after five minutes of stimulation (Mustafa *et al*. 1997). Downstream components of the signal pathway, especially transcription factors (TFs), transcription regulating factors responsible for *T.b.b.* growth promotion and TLTF expression, have not been identified to date.

Gene transcription in *T.b.b.* has been studied extensively in order to understand the molecular events leading to the expression of different variant surface glycoprotein (VSG) genes. These studies involved gene expression for VSG and procyclic acidic repetitive protein (PARP). In contrast to most eukaryotic cells, transcription in trypanosomes appears to encompass more than one gene in a polycistronic way of gene transcription that generates many pre-mRNAs carrying the information for more than one protein (Johnson *et al*. 1987). Three trypanosomal RNA polymerases analogous to eukaryotic RNA polymerases direct gene transcription in these primitive eukaryotes (Marchetti *et al*. 1998), and the gene expression of VSG and PARP are directed by RNA polymerase I (Clayton *et al*. 1990, Zomerdijk *et al*. 1991, Rudenko *et al*. 1992). It was also demonstrated that two reactions are required for processing the pre-mRNA in the trypanosomes, *trans*-splicing to add the cap at the 5' end, and completing the 3' end by polyadenylation (Murphy *et al*. 1986).

Transcription in eukaryotes involves the assembly of DNA binding proteins called TFs and other transcription regulating factors. TFs are proteins that bind to *cis*-acting elements of DNA and guide the binding of RNA polymerase II to start the transcription of specific mRNA (Szabo *et al*. 2000).

MATERIALS AND METHODS

Animals, infection and preparation and cultivation of *T.b.b*

Five DA rats were injected intraperitoneally (i.p.) with 10^5 of *T.b.b.* variable antigen type An Tat 1/1, derived from monomorphic stabilizer EATRO 1125 (WHO central serum bank for sleeping sickness, Antwerp, Belgium). Six days later, blood was collected from the rats by heart puncture in heparin-containing glass tubes. Blood was diluted 3:1 in phosphate buffered saline (PBS) supplemented with 1% glucose, pH 8.0 (PBSG). The suspension was centrifuged at 1,400 rpm for 10 min at 4°C. Trypanosomes were collected from the interface layer. PBSG- equilibrated DEAE columns (Pharmacia, Uppsala, Sweden) were used to separate *T.b.b.* from the remaining red blood cells.

Trypanosomes were collected on ice and their final concentration was adjusted to $5x10^8$/ml. Subsequently, they were incubated in RPMI (GIBCO BRL, Paisley, UK) supplemented with 10% foetal bovine serum (FBS) (GIBCO) for 6 h at 37°C in order to inhibit the *in vivo* activation of the IFN-γ-induced signal pathways.

Trypanosoma brucei brucei were then washed and their concentration was adjusted to $8x10^6$ per ml. They were stimulated with 400U/ml recombinant rat IFN-γ for 15, 60 or 240 min, respectively. A control group remained unstimulated. The stimulation was terminated and *T.b.b.* were recovered by centrifugation at 14,000 rpm. The supernatant was discarded and the pellet was snap-frozen in liquid nitrogen and stored at -85°C.

Infections of the rats are conducted in accordance with accepted ethical practice approved by the ethical committee at Karolinska Institute.

Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared essentially as described (Andrews and Faller 1991). For EMSA analysis nuclear proteins corresponding to 1×10^6 trypanosomes were incubated with 16 fMol double stranded oligonucleotides containing consensus binding sites for the transcription factors NF-κB (5'-AGT TGA GGG GAC TTT CCC AGG C-3') and AP1 (5'-CGC TTG ATG ACT CAG CCG ATC-3') as well as hSIE for STAT-like factors (5'- GTC GAC ATT TCC CGT AAA TCG TCG A) and E74 for ETS-like factors (5'-GAA TAA CCG GAA GTA AC-3'). The oligonucleotides were end-labeled with [γ-^{32}P]-ATP (Amersham Pharmacia Biotech, Freiburg, Germany) by polynucleotide kinase. The shift assays were performed in a total volume of 20 µl of the following buffer: 10 mM Tris-HCl, pH 7.5; 50 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mg/ml bovine

serum albumin (BSA), 5% glycerol, 0.1% NP40, 1 µM Pefabloc (Roche Molecular Biochemicals, Mannheim, Germany). The reactions, also containing poly(dI-dC) (1 mg/ml; Roche), were performed at room temperature for 30 min and initiated by the addition of nuclear extract. Complexes were analyzed by electrophoretic separation on a 6% polyacrylamide gel in 0.25 × TBE buffer. Dried gels were exposed to X-ray film for autoradiographic analysis.

Immunocytochemical staining of IFN-γ stimulated *T.b.b.*

Trypanosoma brucei brucei were purified from rat blood as described above and cultured at 37°C for 6 h. They were then stimulated with 400 U/ml recombinant rat IFN-γ, for 15, 60 or 240 and 480 min, respectively, or were un-stimulated (control group). They were mounted on gelatine-coated slides and air-dried. *T.b.b.* were fixed with 6% paraformaldehyde in PBS for 30 min, and after 4 washes in 0.1% PBS/saponin endogenous peroxidase was blocked using an avidin-biotin blocking kit (Vector Laboratories Inc. Burlingame, CA). Slides were then blocked in 10% normal goat serum (1h at room temperature) before challenge with 2.5 µg/ml of anti-c-fos polyclonal antibody (Santa Cruz Biotechnology Inc., CA, USA). After 4 washes in PBS, biotinylated anti-rabbit antibody (Jackson ImmunoResearch Laboratories, PA, USA) at a dilution of 1:300 was applied. The slides were incubated with avidin-biotin-peroxidase complex (Vector). Staining was performed using a DAB-staining kit (Vector). As control, primary antibodies were omitted or irrelevant primary antibody was used.

SDS-PAGE and Western blotting of IFN-γ stimulated *T.b.b.* lysates

Using the same conditions of stimulation as described above, *T.b.b.* lysates were prepared from the pellet by adding lysis buffer containing 20 mM TRIS-HCl (pH 7.5), 137 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 10% glycerol, 2 mM EDTA, 1 mM phenylmethylsulphonylfloride (PMSF), 0.15 U/ml aprotinin, and 1 mM sodium orthovanadate. After 3-4 min on ice supernatants were removed and kept at -80°C. The protein content in the cytoplasmic extracts was determined using the protein assay of BioRad Hercules, CA). The samples from the cytoplasmic extracts were fractionated by SDS-PAGE. The separated proteins were transferred electrophoretically to a nitrocellulose membrane (BioRad). Membranes were blocked in 5% dry milk in TBST - to prevent non specific binding - in a shaker for 1 h. The primary antibody polyclonal anti-c-fos (diluted 1:1000) was added for 1h. After washing, the membranes were incubated for 1h with the secondary antibody (horseradish peroxidase-conjugated anti-rabbit immunoglobulins (DAKO A/S, Glostrup, Denmark, diluted 1:2000). The protein was detected using enhanced chemiluminescence system (Amersham Pharmacia Biotech) on an autoradiographic film.

RESULTS

EMSA investigation shows that the TFs AP-1 and NF-κB, an ETS-like factor binding to E74 and a STAT-like factor binding to hSIE are all constitutively expressed in the nuclei of the bloodstream form of *T.b.b.* at low levels, as depicted in Fig. 1. The expression of AP-1, E74 and hSIE was up-regulated after 15 min (for AP-1 and E74) to 60 min (for hSIE) of IFN-γ stimulation, whereas NF-κB expression remained at the same level. Maximum expression of AP-1as observed in the long and short exposure, E74 and hSIE was detected after 240 min of IFN-γ stimulation. STAT expression was not detectable by the applied method (data not shown). The expression of the classical immediate early gene c-fos, a component of the AP-1 family, was further investigated by immunocytochemistry and Western blot. Immunocytochemical staining demonstrated the translocation of c-fos to trypanosome nuclei (Fig. 2). In the unstimulated trypanosomes c-fos staining was only observed in the cytoplasm (Fig. 2B), while upon IFN-γ stimulation this factor started to be recruited to the nucleus (Figs 2C, D). The expression pattern of c-fos in Western blot of *T.b.b.* lysates shown in Fig. 3 confirms the suggested activating properties of IFN-γ for this factor.

DISCUSSION

In the present study we investigated the changes caused by IFN-γ stimulation of *T.b.b.* with regard to the expression of the TFs: AP-1 and NF-κB as well of the DNA binding-sites E74 for a ETS-like factor and hSIE for a STAT-like factor.

In eukaryotes, the assemblies of TFs bound to DNA direct RNA polymerase binding to initiate mRNA synthesis. RNA polymerases alone cannot bind efficiently to DNA. TF activities are regulated through different mechanisms such as phosphorylation (de Groot *et al*. 1993). In addition, transcription activity is influenced by several other DNA-binding proteins like those binding to E74 and hSIE sites. An important physiological function of these factors is the control of cell proliferation, achieved through their ability to regulate the expression and function of cell cycle regulators (Shaulian and Karin 2001).

Transcription in *T.b.b.* differs in some aspects from those in multicellular eukaryotes (Marchetti *et al*. 1998). Three types of RNA polymerases direct transcription in the parasite, but details of this process are still unclear. In the present study we demonstrate DNA binding activities of three transcription regulating factors among the nuclear proteins of IFN-γ-stimulated trypanosomes, which are not primarily involved in IFN-γ dependent signalling in multicellular eukaryotes, whereas the con-

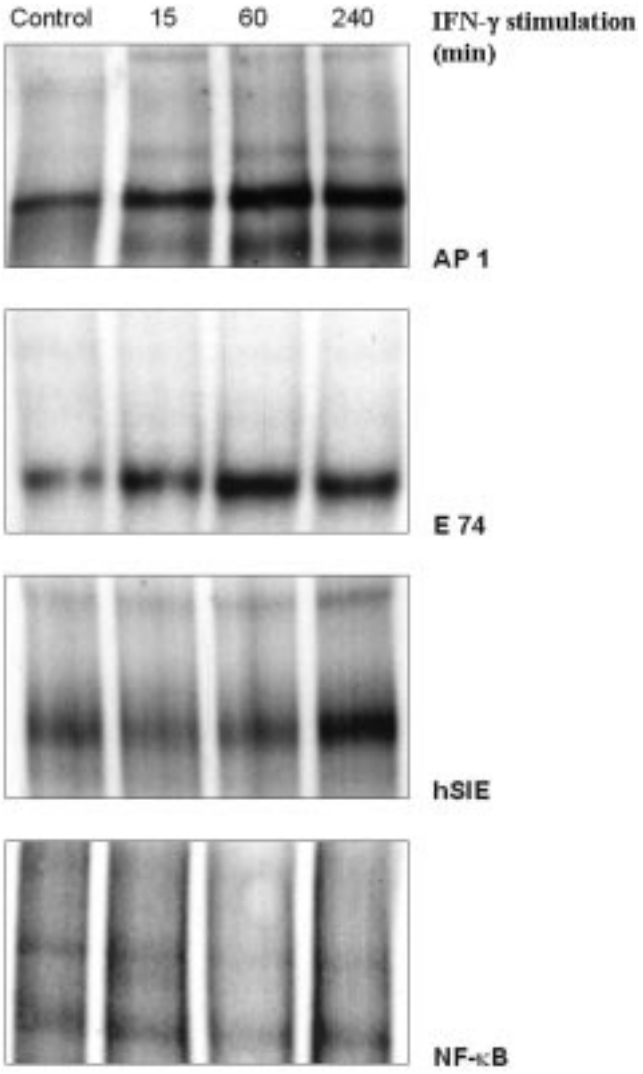

Fig. 1. DNA binding activities of AP-1, E 74, hSIE, and NF-κB are detected by EMSA using nuclear proteins of IFN-γ stimulated *Trypanosoma brucei brucei* Time points indicate time (min) of exposure of *T.b.b.* to IFN-γ after the parasites were adapted to the culture medium for 6h in order to inhibit *in vivo* activation of signal pathways.

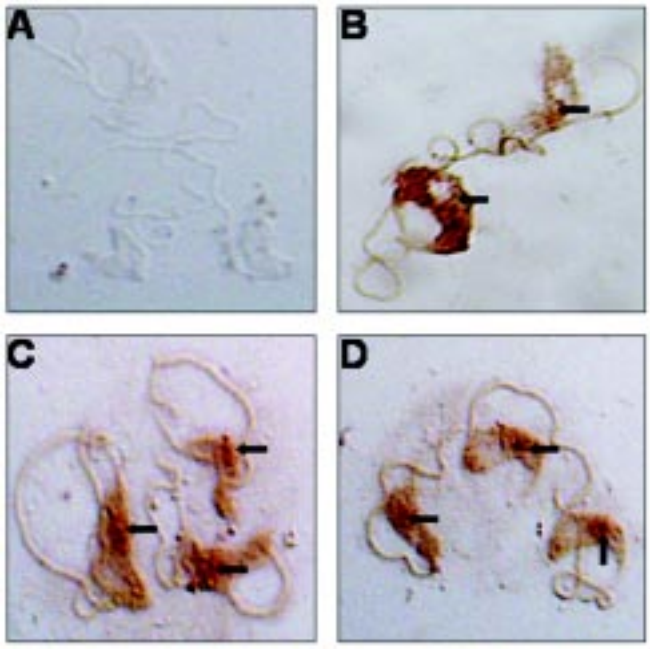

Fig. 2. Detection of c-fos by immunocytochemical staining of IFN-γ stimulated *Trypanosoma brucei brucei* **A:** depicts the negative control omitting the primary antibody. **B:** depicts the expression of c-fos under normal growth condition of the unstimulated trypanosomes. **C:** demonstrates the recruitment of c-fos to the nuclear area. **D:** shows the translocation of c-fos to *T.b.b.* nuclei, which are indicated by arrows.

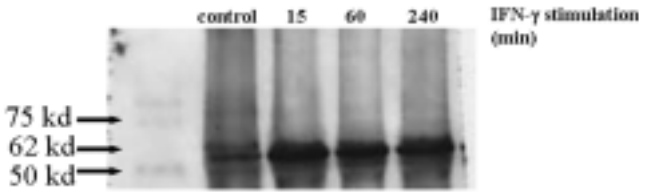

Fig. 3. Western blot detection of c-fos in *Trypanosoma brucei brucei.* The 62kD band represents c-fos protein expression after the indicated time (min) of exposure of the parasites to IFN-γ. The parasites were previously adapted to the culture medium for 6 h in order to inhibit *in vivo* activation of signal pathways.

ventional TFs of the IFN-γ induced signal pathway STAT 1 and 2 were not detected (data not shown). Out of the detected transcription regulating factors AP-1 comprises a family of TFs consisting of homodimers and heterodimers of Jun and Fos, and has been shown to control rapid responses of mammalian cells to stimuli that induce proliferation, differentiation, and transformation (Sharma and Richards 2000). E74 is the DNA binding site of a member of the external transcribed spacer ETS family. The physiological functions of this family were first characterized during *Drosophila* development. DNA binding activity to E74 is induced by the hormone 20-hydroxyecdysone (ecdysone). This activity enhances changes in gene transcription, cell physiology, and tissue organization (Hsu and Schulz 2000). The high-affinity serum-inducible element hSIE and the TF nuclear factor protein-κB NF-κB are involved in different cellular processes such as cell growth, development and apoptosis. NF-κB is induced to control a variety of physiological aspects of immune and inflammatory responses. This protein is regulated by the interaction between Rel and IκB.

The prolonged up-regulation of AP-1 suggests that it might play a role in trypanosome developmental pro-

cesses, since it was reported to control proliferation and differentiation (Sharma and Richards 2000). As it was shown by EMSA the factor was activated to bind to trypanosomes DNA in IFN-γ stimulated parasites, while this activation was not observed in unstimulated parasites, suggesting a role for the cytokine IFN-γ in enhancing the gene transcription process in the trypanosomes.

IFN-γ is a potent activator of the STAT signalling pathway in multicellular eukaryotic cells (Alsayed *et al*. 2000), and it induces a rapid and strong tyrosine phosphorylation in *T.b.b.* (Mustafa *et al*. 1997). Our findings suggest that subsequent to tyrosine phosphorylation the transcription regulating factors AP-1, ETS-like factor (binding to E74), and STAT-like factor (binding to hSIE), but not NF-κB are induced in *T.b.b.* In multicellular eukaryotic cells the binding of IFN-γ to its stimulated response element ISREs induces the expression of different factors, which initiate gene transcription for this cytokine or other proteins (Imam *et al*. 1990). The main signal pathway induced by IFN-γ in multicellular eukaryotes is the Janus kinase (JAK)-STAT pathway, for which no evidence has been found in IFN-γ stimulated *T.b.b.* to date. The induction of the transcription regulating factors AP-1, ETS-like factor (binding to E74) and STAT-like factor (binding to hSIE) in *T.b.b.* was weaker, but prolonged as compared to the common TF induction in multicellular eukaryotes. Their prolonged up-regulation may compensate for the lower level of factor expression in order to achieve efficient mRNA production of IFN-γ-induced target genes.

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