Nuclear levels of NF-κB correlate with syncytium-forming capacity of 8e51 cells, expressing a defective HIV virus

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Abstract The double NF-κB site identified in the LTR of the human immunodeficiency virus-1 (HIV-1) has been demonstrated to be necessary for efficient viral transcription. In this report we present the characterisation of NF-κB subunits engaged in complexes binding to the HIV-1 NF-κB site in human 8e51 T-cells, that harbour a defective HIV-1. At least four different specific NF-κB complexes are present in the nucleus of these cells. With the use of specific antibodies we have determined the composition of each complex using electrophoretic mobility shift assays. The results show the presence of several NF-κB family members, with the transactivating RelA being engaged in multiple complexes. The importance of NF-κB complexes in viral functions has been established comparing the level of NF-κB DNA-binding complexes with syncytia-forming activity of 8e51 cells. In fact, 8e51 cells that had almost lost their syncytia-forming capacity were found to contain at least 10 times less active NF-κB DNA-binding complex than the actively fusing cells. The correlation is specific as the level of at least three other transcription factors did not change.

Key words: NF-κB; HIV; Transcription

1. Introduction

Several *cis*-acting elements in the LTR of the human immunodeficiency virus-1 (HIV-1) influence viral gene expression by interacting with viral or cellular proteins. Regulatory motifs (Sp1, TATA) bind cellular proteins that are constitutively expressed in most cells [1,2]; these sequences appear to be critical for basal HIV gene expression. Other LTR elements (NF-κB) interact with factors synthesised or activated in lymphocytes or macrophages [3]. The interaction of virus-encoded proteins with cellular proteins results in productive infection of the HIV-1. Several reports have demonstrated the importance of the binding of NF-κB to the HIV-1 LTR [3–6]. HIV-1 infection of human T lymphocytes results in a long latency period, which is followed by clinical manifestations when the virus recovers the transcription and replication activity. Cytokines such as TNF-α and -β have been shown to induce HIV-1 expression in latently infected T lymphocytes as a function of their ability to activate NF-κB [7]. A Jurkat derived T cell line chronically infected with an infectious clone of HIV-1 deleted of the NF-κB-binding site in the LTR failed to respond to TNF-α stimulation in terms of increased virus expression [8].

The transcription factors of the Rel-NF-κB family bind to κB elements in several target genes involved in immediate early events of the immune, inflammatory and acute phase responses, as well as to the enhancers of viruses such as HIV-1 and SV40. Members of the mammalian NF-κB/Rel family include NFKB1 (p50), NFKB2 (p52), RelA (p65), cRel and RelB [9]. These proteins are capable of homo- and hetero-dimerization, forming complexes with distinctive transcriptional activity, DNA sequence specificity, and cell type distribution [9]. In most cell lines, NF-κB proteins are present in a cytosolic inactive state in complex with inhibitor proteins of the IκB-family of which there are at least five different species in vertebrates [9]. IκB proteins prevent nuclear translocation of NF-κB and inhibit DNA binding of NF-κB complexes [10,11]. The role of the individual NF-κB dimers active in HIV-1 transcriptional regulation is not clear: first, only some members of the family possess a transactivation domain and, secondly, different complexes have different activity on different NF-κB sites [12].

To obtain more precise information on the cell/viral protein interactions occurring during a productive HIV-1 infection, we have analysed an HIV-1-positive cell line and correlated Rel-related proteins with HIV-1 expression. We have chosen the 8e51 cell line [13] that was derived from an HIV-infected continuous human T cell line (A3.01). The 8e51 cell line has a single integrated copy of a *pol* mutant-HIV-1, which does not replicate, expresses all viral proteins except p64 and hence produces non-infectious viral particles [13]. The use of this cell line therefore allows an analysis of HIV-1 transcription in the absence of viral replication, possibly simplifying the interpretation of the results. These cells, when co-cultured with a $CD4^+$ lymphoblastic T cell line, will produce syncytia, an indication of viral gene expression. Furthermore, this cell line undergoes occasional crisis in the expression of viral proteins so that it no longer forms syncytia, an event not yet correlated with any discernible morphological or physiological change in the cell population. This peculiar phenotype can thus be used to correlate the presence of cellular transcription factors with functional virus expression.

2. Materials and methods

2.1. Cell cultures and transfection conditions

8e51 cells and MOLT-3 ($CD4^+$) cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum. HeLa cells were grown in DMEM medium supplemented with 10% calf serum.

2.2. Protein extracts and oligonucleotides for electrophoretic mobility shift assay (EMSA)

Total nuclear extracts were prepared from 8e51 and HeLa cells by standard procedures [14]. Double stranded probes were labelled with ^{32}P with T4 polynucleotide kinase. Binding reactions were carried out in 20 μl of binding buffer containing 10% glycerol, 0.5 mM EDTA, 50 mM KCl, 1 mM $MgCl_2$, 1 mM DTT and 0.5 mM PMSF. After

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incubation of nuclear extracts (10 μg proteins) for 10 min on ice with 2 μg of poly(dI-dC), the probe, the cold competitor and the buffer were added, and incubations were continued for 20 min at room temperature. Samples were then run on a 5% native polyacrylamide gel in 0.25×Tris-borate-EDTA. When antibodies were used the extracts were pre-incubated for 1 h at 4°C with 0.5 μl of each antibody (100 μg/ml). All antibodies were purchased from Santa Cruz Biotechnology (California). Cytosolic extracts were treated with CHAPS and sodium deoxycholate for 10 min on ice to release NF-κB from IκB, as previously described [14]. Oligonucleotides were synthesised with an Applied Biosystem 381 DNA synthesiser. The sequence of the oligonucleotide encompassing the two NF-κB sites of the HIV-1 virus LTR is: TAGGGACTTTCCGCTGGGGACTTTCCAG [3].

The sequence of the o-17 oligonucleotide is: CAGCAATCAGCATGACAGCCTCCAGC [15].

2.3. Syncytium-forming assay

8e51 and MOLT3 cells were collected and washed twice with PBS. Cells were counted and resuspended at 2 million/ml for the MOLT3 and at 1.2 million/ml for the 8e51; 50 μl of each cell suspension was plated in a 96 conical well plate at 37°C for 30 min in 5% CO_2 and then spun for 5 min at 1000 rpm. After 3 more hours at 37°C in 5% CO_2, the cells were transferred in flat-bottom 96-well plate to count syncytia at the inverted microscope [17].

3. Results

3.1. 8e51 nuclear and cytosolic extracts contain specific complexes binding to the HIV-1 NF-κB site

In order to identify which, if any, of the NF-κB family dimers are present in the 8e51 cells we prepared cytosolic and nuclear extracts and used them in a gel mobility shift experiment with an oligonucleotide spanning the HIV-1 NF-κB site (Fig. 1). CHAPS-activated cytosolic extracts showed one very faint and one stronger, faster, retarded band that were competed by excess unlabeled specific oligonucleotide. These two bands had identical mobility to those present in the HeLa cells cytosol, that we previously showed to be the RelA/c-Rel and the RelA/NFKB1 dimers, respectively [14,16]. The 8e51 nuclear extracts showed four distinct bands that were again competed by increasing amount of excess unlabeled oligonucleotide; two other bands of faster mobility were non-specific. These results show that 8e51 cell nuclei contain constitutively activated NF-κB factors.

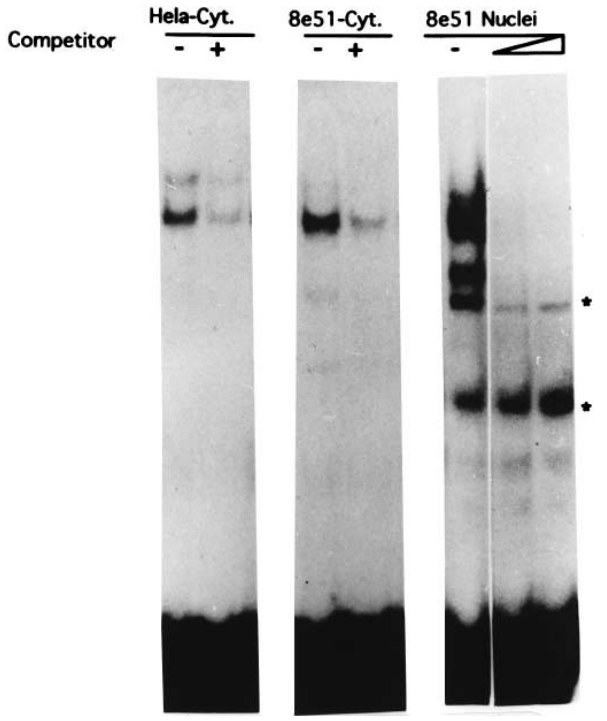

Fig. 1. 8e51 nuclear and cytoplasmic extracts contain specific complexes binding to the HIV-1 NF-κB site. Electrophoretic mobility shift assay (EMSA) with a ^{32}P-labelled HIV-1 NF-κB oligonucleotide. CHAPS activated cytosolic extracts from HeLa cells (first and second lane); cytosolic (third and fourth lanes) and nuclear extracts from 8e51 cells (fifth, sixth and seventh lanes). All lanes: 5 μg proteins. The competitor was the NF-κB oligonucleotide added in a 50- and 100-fold excess (triangle shows increasing amount of cold competitor, sixth and seventh lanes, respectively). The * shows non-specific, that are non-competed bands. Complexes were separated on a 5% polyacrylamide gel.

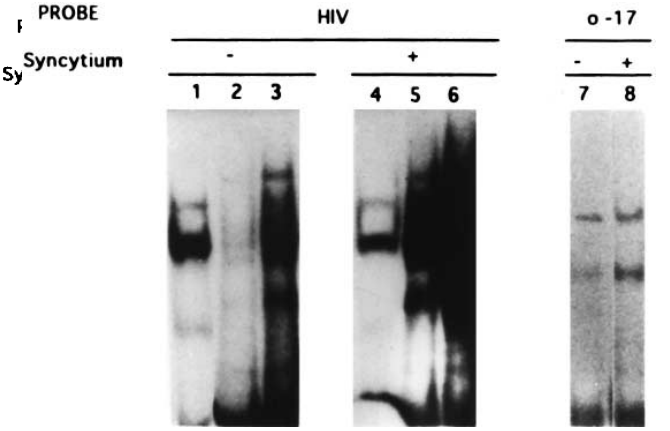

Fig. 2. Immunological characterisation of cytosolic and nuclear complexes binding to the HIV-1 NF-κB site. 8e51 CHAPS-activated cytosolic (lanes 1–5) and nuclear extracts (lanes 6–11) were pretreated with different antibodies prior to the addition of the ^{32}P-labelled HIV-1 NF-κB probe to determine the composition of each of the retarded bands. Lanes 1 and 6: no antibody. Lanes 2 and 7: anti-RelA antibody; lanes 3 and 8: anti-NFKB1 (p50) antibody; lanes 4 and 9: anti-c-Rel antibodies recognizing the amino-terminus of the protein; lane 10: anti-c-Rel antibodies recognizing the carboxyl-terminus of the protein. Lanes 5 and 11: anti-RelB antibody. The * identifies non-specific bands.

3.2. Immunological characterisation of cytoplasmic and nuclear complexes binding to the HIV-1 NF-κB site

To determine the composition of each of the cytoplasmic and nuclear complexes binding to the HIV-1 NF-κB site, we used antibodies specific for each member of the NF-κB family. The two complexes present in the cytosol indeed represent, as previously shown in the HeLa cell cytosol, NFKB1/RelA and RelA/c-Rel (Fig. 2). The slower migrating complex was composed of RelA/cRel dimers since it was supershifted by RelA (lane 2) and inhibited by c-Rel antibodies (lane 4) but was not affected by NFKB1 and RelB antibodies (lanes 3 and 5). The faster migrating band was supershifted by RelA and NFKB1 antibodies (lanes 2 and 3) but not by c-Rel and RelB antibodies (lanes 4 and 5) thus it contained RelA/NFKB1 heterodimers. A similar analysis showed that the four specific complexes present in the 8e51 nuclear extracts (lane 6) are (from top to bottom); RelA homodimers, RelA/NFKB1, NFKB1/RelB heterodimers and NFKB1 homodimers. In fact, the upper band was supershifted by RelA (lane 7) but not other antibodies. The second band (identified on the right as NF-κB) was supershifted by both RelA and NFKB1 antibodies (lanes 7 and 8, respectively), and hence represents the RelA/NFKB1 heterodimer. The third band was affected in its

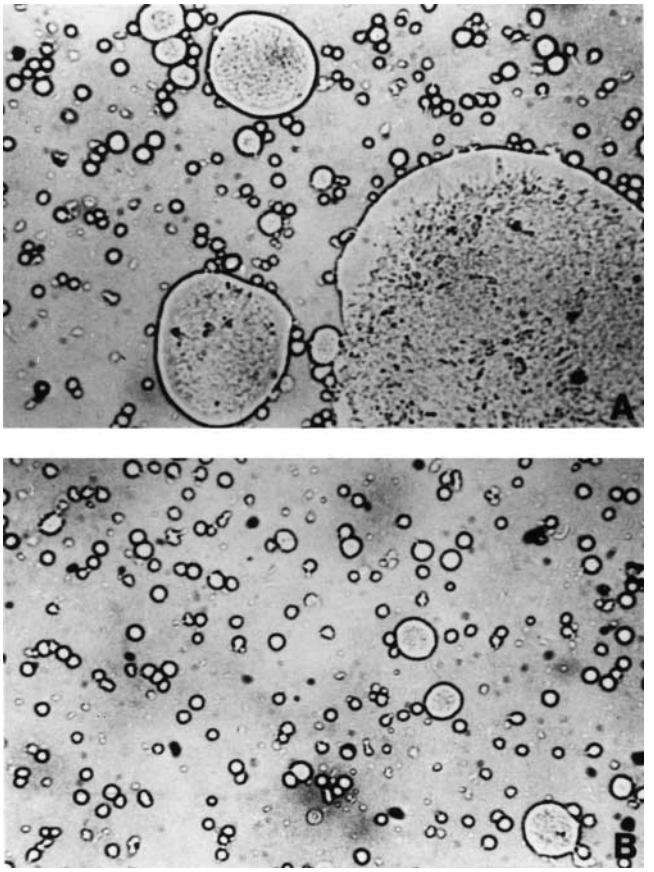

Fig. 3. Syncytium-forming and syncytium-defective activity of 8e51 cells. To monitor HIV-1 gene expression the 8e51 cells were co-cultured with the $CD4^+$ MOLT3 cells; 8e51 cells in which the virus proteins are expressed will form syncytia with the MOLT3 cells (A: syncytium-forming cells) while those in which the viral proteins are not expressed fail to form syncytia with the MOLT3 cells (B: syncytium-defective cells).

migration by NFKB1 and RelB antibodies (lanes 8 and 11, respectively) while the fourth band was supershifted only by the NFKB1 antibody (lane 8). The migration of none of the complexes was affected by c-Rel antibodies raised against either the amino- (lane 9) or the carboxy-terminus (lane 10) of c-Rel.

3.3. Correlation of HIV-1 production in 8e51 cells with the presence of constitutively activated NF-κB

To assess the presence of a transcriptionally competent HIV-1 the 8e51 cell line can be co-cultured with the lymphoblastic T cell line MOLT3 ($CD4^+$). When the two cell types are plated together, the 8e51 cells in which the HIV-1 replicates will produce syncytia with the MOLT3 cells (Fig. 3A), an indication of expression of viral genes [17]. The 8e51 cells, however, undergo periodical crisis in their ability to form syncytia (Fig. 3B). In order to test whether this deficiency correlates with the activity of NF-κB, we compared the DNA-binding activity toward the HIV-1 κB by EMSA of cytosolic and nuclear extracts from syncytium-forming and syncytium-defective 8e51 cells (Fig. 4). Same amount (5 μg) of CHAPS activated cytosolic extracts from the two sets of cells showed no difference in composition and relative abundance of the bands retarded by the HIV-1 probe (lanes 1 and 4). In the case of nuclear extracts, however, when 5 μg of nuclear proteins from the syncytium-defective cells were used, we could not detect any of the specific bands formed with the HIV-1 probe (lane 2) while the same amount of nuclear proteins from syncytium-forming cells gave a very strong signal (lane 5). When the amount of proteins was increased to 15 μg we could detect some binding to the HIV-1 probe from the syncytium-defective cells (lane 3) but the signal obtained with the nuclear extract from syncytium-forming cells was extremely strong (lane 6). To determine whether the decrease of the NF-κB transcription factors observed in syncytium-defective cells was reflecting a general decrease of cellular transcription factors, we tested the same nuclear extracts in a gel shift experiment with an unrelated oligonucleotide (o-17). This sequence binds transcription factors essential for the transcriptional activity of the urokinase gene [15]. When 5 μg of nuclear extracts from syncytium-defective and syncytium-forming cells was compared, essentially no difference was observed (Fig. 4, lanes 7 and 8). These data suggest that the decrease of binding of the NF-κB transcription factors from the syncytium-defective cells to the HIV-1 probe can be cor-

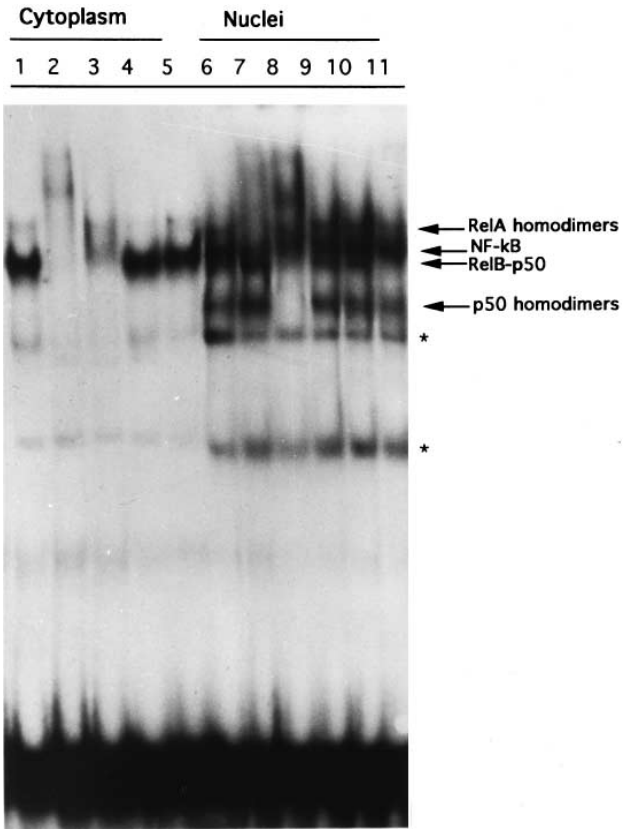

Fig. 4. NF-κB DNA-binding activity of syncytium-forming and syncytium-defective 8e51 cells extracts. Cytosolic and nuclear extracts from syncytium-defective and from syncytium-forming 8e51 cells were compared by EMSA with the ^{32}P-labelled HIV-1 NF-κB oligonucleotide (lanes 1–6) or with a ^{32}P-labelled control o-17 oligonucleotide recognizing the COM-binding proteins of the human urokinase gene (lanes 7 and 8). CHAPS activated cytosolic extracts (5 μg) from syncytium-defective cells (lane 1) and from syncytium-forming cells (lane 4) retarded identical complexes. In nuclear extracts (5 μg) from syncytium-forming cells (lane 5) and from syncytium-defective cells (lane 2) the abundance of the complexes was strikingly different. In lanes 3 and 6, 15 μg of proteins from the nuclear extracts of syncytium-defective and syncytium-forming cells. In the case of control o-17 oligonucleotide 5 μg of nuclear extracts from the two cell types were used (lanes 7 and 8).

related to the inability of the HIV-1 pro-virus to be expressed in these cells, and do not reflect a general decrease of cellular transcription factors.

4. Discussion

Transcription and replication of the HIV-1 virus are intricately tied and binding of the NF-κB family transcription factors to the HIV-1 κB site is crucial for both transcription and replication. While deletions of the κB or Sp-1 sites in the HIV-1 LTR DNA reduce or abolish viral replication depending on the T cell line, the simultaneous deletion of both κB and Sp-1 sites abolishes HIV-1 replication in all target cells [18,19]. NF-κB nuclear factors positively affects transcription of both cellular and viral genes by binding to the enhancers of different transcriptional units. However, different members of each protein family may have subtly different properties that allow fine tuning and cell-specific regulation of transcription. In fact, discrete protein-binding sites function in enhancers as modular units [20,21], which can substitute for one another in a cell-specific way. It is now becoming more and more clear that, although some transcription factors are extremely important or essential for some transcriptional units, cell-specificity of transcription heavily relies on the interaction between different protein factors, differently expressed in different cells. It is therefore essential to know the full complement of relevant transcription factors that are expressed in a given cell line before concluding on the specific role of one given factor.

The long latency periods observed in HIV-1-positive individuals and in infected T cell cultures are among the unsolved problems preventing a complete understanding of AIDS. Obviously, this phenomenon depends on the interaction between the infected cell and the virus, and hence also on the presence or absence of specific transcription factors, or on their balance. To better understand this process and the involvement of cellular components, it is important to define which cellular proteins can affect viral transcription in a given cell. In this paper we have characterised the protein complexes binding to the HIV-1 κB site in a HIV-1-producing cell line.

8e51 cells contain NF-κB proteins in both the cytoplasm and nucleus (Figs. 1 and 2). In the cytoplasm, inactive NFKB1/RelA and RelA/cRel complexes were observed. Nuclear extracts contained high levels of several DNA-binding NF-κB complexes: our data show that RelA and NFKB1 are very prominent, possibly evident as NFKB1/RelA and NFKB1/RelB heterodimers. Interestingly, RelA homodimers also appear to be present.

A specific quantitative difference in NF-κB factors between syncytium-forming and -defective cells was demonstrated (Fig. 4), with an at least 10-fold higher expression in the nuclei of syncytium-forming cells. No difference was noticed on the levels and nature of inactive cytoplasmic components. When we used syncytium-defective nuclear extracts, we could still detect DNA-binding activity to the HIV-1 probe at higher protein concentrations (see Fig. 4). This is probably due to the presence of a minority of the cells population which retains syncytium-forming activity. Indeed during the crisis period, the 8e51 cells do not totally lose their syncytium-forming ability, but only decrease it by about 90% (not shown).

In conclusion, the analysis of the 8e51 cell line while in agreement with the already available information on the potential role of the NF-κB factors in HIV-1 expression expands our knowledge on the NF-κB complexes required for a productive HIV-1 expression. The quantitative difference in NF-κB DNA-binding activities found in syncytium-forming and syncytium-defective cells underlines the importance of these factors in HIV-1 expression.

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