Biochimie 93 (2011) 1592–1600

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

# Structural and functional insights into IκB-α/HIV-1 Tat interaction

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ARTICLE INFO

*Article history:*
Received 7 March 2011
Accepted 24 May 2011
Available online 31 May 2011

*Keywords:*
Surface plasmon resonance
CD spectroscopy
Luciferase assay

ABSTRACT

Protein–protein interactions play fundamental roles in physiological and pathological biological processes. The characterization of the structural determinants of protein–protein recognition represents an important step for the development of molecular entities able to modulate these interactions. We have recently found that IκB-α (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha) blocks the HIV-1 expression and replication in a NF-κB-independent manner by directly binding to the virus-encoded Tat transactivator. Here, we report the evaluation of the entity of binding of IκB-α to Tat through *in vitro* Surface Plasmon Resonance assay. Moreover, by designing and characterizing a set of peptides of the C-terminus region of IκB-α, we show that the peptide corresponding to the IκB-α sequence 262–287 was able to bind to Tat with high affinity (300 nM). The characterization of a number of IκB-α-based peptides also provided insights into their intrinsic folding properties. These findings have been corroborated by mutagenesis studies on the full-length IκB-α, which unveil that different IκB-α residues are involved in NF-κB or Tat recognition.



## 1. Introduction

The innovative concept that proteins exert their function by establishing intricate contact networks rather than acting as independent entities is of outstanding importance both in the comprehension of molecular mechanisms underlying biological processes and in modern drug discovery [1]. Protein–protein interactions are the ensemble of fine tuned recognition events that take place at protein surfaces [2]. These contacts frequently involve large protein surface areas, which comprise several contact sites. Alternatively, they can be mediated by "hot spots" represented by few crucial residues. Recent investigations have highlighted the role of peptide molecules as powerful tools for the characterization [3,4] and the regulation [5,6] of these interactions. Peptides are particularly suitable as models for proteins that are not fully folded in their isolated states but that achieve conformational stability upon the formation of complexes with other partners [7].

Protein interactions play crucial roles in host–pathogen recognition in infectious diseases [8]. In the case of HIV-1 infection, the HIV-1-encoded Tat protein is essentially required for HIV-1 expression and replication through the interaction with the HIV-1 Long Terminal Repeats (LTR) and host cell functions. In fact, Tat increases the HIV-1 gene expression by binding to the trans-activation-responsive region (TAR) at the 5′ leader HIV-1 RNA (nucleotides +1 to +59) and enhances the viral transcription initiation and elongation [9]. In particular, Tat promotes the assembly of the pre-initiation complex and the nucleosomal remodeling through the interaction with several transcription factors and cofactors including TBP [10], NF-κB [11], Sp1 [12] and the histone acetyltransferases p300/CBP and P/CAF

*Abbreviations:* TIS, triisopropylsilane; TFA, trifluoroacetic acid; DMF, dimethylformamide; DCM, dichloromethane; HBTU, 1-H-benzotriazolium, 1-[bis(dimethylamino)methylene]-hexafluorophosphate(1-),3 oxide; HOBt, N-hydroxybenzotriazole; DIEA, di-isopropylethylamine; Fmoc, fluorenylmethoxycarbonyl; TCEP, tris(2-carboxyethyl) phosphine; HPLC, high performance liquid chromatography; LC–MS, liquid chromatography mass spectrometry; EDC, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride; NHS, N-hydroxy-succinimide.

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doi:10.1016/j.biochi.2011.05.025

[13–15]. Furthermore Tat interacts with the cell cycle regulators p53 [16], E2F-4 [17] and p73 [18] and with the enzymes PP-1 [19] and DICER [20].

We have recently found that IκB-α (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha), which is the best characterized member of the Inhibitors of NF-κB (IκB family [21,22]), physically interacts with Tat and determines its nuclear export to the cytoplasm, which results in the inhibition of Tat-dependent HIV-1 expression [18]. These data indicated that IκB-α was a potent repressor of HIV-1 transcription through the inhibition of NF-κB and Tat transcriptional activities, which are both required for optimal trans-activation of the HIV-1 LTR [23–26]. Consistently, recombinant HIV-1 and SIVmac239 retroviruses that expressed the proteolysis-resistant IκB-αS32/36A mutant were highly attenuated in cell culture [27–29]. In this regard, the complex Tat-IκB-α represents an appealing molecular target for the treatment of pathological conditions associated with HIV-1.

The inhibitory activity of IκB-α is signaling-regulated. In unstimulated cells, IκB-α binds extremely tightly to the p50/p65 NF-κB complex preventing its nuclear accumulation and association with DNA [30]. In response to different stimuli, IκB-α is phosphorylated at serines 32 and 36, and subsequently ubiquitinated at lysines 21 and 22, which results in its proteasome-mediated degradation with the release of the NF-κB complex [31,32].

From a molecular point of view, IκB-α is a 36 kDa protein composed of: a) a N-terminal signal response region, where phosphorylation and ubiquitination occur in response to NF-κB activation signals; b) an ankyrin (AR)-rich region made of six ankyrin repeats; c) a C-terminal PEST sequence rich in proline, glutamic acid, serine, and threonine that is involved in basal degradation of free IκB-α [22,33,34] (Fig. 1A). Each AR of IκB-α has a length of approximately 35 residues, and is composed of a β-hairpin followed by two anti-parallel α-helices and a variable loop (Fig. 1B). ARs constitute a protein–protein interaction domain that is shared by different proteins with highly varied functions [35,36]. In fact, IκB-α binds to the NF-κB dimer via its AR domain (spanning residues 67–287) interfering with the NF-κB interaction with DNA. The binding affinity of IκB-α to p50/p65 hetero- and p65/p65 homo-dimers is in the picomolar range, and recent studies revealed that binding interface has two hot spots one at either end of the interface [37,38]. Notably, in contrast to its remarkable stability in the NF-κB-bound state, free IκB-α is intrinsically unstable and is rapidly degraded through a process that does not require phosphorylation and ubiquitination [39]. In free state, ARs 1–4 of IκB-α are compactly folded while ARs 5–6 are presumably weakly folded and highly flexible; however, ARs 5–6 adopt a fully folded conformation when IκB-α binds to NF-κB [40–42]. The coupled folding and binding of ARs 5–6 in IκB-α has been proposed to modulate the binding affinity of IκB-α to NF-κB [43] as well as the switch between the basal and stimulated degradation mechanisms [41]. Furthermore, ARs 5–6 appear to be involved in the mechanism by which IκB-α increases the dissociation rate of NF-κB from the DNA [44].

In this study, we report a quantification of the binding between IκB-α and Tat proteins through *in vitro* Surface Plasmon Resonance (SPR) experiments. Moreover, we designed and characterized IκB-α-based peptides that are able to bind Tat with high affinity. The characterization of IκB-α peptides has provided novel insights into the intrinsic properties of IκB-α fragments and structural determinants of IκB-α/Tat recognition. These findings have been corroborated by mutagenesis studies of full-length IκB-α protein.

## 2. Materials and methods

### 2.1. Protein and peptide synthesis

Reagents for peptide synthesis (Fmoc-protected amino acids and resins, activation and deprotection reagents) were from Novabiochem (Laufelfingen, Switzerland) and InBios (Napoli, Italy). Solvents for peptide synthesis and HPLC analyses were from Romil (Dublin, Ireland); reversed phase columns for peptide analysis and the LC–MS system were from ThermoFisher (Milan, Italy). Solid phase peptide syntheses were performed on a fully automated multichannel peptide synthesizer Syro I (Multisyntech, Germany). Preparative RP-HPLCs were carried out on a Shimadzu LC-8A, equipped with a SPD-M10 AV detector and with a Phenomenex C18 Jupiter column (50 × 22 mm ID; 10 μm). LC–MS analyses were carried out on a LCQ DECA XP Ion Trap mass spectrometer equipped with a OPTON ESI source, operating at 4.2 kV needle voltage and 320 °C with a complete Surveyor HPLC system, comprised of MS

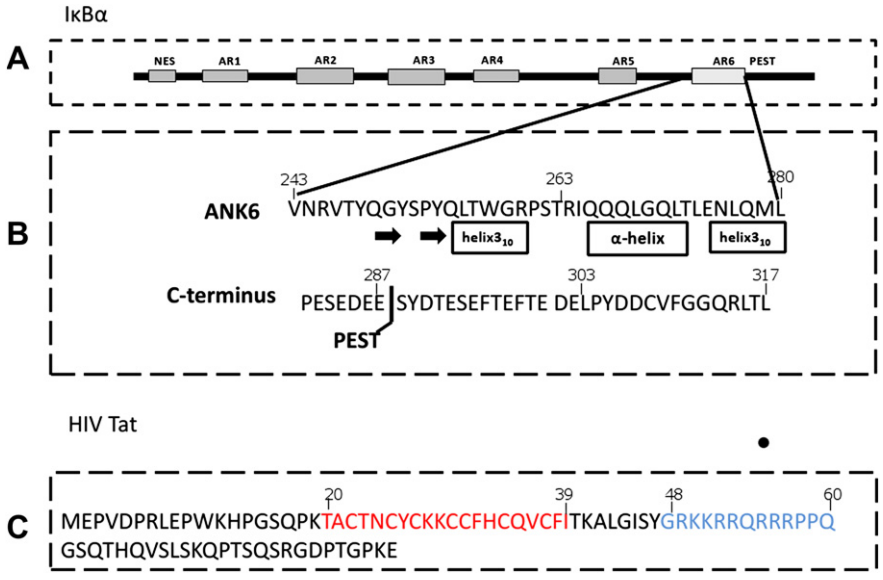


**Fig. 1.** HIV Tat and IκB-α proteins. (A) Schematic representation of modular structure of IκB-α protein; Primary structures of (C) Ankyrin 6 and C-terminus of IκB-α protein (herein some C- and N-termini of IκB-α-based designed peptides are evidenced (Table 1)), and of (B) HIV Tat BH10 protein, in red is reported Cys-rich domain and in blue Arg-rich domain. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)