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NON-CONVENTIONAL INTERACTIONS BETWEEN THE NEURONAL TRANSCRIPTION FACTOR N-OCT-3 AND THE HIV-1 TAR AND RRE RNAS: A RATIONALE FOR NEUROPROTECTION?

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RESEARCH ◊ HYPOTHESIS

ABSTRACT. WE PROPOSE A NOVEL INTERACTION PATTERN for the neuronal transcription factor N-Oct-3 which could be of relevance to HIV-1 replication restriction in the central nervous system. Whereas a significant number of AIDS patients develop a severe form of neurological impairment known as HIV-associated dementia, neuronal damage in fact occurs through indirect molecular mechanisms. Indeed, only the brain macrophages and microglia are sites of productive infection. Bearing in mind the established correlations between HIV-1 propagation inhibition and neurotypic cell differentiation, we hypothesized that N-Oct-3, a key-player of neuronal cell differentiation, might be one of the neuro-specific molecules restricting HIV-1 replication. By using circular dichroism and molecular modeling, we demonstrate here that N-Oct-3 has the ability to interact with the HIV-1 TAR and RRE RNAs, in a way which would prevent the binding of their respective viral protein partners, Tat and Rev, thus impeding the two major regulatory pathways of HIV-1 replication. The main determinant of specificity in both cases is the tight fitting of the N-Oct-3 homeodomain rigid arm in the respective RNA minor grooves. These predictions are a powerful incentive to design peptidomimetics able to synergistically inhibit HIV-1 Tat and Rev functions.

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1. INTRODUCTION

Early in the acquired immune deficiency syndrom (AIDS) epidemic, the interplay between the human immunodeficiency virus type 1 (HIV-1) and the central nervous system (CNS) appeared quite paradoxical. Whilst as many as 25 % of the AIDS patients developed the severe form of neurological disability which is now known as 'HIV-associated dementia' (HAD) [1,2], no significant viral load could be detected in the infected brains. Subsequent studies, benefiting from improvements in HIV-1 detection techniques, determined that, whereas neurons and macroglia were primarily non-infected, HIV-1 mediated a productive infection of brain macrophages and microglia [3]. In addition, a sub-population of astrocytes were consistently infected [4], although yielding little progeny virus [5]. Thus, understanding the different mechanisms of neuroprotection and neuropathology in AIDS disease is an important challenge.

The major steps of the HIV-1 life cycle have been elucidated. The virions bind to the CD_4 receptors and their associated co-receptors at the surface of susceptible cells, such as macrophages and CD_4 T cells, and enter the cytoplasm by the way of membrane fusion. Understandably, much effort has been devoted to designing inhibitors of these critical interactions (for a review, see [6]). However, absence of CD_4 receptors at the surface of several neural cell-lines is not the only factor leading to relative protection from HIV-1. A very recent study demonstrates that astrocytes can in fact use the mannose receptor to allow virion entry, thus bypassing the lack of CD_4 receptors [7]. On the other hand, astrocytes have developed several lines of defence to restrict HIV-1 replication, both at the level of transcription and translation (for a detailed account, see [8,9] and references therein).

Once in the host-cell, the viral RNA is first retro-transcribed into proviral DNA to be integrated in the cellular genome, this process being facilitated by repetitive DNA sequences called long terminal repeats (LTRs) that flank all retroviral genomes. The HIV-1 genome is then expressed through elaborate mechanisms to which both viral nucleoproteins and the cellular machinery take part. Two early viral proteins, Tat and Rev, are essential to promote HIV-1 replication, by acting respectively at the level of transcriptional activation and splicing regulation. Tat, in complex with cyclin T and CDK9, interacts with the so-called 'Transcriptional Activation Region' TAR, an RNA structure at the 5'-end of the HIV-1 mRNA, and thereby regulates transcription by stimulating RNA polymerase processivity through the phosphorylation of its C-terminal tail [10,11] (FIG. 1A). Rev interacts with the 'RNA-Responsive Element' RRE, an RNA structure within the viral mRNA, and is thus involved in the nuclear export of unspliced or singly-spliced RNAs [12] (FIG. 1B). At the mechanistic level, Tat and Rev recognize their respective RNA targets by an arginine-rich motif. We have shown that TRBP, a human cellular protein, can act as a helper protein, facilitating both the Tat/TAR and Rev/RRE regulatory pathways ([13,14] and unpublished data). Obviously, these two pathways are the targets of intensive therapeutic research.

A number of early studies have indicated that neuronal differentiation may be a key-factor in restricting HIV-1 replication (for a review, see [15]). Most importantly, Vesanen et al. [16] have demonstrated that HIV-1 can infect human neuroblastoma cells and tumor cells of neuroectodermal origin, but that viral propagation is inhibited by neurotypic cell differentiation. N-Oct-3 is a neuronal transcription factor, found to be widely expressed in the developing mammalian central nervous system, and shown to be necessary to maintain neural cell differentiation. It has also been detected in fully differentiated type-I astrocytes [17-19].

In the present study, we have explored the capacity of N-Oct-3 to interact with the HIV-1 TAR RNA, known to be the target of a number of cellular proteins [20]. Bearing in mind the interchangeability of TAR and RRE RNAs with respect to a number of small molecules and protein ligands [21,22] we have also monitored the N Oct-3 binding to RRE. We demonstrate for the first time the capacity of the neuronal transcription factor N-Oct-3 to specifically interact with TAR and RRE, the two major regulatory HIV-1 RNAs. We discuss the possible implications of these novel interactions in the context of HIV-1 replication restriction in neural cell-lines.

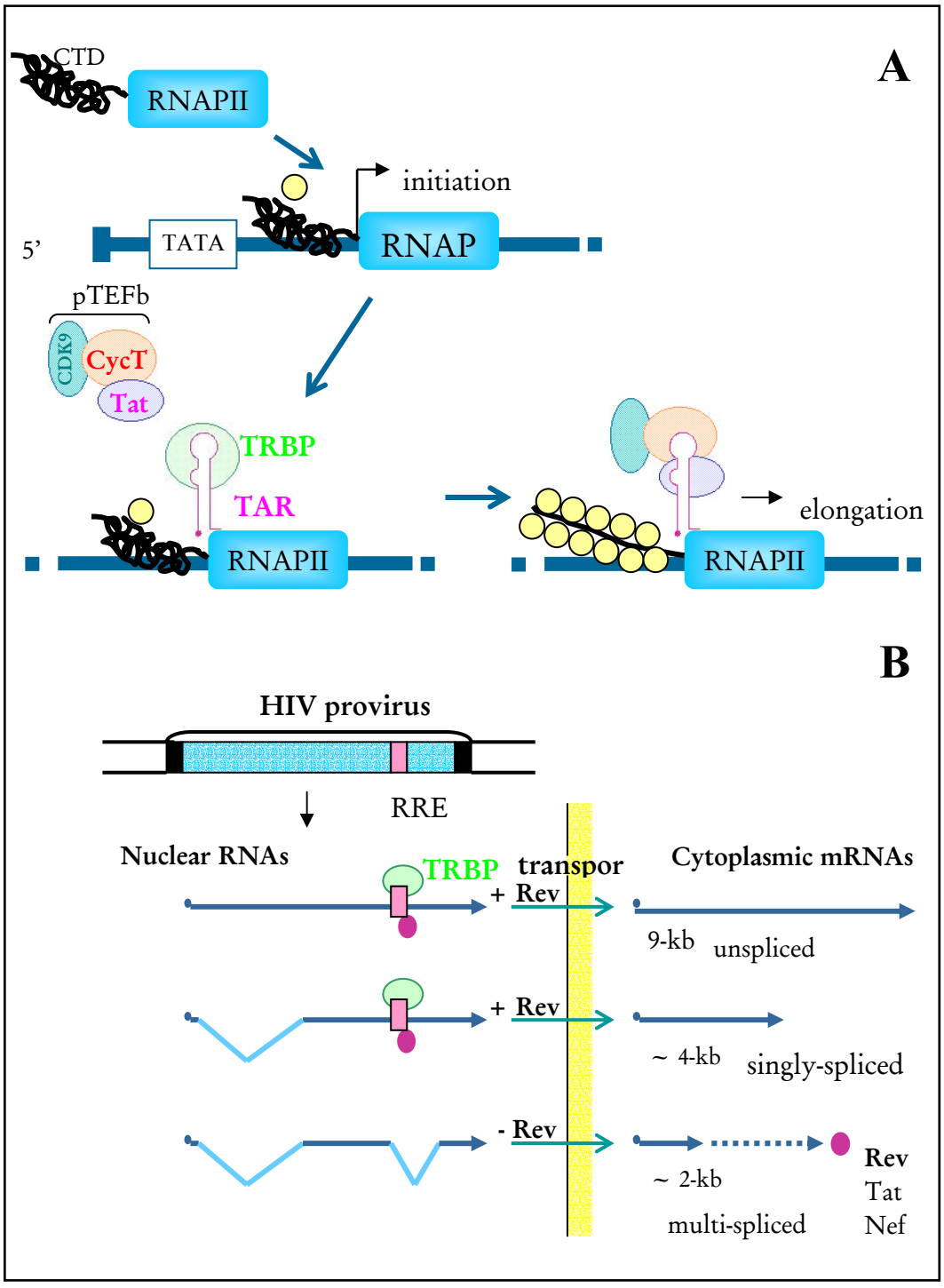

FIGURE 1. TWO MAJOR PATHWAYS REGULATING HIV-1 REPLICATION. (A) The viral Tat protein regulates transcription by stimulating RNA polymerase processivity. In pre-initiation complexes formed at the HIV-1 LTR, the C-terminal domain (CTD) of RNA polymerase II (RNAPIIa) is rapidly phosphorylated by TFIIH. Tat, in complex with cyclin T and CDK9 (the kinase subunit of pTEFb), is then recruited to the transcription complex via its interaction with the trans-activation responsive region TAR, a 59 residue stem-loop RNA found at the 5'-end of all viral transcripts. Tat can thus stimulate additional CTD phosphorylation in elongation complexes. (B) Early gene expression generates the regulatory proteins Tat and Rev from double-spliced mRNA. Once levels are sufficiently high, Rev binds to the Rev-response element (RRE) within the unspliced or single-spliced mRNAs, both stabilizing them and controlling their export into the cytoplasm. These will give rise to full-size genomic viral RNA and to various viral proteins.

2. MATERIALS AND METHODS

2.1. PEPTIDE AND PROTEIN SYNTHESIS

The N-Oct-3 P peptide GRKRKKRTSIEVSVK was purchased from Neosystem. It was synthesized, HPLC-purified and the sequence confirmed by mass spectrometry following standard procedures. The N-Oct-3 and Oct-1 DNA-binding domains (DBDs) were expressed in Escherichia coli and purified as previously reported [23, 24].

2.2. CIRCULAR DICHROISM

Circular dichroic spectra were recorded at 20°C with a Jobin-Yvon VI dichrograph. Quartz cells of either 1-mm or 8.8-mm optical path-lengths were used to record spectra of RNA and polypeptide/RNA complexes at RNA concentrations ranging from 20 µg/ml to 500 µg/ml in 0.15 M NaCl/20 mM sodium phosphate, pH 7.4 in the 200-320 nm spectral region. The results are presented as normalized $\Delta\varepsilon$ values on the basis of the nucleotide mean residue mass of 330 Da. Taking into account the sensitivity of the apparatus $\delta(\Delta A)=10^{-6}$, the nucleotide concentration and the optical path-length of the cell, the precision of the measurements is $\delta(\Delta\varepsilon) = \pm 0.03\ dm^3.mol^{-1}.cm^{-1}$.

2.3. RNA SECONDARY STRUCTURE PREDICTION

RNA secondary structure prediction was performed using the Mfold web server [25] and applied to the 29- and 34-nucleotide sequences corresponding to the functional portions of the respective TAR and RRE RNAs.

2.4. MOLECULAR MODELING

Models were generated using the Accelrys modules Insight-II, Biopolymer, Discover, Docking and Homology (version 2000.1), run on a Silicon Graphics Fuel workstation.

The structure of the complex between the N-Oct-3 POU DBD and the octamer ATGCAAAT was modeled as previously described [24]. The structures of the N-Oct-3 POUs and POUh sub-domains were first built by homology with those of Oct-1 (PDB code: 1OCT; [26]), then prepositioned in register with their respective tetramers, ATGC and AAAT as in the Oct-1 DBD/octamer complex. Both secondary structure prediction and experimental constraints helped building the structure of the linker [23,24]. The fine docking was performed by using the Affinity program. The final interaction energy was: -39.6 kcal/mol for the van der Waals component and -717.9 kcal/mol for the electrostatic component.

To model the structures of the N-Oct-3 DBD/HIV-1 RNA complexes, we used the octamer-induced N-Oct-3 DBD predicted structure on the one hand, and the NMR-derived structures of HIV-1 TAR and RRE RNAs on the other hand (respective PDB codes: 1ANR [27] and 1ETF [28]). The N-Oct-3 POUh arm was first anchored into either the TAR or the RRE RNA minor-groove and the fine docking was completed using the Affinity program. In the case of the N-Oct-3 DBD/TAR complex, the final interaction energy was: 47.3 kcal/mol for the van der Waals component and -581.4 kcal/mol for the electrostatic component. In the case of the N-Oct-3 DBD/RRE complex, the final interaction energy was: -148 kcal/mol for the van der Waals component and -18.9 kcal/mol for the electrostatic component.

3. RESULTS AND DISCUSSION

3.1. N OCT-3, A NEURONAL POU PROTEIN WITH SINGULAR DNA-BINDING PROPERTIES

The POU (acronym of Pit, Oct, Unc) family of transcription factors is defined on the basis of a common DNA-binding domain (DBD) of approximately 160-residues, first identified in the mammalian proteins Pit-1 and Oct-1 and the nematode factor Unc-86 (for a review, see [29]). The POU DBD is made of two distinct, highly conserved sub-domains, termed 'POUs' and 'POUh', which contain respectively four and three α-helices and are connected by a linker, variable in sequence and length (FIG. 2A, 2B, 2C). All the POU domains bind specifically to the prototypic octamer ATGCAAAT. However, due to the flexibility of the linker between the two sub-domains, they can also recognize various AT-rich sequences. The crystallographic structure of the complex between the POU domain of the ubiquitous protein Oct-1 and the octamer has revealed that POUs interacts with

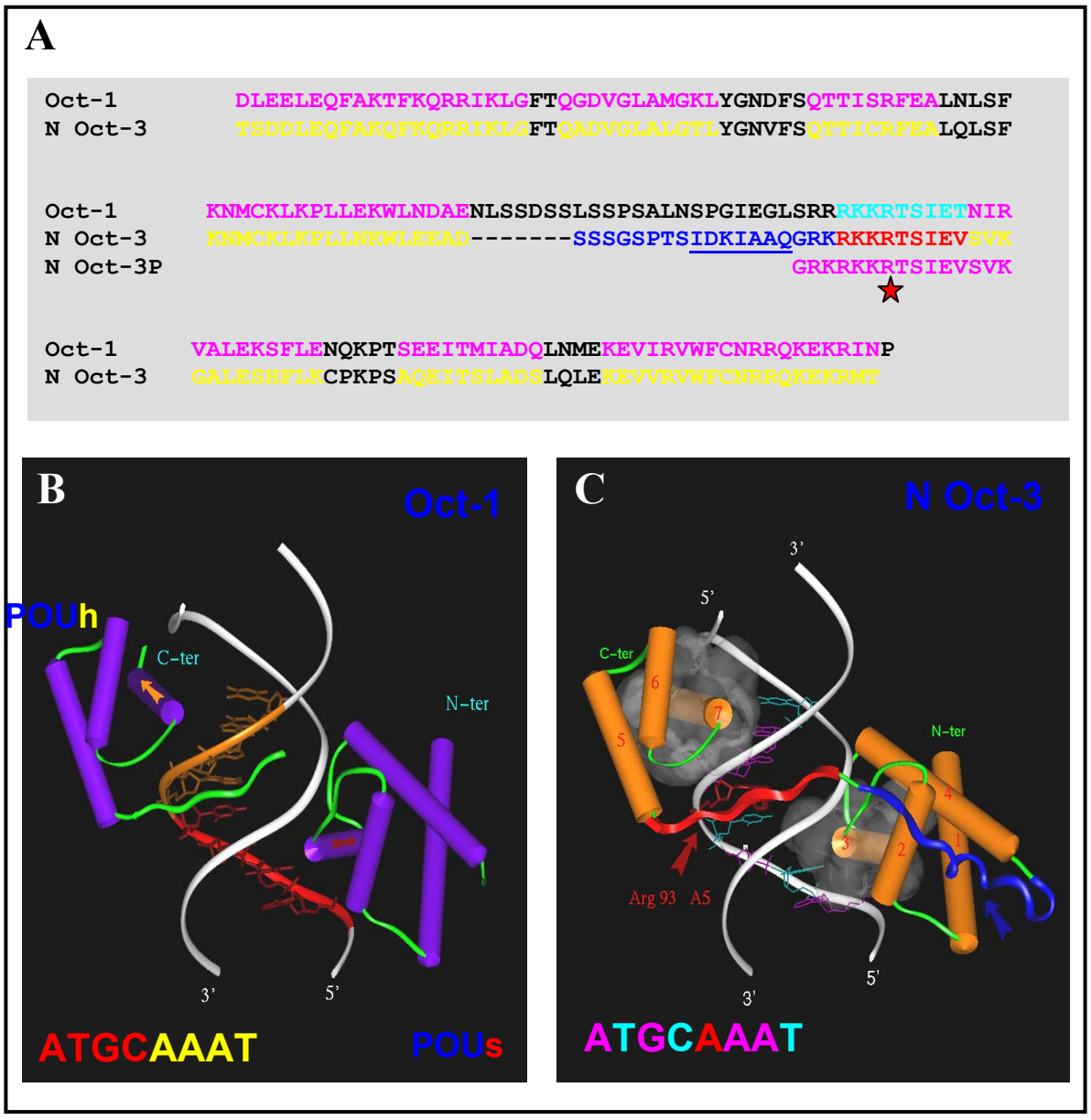

FIGURE 2. SINGULAR DNA-BINDING PROPERTIES OF THE NEURONAL POU PROTEIN N-OCT-3. A: Alignment of the sequences of the Oct-1 and N-Oct-3 POU DBDs. The seven α-helical fragments are color-coded in indigo for Oct-1 and light brown for N-Oct-3. The 4-α-helical POUs sub-domains are separated from the 3-α-helical POUh sub-domains by distinct linker regions. The portion of the N-Oct-3 DBD linker (blue-colored) capable of adopting an α-helical secondary structure is underlined. The N-terminal rigid arms of the Oct-1 and N-Oct-3 POUh sub-domains are color-coded in green and red respectively. The conserved Arg 93 is marked with a star. The sequence of the synthetic N-Oct-3 P peptide is aligned with the N-Oct-3 DBD sequence (magenta-colored). B: Crystallographic structure of the Oct-1 DBD/octamer DNA complex. Same color-coding for the Oct-1 DBD as in A). The two recognition α-helices of the POUs and POUh subdomains, inserted within the ATGC (red) and AAAT (brown) tetramer major grooves, are marked with red and brown arrows respectively. C: Modeled structure of the N-Oct-3 DBD/octamer DNA complex (see text). Same color-coding for the N-Oct-3 DBD as in A). The red arrow indicates the hydrogen-bond between Arg 93 and Ade 5 of the octamer DNA. In the ATGCAAAT sequence the pyrimidine nucleotides are displayed in turquoise whilst the purine nucleotides are displayed in pink (or red for Ade 5). The blue arrow indicates the portion of the DBD linker which adopts an α-helical structure in the complex.

the tetramer ATGC in a way similar to the phage repressors, whereas POUh interacts with the tretramer AAAT as an homeodomain [26]. Both sub-domains insert their third α-helix, the so-called « recognition helix », into the DNA major groove. In addition, POUh inserts its rigid N-terminal arm into the DNA minor groove (FIG. 2B) via a specific contact between a highly conserved arginine residue (marked with a star in FIG. 2A) and the first adenine nucleotide of the AAAT sequence.

Within the Oct-1 POU domain, the linker is so flexible that its structure could not be resolved by crystallography [26]. By contrast, we have shown that on binding to a number of DNA targets, a portion of the linker of the N Oct-3 POU domain adopts an α-helical conformation [23,24]. Indeed, when building the model of the N-Oct-3 DBD/octamer complex (see Materials & Methods for a full account), it was necessary to incorporate this short fragment of α-helix as a steric constraint when building the linker (FIG. 2C). We have experimental evidence that this structuration of the linker strengthens the interaction between N Oct-3 and its targets (unpublished data), most likely by stabilizing the insertion of POUh into the DNA minor groove.

3.2. NON-CONVENTIONAL INTERACTION BETWEEN THE NEURONAL TRANSCRIPTION FACTOR N OCT-3 AND THE HIV-1 TAR RNA: POSSIBLE INTERFERENCE WITH THE TAT/TAR AXIS

The viral Tat/TAR complex and its associated cellular proteins play a pivotal role in trans-activation, leading to high-level transcription of the HIV-1 genome in lymphocytes [30]. We have shown that TRBP acts as a positive effector of trans-activation [13] by maintaining the TAR RNA in a conformation appropriate to Tat binding (unpublished data). In an attempt to shed light on some of the molecular mechanisms causing HIV-1 replication restriction in the CNS, we looked for a protein specific of neural cell-line differentiation and capable of acting as a negative factor towards the trans-activation complex - for example by locking TAR in a 'closed' conformation with respect to Tat binding. As a neuronal transcription factor, N Oct-3 could indeed be such a candidate, provided that it can also bind the TAR RNA.

We therefore examined whether the N Oct-3 DBD could interact with the so-called 'minimal TAR', which corresponds to the 29-residue TAR fragment necessary for trans-activation (FIG. 3A). Circular dichroism (CD) spectroscopy has been used extensively in the study of protein-DNA(/RNA) interactions since two distinct wavelength windows provide information about the conformation of each of the partners in nucleoprotein complexes (for a review, see [31]). In the 200-240 nm range, CD spectra are dominated by the amidic contributions of the protein backbone, whereas, in the spectral region above 240 nm, nucleic acids have strong CD bands by comparison with the relatively weak bands of the protein aromatic side-chains. As can be seen in FIG. 4A, the N-Oct-3 DBD interacts with the minimal TAR RNA, causing a decrease in $\Delta\varepsilon$ at 265 nm of 1.40 ± 0.03, a plateau value already reached for a 1/1 stoichiometry. This decrease in the CD signal, similar to that obtained when Tat binds to TAR [32], indicates nucleotide unstacking and backbone conformational change.

We next wanted to determine whether the binding pattern of N Oct-3 DBD to TAR presented features similar to those characteristic of its complex with the prototypic octamer DNA target. In particular, was the N Oct-3 POUh arm anchored into the TAR minor groove? By using CD and gel-shift experiments (data not shown), we showed that the N Oct-3 DBD could efficiently compete with neomycin B, known to bind to the TAR minor groove [33]. We then directly checked the ability of the 15–residue peptide, 'N-Oct-3 P' (FIG. 2A), which encompasses the N Oct-3 POUh arm, to interact with TAR by CD analysis. A similar effect, i.e., a decrease in $\Delta\varepsilon$ at 265 nm of 1.40 ± 0.03, was obtained when N-Oct-3 P was complexed with TAR (FIG. 4B). Thus the insertion of the N-Oct-3 POUh arm into the minimal TAR minor groove appears to be the main structural determinant of the interaction.

Finally, we predicted the structure of the N-Oct-3 DBD/TAR complex by molecular modeling. We pre-positioned the DBD modeled structure (see Materials and Methods) by anchoring its POUh into the RNA minor groove, using as a steric constraint

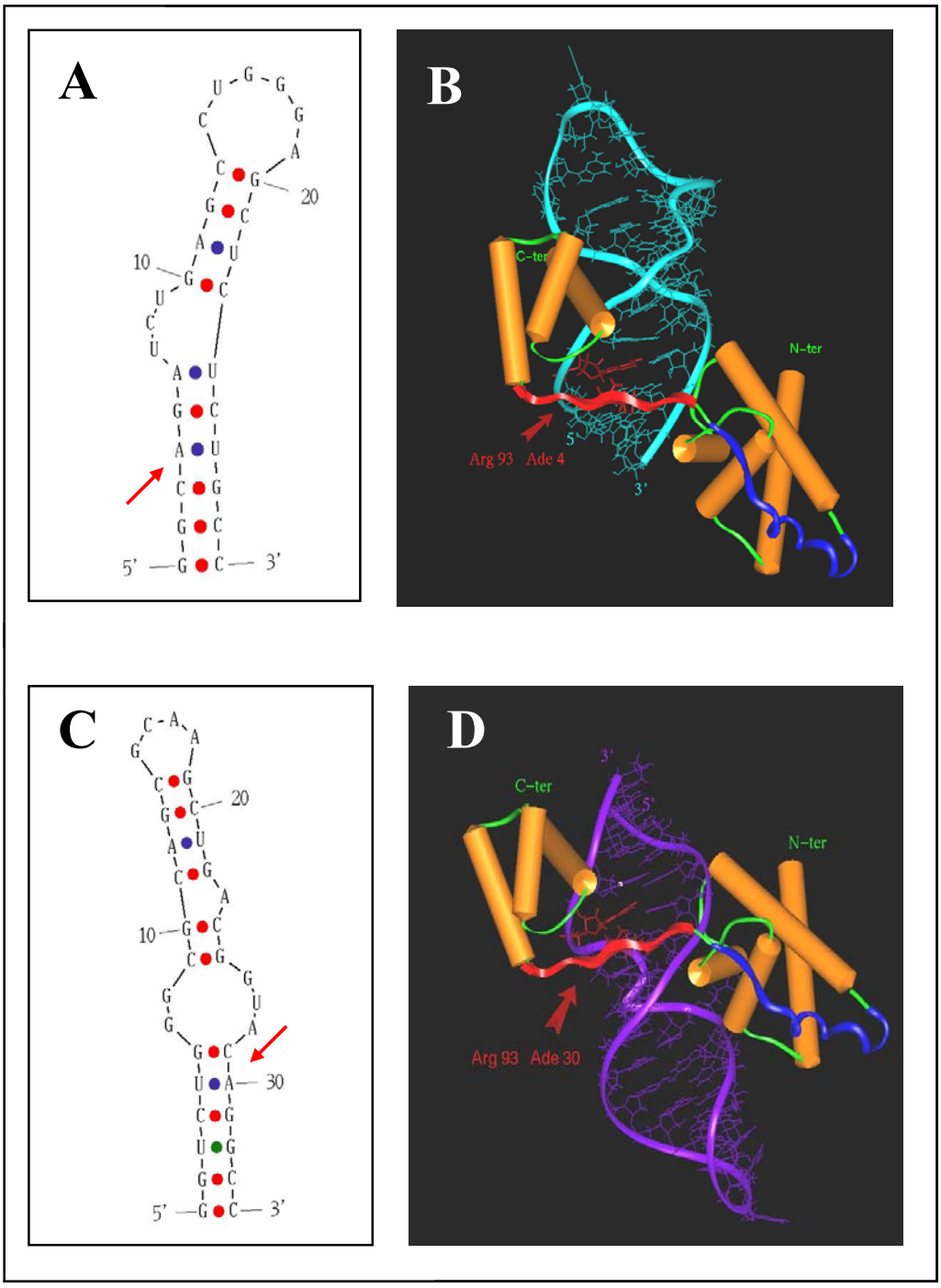

FIGURE 3. COMPUTER-MODELING OF THE INTERACTION BETWEEN THE N-OCT-3 DBD AND ITS HIV RNA TARGET. A: Secondary structure prediction of the 29-residue fragment corresponding to the 'minimal' TAR. B: Docking of the octamer-induced N-Oct-3 DBD predicted structure into the TAR RNA NMR-derived structure (see text). C: Secondary structure prediction of the 34-residue RRE fragment. D: Docking of the octamer-induced N-Oct-3 DBD predicted structure into the RRE RNA NMR-derived structure (see text). The red arrows indicate the critical adenine-anchors in A and C and the hydrogen-bonds between these critical adenine and the conserved Arg 93 in B and D.

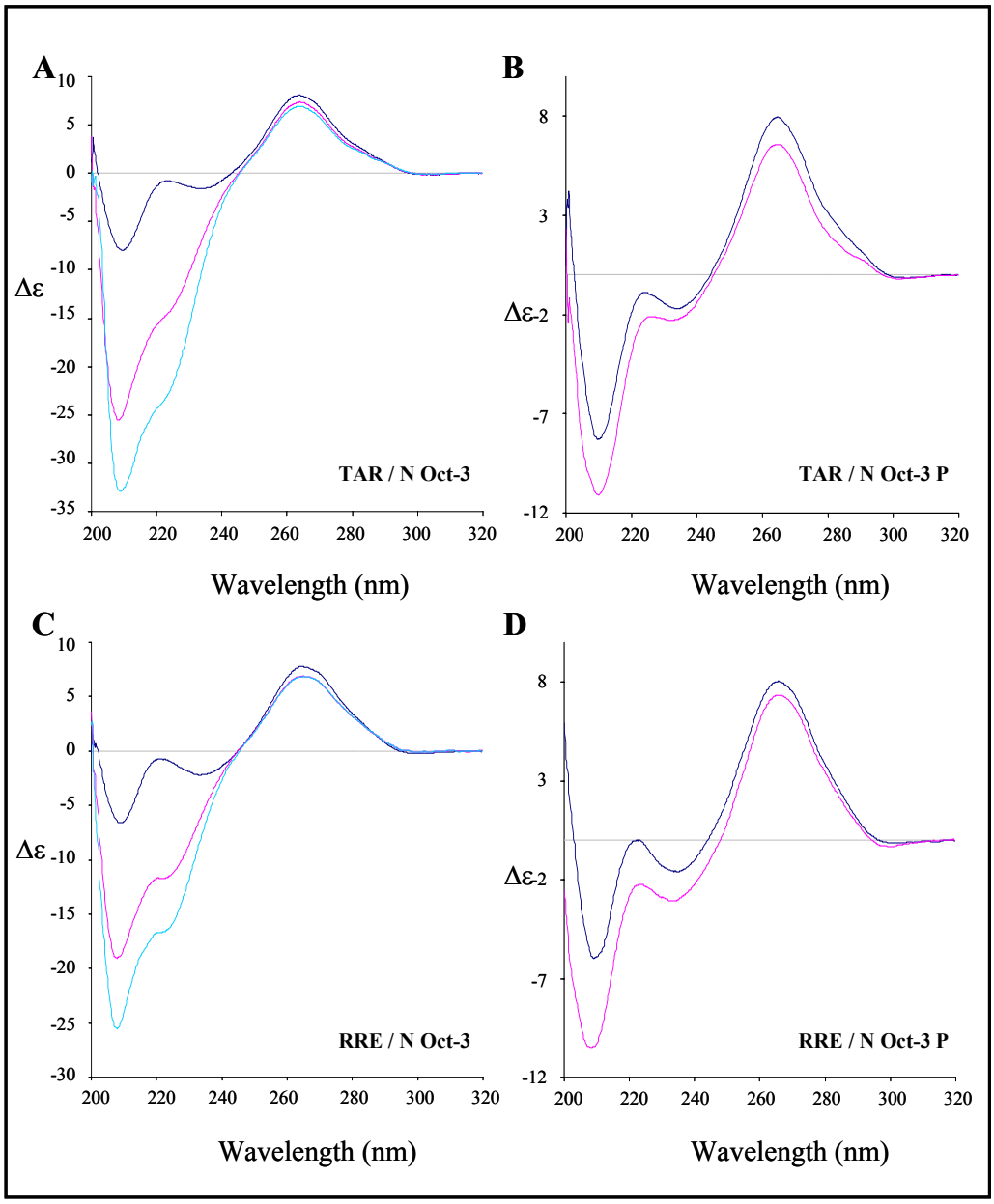

FIGURE 4. CIRCULAR DICHROISM ANALYSIS OF THE INTERACTIONS BETWEEN N-OCT-3 AND ITS HIV RNA TARGET. A and B: Interaction between the HIV-1 TAR RNA and either the N-Oct-3 DBD (A) or the peptide N-Oct-3 P (B). C and D: Interaction between the HIV-1 RRE RNA and either the N-Oct-3 DBD (C) or the peptide N-Oct-3 P (D). The polypeptide/RNA stoichiometries are: 0 (black curve); 1 (red curve); 2 (blue curve).

the characteristic homeodomain hydrogen-bonding between the amino-group of the conserved Arg 93 residue (FIG. 2A) and the N3 atom of a specific adenine, here Ade 4 (FIG. 3B). To identify this adenine, we applied a structural filter derived from a detailed analysis of Oct-1 DBD/DNA complex crystallographic structures (PDB codes: 1OCT and 1HF0; [26,34]), which showed that the critical adenine anchor is embedded in a Pur-Pyr-Ade-Pur-Pur-Pyr motif. Indeed, the so-called 'pyrimidine/

purine steps' are notorious sites of bending [35] and increased nucleotide accessibility. The fine docking of the pre-positioned DBD/RNA complex led to a structure without steric clash, in which both the minor and the major grooves of TAR are occupied by the protein components. We propose that this steric hindrance along with the RNA conformational change detected by CD indicate the ability of the N-Oct-3 DBD to interfere with the HIV-1 Tat/TAR regulation pathway.

3.3. NOVEL INTERACTION BETWEEN N-OCT-3 AND THE HIV-1 RRE RNA: POSSIBLE INTERFERENCE WITH THE REV/RRE PATHWAY

Taking into account the interchangeability of some of the TAR and RRE ligands [21,22], we now asked whether N-Oct-3 could also interact with RRE, the second regulatory HIV-1 RNA (FIG. 3C). By using the same CD approach (FIG. 4C), we were able to conclude that the N-Oct-3 DBD is also able to bind to the RRE RNA at a 1/1 stoichiometry, inducing a decrease in $\Delta\varepsilon$ of 0.70 ± 0.03. Again, the insertion into the RNA minor groove was a major element of the complex, since the interaction between the N-Oct-3 P peptide and RRE virtually led to the same $\Delta\varepsilon$ decrease (FIG. 4D). The structure of the N-Oct-3 DBD/RRE complex was predicted following the same protocol (FIG. 3D) as in the case of the N-Oct-3 DBD/TAR complex.

3.4 SPECIFICITY OF THE INTERACTIONS BETWEEN N OCT-3 AND THE HIV-1 TAR AND RRE RNAS

N-Oct-3 can thus be added to the list of transcription factors which can also bind RNA, such as TFIIIA, p53 and WT-1 [36]. The lower limit of the HIV-1 TAR and RRE RNA concentrations used in our CD experiments allowed us to estimate Kd values of N-Oct-3 DBD for its respective targets in the nanomolar range. The main determinant of specificity is in both cases the tight fitting of the POUh arm in the RNA minor groove.

Bearing in mind the high degree of conservation of the POU domains, it was necessary to assess the specificity of these two novel interactions. We thus performed the same CD experiments replacing the N-Oct-3 DBD by the Oct-1 DBD. Strikingly, the Oct-1 POU domain did not show any significant binding to either HIV-1 RNA. By analogy with the N-Oct-3 POU/DNA complexes described earlier, N-Oct-3 DBD anchoring to its RNA targets is likely to be stabilized by the capacity of its linker to adopt a partial α-helical structure upon complex formation. Significantly, this stabilization is not possible with the Oct-1 DBD. In conclusion, the non-conventional interactions between the N Oct-3 DBD and the two HIV-1 RNAs are specifically neuronal.

3.5. IMPLICATIONS FOR HIV REPLICATION RESTRICTION IN THE CENTRAL NERVOUS SYSTEM

HIV-1 infection results in brain injury in a significant number of AIDS patients. Although most affected, neurons are rarely infected, which suggests that : (i) they possess specific molecules which restrict HIV-1 replication and (ii) they are nevertheless damaged by indirect mechanisms. Indeed, the activation of brain macrophages and microglia following their infection by HIV-1 can now be considered the major factors leading to neurotoxicity [15,37].

We demonstrate here that the neuronal POU factor N-Oct-3 has the ability to interact with the HIV-1 TAR and RRE RNAs, in a way which would prevent the binding of their respective protein partners, Tat and Rev, thus impeding two major regulatory pathways of HIV-1 replication. We suggest that N-Oct-3 might be one of the neuro-specific molecules restricting HIV-1 replication. Interestingly, the ubiquitous POU protein Oct-1 would not fulfil the same role because of its inability to bind as tightly to RNA minor grooves. Conversely, other neuronal POU proteins with the same type of linker within their DBD as N-Oct-3 [23] might also be able to interfere with both Tat/TAR and Rev/RRE complex formation.

Our results are an incentive to design peptidomimetics able to synergistically inhibit the functions of HIV-1 Tat and Rev by binding tightly to both TAR and RRE minor grooves. Such inhibitors would be effective on a wide spectrum of cells, including the brain macrophages and microglia which are essentially responsible for

HAD, irrespective of intrinsic neuronal protection mechanisms.

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ABBREVIATIONS USED:

AIDS: acquired immune deficiency syndrome
CNS: central nervous system
HAD: HIV-associated dementia
LTRs: long terminal repeats
DBDs: DNA-binding domains
CD: circular dichroism

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