The Intrinsically Disordered Cytoplasmic Domain of the T Cell Receptor ζ Chain Binds to the Nef Protein of Simian Immunodeficiency Virus without a Disorder-to-Order Transition[†]

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ABSTRACT: Intrinsically disordered proteins are thought to undergo coupled binding and folding upon interaction with their folded partners. In this study, we investigate whether binding of the intrinsically disordered T cell receptor ζ cytoplasmic tail to the well-folded simian immunodeficiency virus Nef core domain is accompanied by a disorder-to-order transition. We show that ζ forms a 1:1 complex with Nef and remains unfolded in the complex. Thus, our findings oppose the generally accepted view of the behavior of intrinsically disordered proteins and provide new evidence of the existence of specific interactions for unfolded protein molecules.

There is a rapidly growing number of proteins that have been shown to have little or no ordered structure under physiologic conditions and are termed intrinsically disordered (natively unfolded, intrinsically unstructured) proteins (IDPs)[1] (*1*). Recently, it has been shown that IDPs play an important role in cell signaling (*2*) and that protein phosphorylation, one of the critical and obligatory events in cell signaling, predominantly occurs within these regions (*3*). In line with these observations, the cytoplasmic domains of the signaling subunits of multichain immune recognition receptors (MIRRs) (*4*) found on immune cells have been shown recently to represent a novel class of IDPs (*5*). In addition, these domains all contain one or more copies of an immunoreceptor tyrosine-based activation motif (ITAM), tyrosine residues of which are phosphorylated upon receptor triggering.

Among the MIRR signaling subunits, the T cell receptor (TCR) ζ chain is particularly interesting due to its crucial role in T cell activation and the presence of three ITAMs in its cytoplasmic tail (ζ_{cyt}), allowing for multiple phosphorylation states (*6*). The 13 kDa random coil ζ_{cyt} is known to interact with SIV pathogenicity factor Nef (*7*), providing a possible structural basis for viral modulation of TCR signaling (*8*). This interaction could promote TCR clustering in human immunodeficiency virus (HIV)/SIV-infected T cells, potentially activating these cells or lowering their activation threshold (*9*).

The SIV Nef−ζ_{cyt} complex represents a novel system for studying the interaction of an IDP with a well-structured partner. With only a few such complexes known to date, the generally accepted view is that binding of IDPs to folded proteins is coupled with their folding on their partners (*10*). In those complexes, the binding interface is characterized by specific interactions between structured protein domains. In contrast, we have shown that several IDPs, including ζ_{cyt}, can homodimerize without a disorder-to-order transition (*5, 11, 12*), thus providing the first evidence of the existence of specific dimerization interactions within an IDP species. More recently, these results have been confirmed for intrinsically disordered products of the *umuD* gene which dimerize without secondary structure induction (*13*). Structural studies of the Nef−ζ_{cyt} complex can assist in answering the fundamentally important question of whether the random coil ζ_{cyt} folds on the well-structured Nef core domain.

In this study, we have investigated binding of ζ_{cyt} to the SIV Nef core domain using purified proteins examined by gel filtration chromatography and multidimensional NMR. We found that under the conditions used, ζ_{cyt} forms a 1:1 complex with SIV Nef and remains unfolded in the complex. These data oppose the generally accepted "coupled binding and folding" paradigm for IDPs (*10*), suggesting the existence of specific protein−protein interactions between the IDP molecule and its folded partner.

Gel filtration (GF) chromatography is a useful technique for monitoring molecular association under native conditions. To study the Nef−ζ_{cyt} interaction and its binding stoichiometry, we used a GF assay developed and optimized as previously described for the ζ_{cyt} dimerization studies (*11*). The GF analysis was performed for Nef and uniformly ^{15}N-labeled ζ_{cyt} alone as well as for the Nef−ζ_{cyt} mixtures at a constant ζ_{cyt} concentration of 15 μM and 1:1, 1:2, and 2:1 molar ratios of Nef to ζ_{cyt} (Figure 1). All experiments were performed in 20 mM phosphate buffer and 100 mM NaCl

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[1] Abbreviations: GF, gel filtration; HIV, human immunodeficiency virus; HSQC, heteronuclear single-quantum coherence; IDP, intrinsically disordered protein; ITAM, immunoreceptor tyrosine-based activation motif; MW, molecular weight; NMR, nuclear magnetic resonance; SIV, simian immunodeficiency virus; SNID, SIV Nef interaction domain; TCR, T cell receptor.

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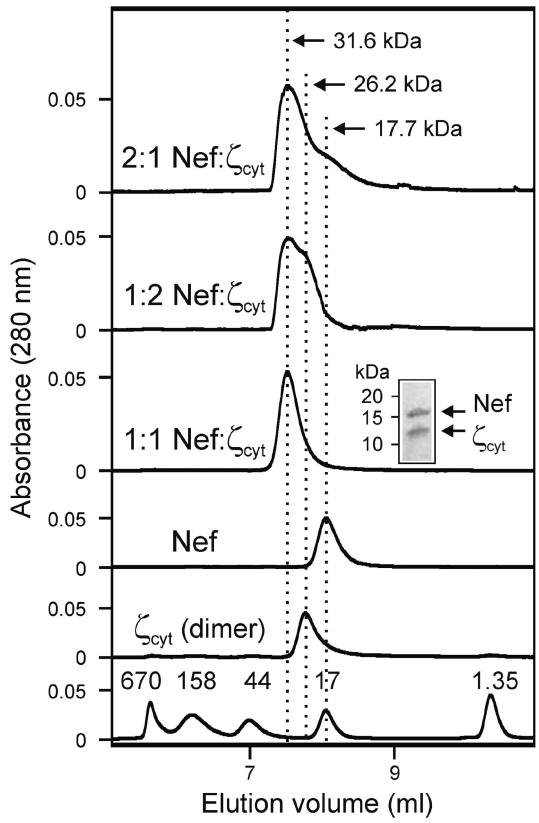

FIGURE 1: SIV Nef breaks the ζ_{cyt} homodimer and forms a 1:1 Nef$-\zeta_{cyt}$ complex. Typical gel filtration profiles of the free Nef and ζ_{cyt} proteins and the Nef$-\zeta_{cyt}$ complex formed at a constant ζ_{cyt} concentration of 15 μM and 1:1, 1:2, and 2:1 molar ratios of Nef to ζ_{cyt}. Apparent molecular masses of homodimeric ζ_{cyt}, Nef, and the 1:1 Nef$-\zeta_{cyt}$ complex (26.2, 17.7, and 31.6 kDa, respectively) were determined by calibration of the column with molecular mass markers (shown at the bottom). The SDS−PAGE (inset) profile of the 1:1 Nef$-\zeta_{cyt}$ complex is also shown.

(pH 6.2) using a range of molecular mass (MM) markers for calibration. The apparent native MM for ζ_{cyt}, determined to be 26.2 kDa, is consistent with that previously reported for a ζ_{cyt} dimer (*11*). The apparent MM of 17.7 kDa determined for the SIV Nef core domain corresponds to that predicted by the protein sequence (16679 Da), while the apparent MM of 31.6 kDa determined for the Nef$-\zeta_{cyt}$ complex is consistent with that predicted for an equimolar complex (Figure 1). The SDS−PAGE profile of the Nef$-\zeta_{cyt}$ complex (Figure 1, inset) also confirms a 1:1 molar ratio. Addition of a molar excess of each constituent results in the appearance of peaks corresponding to the relevant excess protein (Figure 1). Similar results were obtained using unlabeled ζ_{cyt} (data not shown). The reinjection analysis of the collected Nef$-\zeta_{cyt}$ complex revealed the full dissociation of the complex to its protein constituents under these conditions (data not shown). The Nef$-\zeta_{cyt}$ binding affinity apparently is higher than that of ζ_{cyt} dimerization, previously reported to be 10 μM (*11*), and considering the approximate 20-fold sample dilution through the chromatographic column is likely to be ~1 μM. This binding affinity fits our surface plasmon resonance data (W. M. Kim, A. B. Sigalov, and L. J. Stern, unpublished results).

In summary, at the protein concentrations that were used, SIV Nef breaks the ζ_{cyt} homodimer and forms a 1:1 Nef$-\zeta_{cyt}$ complex. Unfortunately, we were unable to study the complex stoichiometry at high protein concentrations because of its low solubility. Nef and ζ_{cyt} alone are soluble at >1 mM, while the complex precipitates at >25 μM. Interestingly, SDS−PAGE analysis reveals that a stoichiometry of the precipitated complex is characterized by a molar excess of ζ_{cyt} (data not shown), indirectly supporting a hypothesis that Nef mediates modulation of TCR signaling by promoting receptor clustering through interaction with ζ in the cytoplasmic milieu (*9*).

Because of the high sensitivity of NMR chemical shifts to subtle changes in protein conformational ensembles, NMR is unequaled in its ability to characterize the structure of unfolded proteins and their complexes. In this work, we used multidimensional NMR to answer the question of whether random-coil ζ_{cyt} folds upon binding to Nef and to characterize the binding site for Nef on ζ_{cyt}. Two SIV Nef interaction domains (SNIDs) on ζ_{cyt} that comprise portions of the first and second of the three ITAMs (Figure 2A) have been previously reported (*7*). The ^{1}H$-^{15}$N HSQC spectra of 15 μM uniformly ^{15}N-labeled ζ_{cyt} were recorded in the absence (blue) and presence (red) of an equimolar amount of Nef at 600 MHz (Figure 2B). The existence of the 1:1 Nef$-\zeta_{cyt}$ complex under the NMR conditions used was confirmed by GF and SDS−PAGE analysis of the NMR sample after completion of the NMR experiments (Figure 1, 1:1 Nef:ζ_{cyt} panel, and Figure S1 of the Supporting Information).

The HSQC spectrum of free ζ_{cyt} (Figure 2B, blue, and ref *11*) has been assigned previously (*14*); all but three of the expected cross-peaks were observed. The low signal dispersion of the ζ_{cyt} backbone amide ^{1}H chemical shifts is typical for IDPs. No substantial changes in the average peak intensity were found in the free and Nef-bound ζ_{cyt} samples, indicating that essentially all of the ^{15}N-labeled ζ_{cyt} in the Nef$-\zeta_{cyt}$ sample is observed under these NMR conditions. Surprisingly, the overlaid spectra of free and Nef-bound ζ_{cyt} show no differences in chemical shift values for all observed ζ_{cyt} residues (Figure 2B). This includes residues in the previously identified SNID regions (Figure 2A). Similar results were obtained for ζ_{cyt} in the presence of a 1.5-fold excess of Nef (data not shown). The lack of chemical shift changes for the residues involved in the Nef$-\zeta_{cyt}$ interaction could be explained by multivalent and cooperative interactions of several subsites, which would not need to be simultaneously and quantitatively populated.

Thus, the lack of Nef-induced changes in cross-peak chemical shifts and intensities provides strong evidence that ζ_{cyt} remains unfolded upon binding to Nef. These findings are in line with our previous NMR studies of a ζ_{cyt} homodimer where no chemical shift changes were observed upon dimerization (*11, 12*), indicating no disorder-to-order structural transition during this process.

In conclusion, our data suggest that the intrinsically disordered TCR ζ_{cyt} binds to the well-structured SIV Nef core domain, forming a 1:1 complex, and remains unfolded upon binding. This finding indicates that in contrast to a generally accepted coupled binding and folding model of interaction of IDPs with their well-folded partners, formation of the Nef$-\zeta_{cyt}$ complex is not accompanied by a disorder-to-order structural transition of the ζ_{cyt} protein molecule (Figure 2C; the monomeric form of free ζ_{cyt} is shown for illustrative purposes).

It is interesting to consider the results of this study in light of accumulating data suggesting the existence of specific

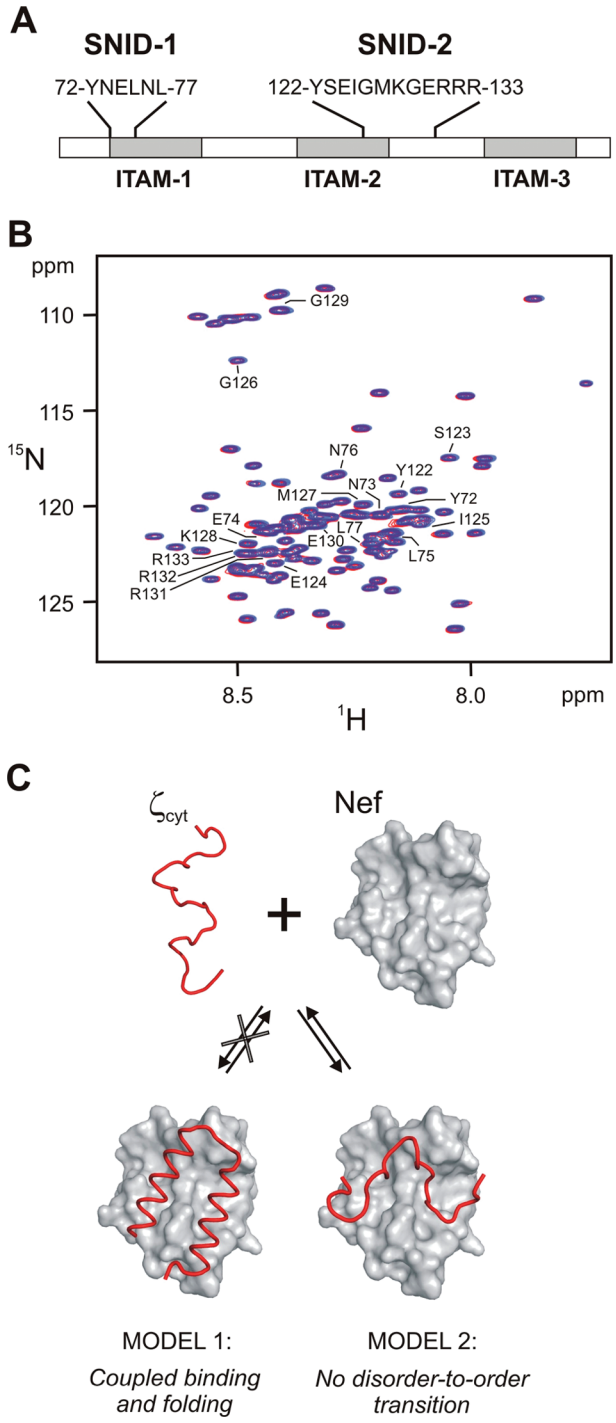

FIGURE 2: Intrinsically disordered ζ_{cyt} does not fold upon binding to SIV Nef. (A) Domain organization of ζ_{cyt}. The two SIV Nef interaction domains (SNIDs) are indicated. ITAM domains are shown as gray rectangles. (B) $^{1}H-^{15}N$ HSQC spectra of 15 μM [^{15}N]ζ_{cyt} in the absence (blue) or presence (red) of an equimolar amount of Nef. Cross-peak positions of SNID residues are marked to highlight the lack of chemical shift changes for these residues upon binding to Nef. (C) Two possible conceptual models of binding of ζ_{cyt} to Nef. Images were created using PyMol (www.pymol.org) from Protein Data Bank entry 1AVV for the HIV-1 Nef core domain (*15*) and using arbitrary idealized structural elements to represent the ensemble of unfolded conformations of ζ_{cyt}.

binding interactions for IDPs (*12, 13*). These interactions could play important roles in cellular signaling and viral pathogenesis (*4, 9*). Thus, defining the nature of these unique interactions and developing ways to control and influence them can have not only fundamental but also practical impact.

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SUPPORTING INFORMATION AVAILABLE

Methods and experimental design. This material is available free of charge via the Internet at http://pubs.acs.org.

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