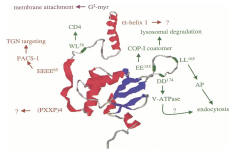
Characterization of domains of E3 ubiquitin ligase involved in chemokine receptor down regulation by Nef protein of HIV

Abstract

CXCR4, the chemokine receptor for CXCL12 and co-receptor for HIV-1, is down regulated by HIV Nef protein. Agonist occupied CXCR4 is ubiquitylated by E3 ligase Atrophin-1 Interacting Protein 4 (AIP4) before endocytosis. We have shown that Nef usurps the same pathway for down regulating many chemokine receptors including CXCR4. The current study aimed to characterize mutual interaction domains of Nef and AIP4 that may be involved in down regulation of CXCR4. Different mutants of Nef (M20A, E62A, P72A, and L164A) were cloned into cerulean vectors upstream of the cerulean fluorophore to express Nef-Cerulean fusion proteins. Confocal microscopy was performed using the Nef-Cerulean constructs to confirm its expression. Flow cytometry was used to determine the ability of the Nef-Cerulean constructs in down regulating CD4 and CXCR4. GST-fusion proteins of AIP4 and mutants of AIP4 were expressed in bacterial host and purified for use in later studies. The results from this ongoing study will assist in revealing some of the yet unidentified mechanism behind the Nef-mediated down regulation of CXCR4.

Nef protein of HIV

- Nef (Negative factor) has a positive role in HIV replication and pathogenesis
- Nef is a 27kD, N-terminal myristoylated accessory protein involved in post integration infection
- HIV positive individuals with apparent deletions within the *nef* gene exhibited no signs of progression to AIDS (Gorry *et al.*, 2007)
- Nef downregulates the expression of the Chemokine receptors CXCR4 and CCR5, thereby potentially enhancing resistance to superinfection
- Downregulation of chemokine receptors is a shared property of many Nef alleles
- Mutagenesis of the SH3 domain or acidic cluster motif abrogates the ability of Nef to downmodulate Chemokine receptors



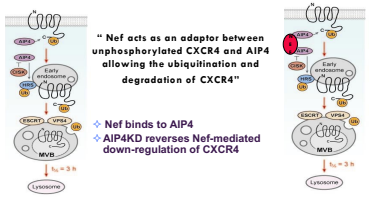
- A putative three-dimensional structure of full-length Nef. Alpha-helices are painted in red, beta-sheets in blue. Items concerning CD4 down-modulation are in green; those important for MHC-I regulation in brown. The myristoylation signal, essential for both functions, is in maroon (Image taken from Doms RW& Trono D., 2000)

Mechanism of CXCR4 down-regulation by Nef- What is known!

CXCR4

- CXCR4 is a co-receptor for X4 and X4R5 HIV
- When bound to its agonist, CXCL12, CXCR4 undergoes endocytosis and ubiquitination by E3 Ubiquitin ligase AIP4 (Marchese *et al.*, 2003)
- AIP4 Binds to a non-canonical domain of CXCR4 via a novel WW domain (Bhandari *et al.*, 2009), characterized by 2 highly conserved tryptophan that bind proline rich motif.

Nef utilizes the pathway of Agonist (CXCL12) driven CXCR4 down regulation (Model proposed by the lab from previous observations)



AIP4 directly binds phosphorylated CXCR4 (Marchese *et al.*, 2008)

Modified image depicting the hypothesized role of Nef during downregulation of CXCR4

Hypothesis

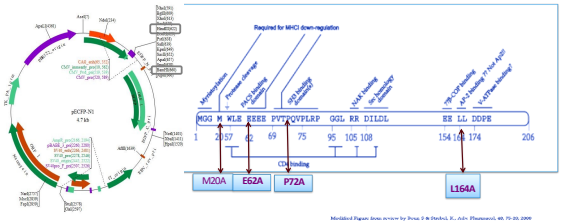
"Since WW domain has high affinity for proline rich motifs, we hypothesize that the WW domain of AIP4 might be crucial to binding Nef PXXP"

Research Question

"What domains of Nef and AIP4 are interacting ?"

Construction of cerulean tagged Nef mutants

Cloning and expression strategy

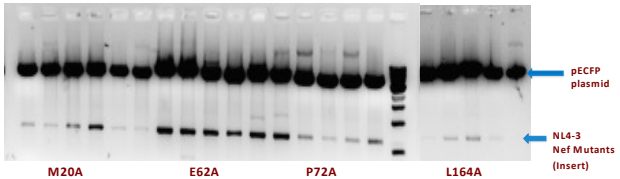


Mutants of Nef used in this study

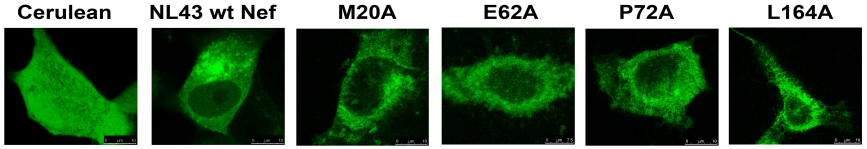
1. M20A: mutation of methonine at the 20th position
2. E62A: mutation of glutamic acid at the 62nd position
3. P72A: mutation of proline at the 72nd position
4. L164A: mutation of lysine at the 164th position

NL4-3 Nef mutants PCR amplification *BamH1* and *EcoR1* digested clones of NL4-3 Nef mutants cloned in pECFP

Annealing Temperature : 60°C
Templates : Plasmids containing cloned NL4-3 and mutants of NL4-3(from lab)

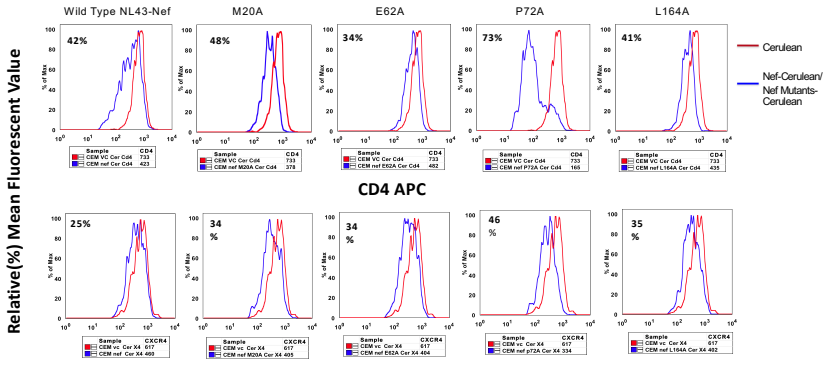


Confocal microscopic visualization Cerulean tagged Nef mutants in Hela cells



Hela cells were transfected with the plasmids encoding Cerulean/ Nef cerulean and its mutants using lipofectamine. The cells were fixed and images were acquired using Leica SP5 confocal microscope.

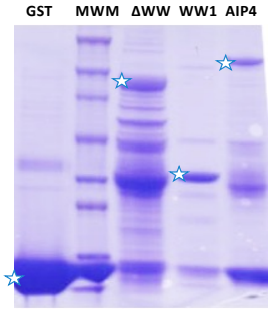
Down-regulation of CD4 and CXCR4 by NL43-Nef cerulean and its mutants



* The numbers in the inlay represents the percentage of receptor down regulation with respect to the plasmid vector

CEM cells were electroporated with plasmids encoding Cerulean/ Nef cerulean and its mutants along with GFP plasmid as the marker for transfection. Expression in the transfected cells were assessed by gating on the GFP positive cells in flow cytometry

Expression of GST-tagged AIP4 and its mutants



Coomassie blue staining of GST fused to amino terminus of full length AIP4(GST-AIP4), WW1 domain alone(GST-WW1), and AIP4 lacking WW1 domain (ΔWW1)

Summary

- The wild type and mutant derivatives of NL4-3 Nef were cloned in pECFP plasmids as Nef-Cerulean fusion proteins. Results were confirmed by restriction digestion and DNA sequencing.
- The expression of Nef-Cerulean constructs was further confirmed by confocal microscopy. The fusion proteins localized to the perinuclear region, a region where Nef proteins are typically found.
- The functionality of the Nef-Cerulean constructs was assessed via flow cytometry by quantifying its ability to down regulate CD4 and CXCR4 expression on the cell surface.
- GST-fusion AIP4 and its mutants were expressed in *E.coli* cells. The fusion proteins were then purified using GST-sepharose beads.

Future Directions

- Co-localization studies of Cerulean tagged Nef mutants with AIP4 to characterize the Nef domains that are interacting with endogenous AIP4
- Pull down experiments using GST-fusion AIP4 and its mutants with cells transfected with Nef mutants to identify the domains of Nef binding to AIP4

References

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