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HIV-1 Nef physically associate with CAMKIIδ – ASK-1 complex to inhibit p38MAPK signalling and apoptosis in infected cells

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ABSTRACT

Human immunodeficiency type 1 virus accessory protein Nef is a key modulator of AIDS pathogenesis. With no enzymatic activity, Nef regulated functions in host cells largely depends on its ability to form multi-protein complex with the cellular proteins. Here, we identified Calcium (Ca^{2+})/Calmodulin dependent protein kinase II subunit delta (CAMKIIδ) as novel Nef interacting host protein. Further, we confirmed that Nef mediated $[Ca^{2+}]_I$ promote formation of Nef-CAMKIIδ - apoptosis signal-regulating kinase (ASK-1) heterotrimeric complex. The assembly of Nef with CAMKIIδ - ASK-1 inhibits the downstream p38MAPK phosphorylation resulting in abrogation of apoptosis. Further, using competitive peptide inhibitors against Nef binding domains to CAMKIIδ, identified in the present study and ASK-1, individually blocked physical interaction of Nef with CAMKIIδ-ASK-1 complex and restored p38MAPK phosphorylation and apoptosis. Altogether, our study indicates that HIV-Nef modulates cytosolic $[Ca^{2+}]_I$ and blocks CAMKIIδ – ASK-1 kinase activity to inhibit apoptosis of infected cells.

1. Introduction

HIV-1 Nef is a key mediator of viral replication and disease progression during Human Immunodeficiency type 1 virus (HIV-1) infection as evident by negligible viral load and reduced clinical symptoms of AIDS in patients infected with non-functional Nef HIV-1 virus [1,2]. Nef is a 27–34 kDa myristoylated accessory protein of HIV-1 which is highly conserved and belongs to the characteristic of primate lentiviruses. HIV-1 Nef is an abundantly expressed protein during early stages of the viral infection cycle [3,4]. Despite of lacking enzymatic activity, the unique structural property of HIV-1 Nef assists its interaction with different host proteins for the regulation of various signalling pathways in host [5–7]. Nef expression has been accounted for depletion of the surface receptors CD4 and MHC I, enhanced viral pathogenicity and modulation of multiple cellular signalling pathways in both CD4 lymphocytes and macrophages during HIV-1 infection [8–12]. Nef has been implicated in the depletion of bystander CTLs *in vivo* leading to immunoparesis, a hallmark of AIDS pathogenesis [13,14]. This is likely due to the widespread pro-apoptotic signals induced by HIV 1-Nef through $CD4^+$T cell signalling upon early infection to circumvent immune surveillance. To date, several different hypotheses has been proposed to regulate apoptotic pathways in HIV-1 infection, including TNF/TNFR [15], Fas/FasL [16], and an interaction between TNF-related apoptosis-inducing ligand (TRAIL) and its receptors [13,17]. However, the infected T cells themselves do not get sensitized to these pro-apoptotic signals, which reflects the ability of Nef in programming escape mechanisms within infected T cells.

Myriad of scientific evidences has unveiled several molecular mechanisms highlighting the ability of HIV-1 Nef to modulate host internal milieu, which confer survival advantage to HIV-1 infected cells for viral

Abbreviations: CaMKIIδ, Ca^{2+}/calmodulin dependent protein kinase II subunit delta; ASK-1, apoptosis signal-regulating kinase 1; $[Ca^{2+}]_I$, calcium influx; HIV-1, human immunodeficiency virus 1; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; AIDS, acquired immune deficiency syndrome; CTL, cytotoxic T lymphocytes; MHC 1, major histocompatibility complex 1; NFAT, nuclear factor of activated T-cells; Cd(2+), cadmium; $CdCl_2$, cadmium chloride; Nef, negative regulatory factor; LTR, long terminal repeat; CaM, calmodulin; TCR, T cell receptor

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replication. Nef mediated selective depletion of MHC class I a, b and CD4 surface receptors has been reported to diminish recognition of HIV-1infected cells by CTLs. The downmodulation of MHC-I allows infected cells to undergo immune evasion which is first line of defence against viral infection whereas down-regulation of CD4 receptors blocks super infection, indispensable for optimum production of pathogenic virus progeny [10,18,19]. Furthermore, Nef has been reported to interact with TCR-ζ chain to augment Fas ligand (FasL) on infected T cells to avoid viral antigen recognition by T cells, which triggers death program in bystander T cells through Fas L/Fas dependent manner [20]. On contrary, the infected cells escape Fas L/Fas cis ligation mediated apoptosis through directly binding apoptosis signal-regulating kinase (ASK-1) and inhibiting stimulus-dependent release of thioredoxin (Trx) from it, thus blocking kinase activity of ASK-1 [21]. By targeting ASK-1 and blocking its kinase activity, Nef also inhibits TNF-α induced apoptosis of infected cells [9]. In support of this hypothesis, we have previously reported constituent minimal essential domain (152 DEVGEANN 159) through which HIV-1Nef interacts with ASK-1 and inhibits its function [9]. Moreover, the study also delineated a novel alternate mechanism underlying HIV-1 Nef mediated ASK1 functional modulation, wherein by reinforcing the inhibitory ser967 phosphorylation of ASK-1, HIV-1Nef negatively regulated ASK-1function. Besides, HIV-1 Nef has been reported to synchronize Bcl family of proteins differently in infected and uninfected cells [20]. During apoptotic signalling pro-survival members of Bcl family (Bcl2, Bcl-XL, Bcl-w etc) were inactivated probably due to homodimerization of Bad, Bax, Bak, Bid *etc.* [22]. In anti-apoptotic signalling Nef promote activation of PI-3 and Akt kinases which subsequently leads to stimulation of Nef-associated PAK kinase to form Nef-PI-3-PAK complex for the phosphorylation of Bad [20,22]. Anti-apoptotic signalling modulated by Nef have been predicted to significantly promote viral particle release indicating its importance for viral replication [20].

HIV-1 has been reported to elevate intracellular free Ca^{2+} concentration in infected CD^{4+}T cells to promote viral replication [23]. Additionally, it had been also shown that the viral load is enhanced by drugs increasing cytoplasmic Ca^{2+} and reduced by drugs decreasing cytoplasmic Ca^{2+}in HIV-1 infected T lymphoblastoid cells [24]. Evidences suggest that transfection of HIV-1 Nef has been demonstrated to increase cytosolic calcium basal levels in Jurkat T cells, lymphoblastoid CEM cells and promyelocytic HL60 differentiated cell lines [25–27]. Nef-induced increase in cytoplasmic Ca^{2+} was shown to initiate Nuclear Factor of Activated T cells (NFAT) (a transcription factor sensitive to low-amplitude cytoplasmic Ca^{2+} changes), which activates HIV-1 LTR to induce transcription and viral replication [28–30]. The activation of calcium signalling by HIV-1 Nef reportedly represent a positive feedback mechanism in the regulation of virus replication. Ample experimental evidence exists in support of ability of Nef to simulate T cell signalling but are not linked directly to viral replication. So far, all the functions mediated by HIV-1 Nef are resultant of its interacting property with different host proteins. Identification of novel host interacting proteins is likely to reveal novel cellular pathways contributing towards viral replication and mechanistic intersections for possible pharmacological interventions.

In the present study we show that the Nef protein of HIV-1, *via* its C terminus ($Nef^{171–178}$), interacts directly with Ca^{2+}/Calmodulin dependent protein kinase II (CAMKIIδ) and ($Nef^{152–159}$) Apoptosis signal regulating kinase-1 (ASK-1). The cytoplasmic increase in Ca^{2+} concentration upon transfection with recombinant Nef in HEK-293T and Jurkat T cells [23], facilitated physical interaction of CAMKIIδ with its downstream ASK-1, forming a hetero-trimeric complex. Although physical interaction of HIV-1 Nef - ASK-1 and CAMKIIδ-ASK-1 for the regulation of the intracellular signal transduction to modulate apoptosis was individually implicated, the direct interaction between HIV-1 Nef - CAMKIIδ has thus far not been indicated. These data support an important role for Nef in controlling apoptosis in infected cells by blocking kinase activity of CAMKIIδ-ASK-1 complex during Ca^{2+} signalling required to promote of viral replication.

2. Materials and method

2.1. Reagents

Anti-CaMKIIδ (ab97531) and anti-HIV-1 Nef (ab42355) antibodies were purchased from Abcam (USA). Anti-P-Thr286CaMKII (3361S), anti-p-p38 (#9215) and anti-p38 (#9212) antibodies were purchased from cell signalling technology (USA). Protein-A (sc-2001) agarose beads, normal IgG rabbit (sc-2027) and IgG mouse (sc-2025) for immunoprecipitation were purchased from Santa Cruz Biotechnology. Cell culture reagents were purchased from Gibco, Life Technologies, unless stated otherwise. Transfection reagent Turbofect and enzymes were purchased from Thermo fisher scientific. Two-Hybrid system of protein–protein interaction was purchased from Promega (USA). Trizma Base, SDS, Glycine and agarose were purchased from Sigma.

2.2. Pull down assay of His-Nef with C. elegans and identification of protein bands by MALDI TOF-TOF

The Pull down assay was carried out following the methodology as described elsewhere [19]. The gel was stained with mass spectrometry compatible Coomassie Brilliant blue R-250 (Sigma Aldrich, C.I. 42660). Similar protocol of the experiment was performed without using Nef protein as negative control.

The protein samples were analyzed using MALDI TOF-TOF (Bencos Research Solution Pvt. Ltd.) as per manufacturer's protocol. Briefly, the protein samples were trypsin digested and peptides extracted according to standard techniques. Peptides were analyzed by mass spectrometer and generated spectra were analyzed to identify protein of interest through Mascot sequence matching software (Matrix Science) with Ludwig NR Database; Taxonomy: *Caenorhabditis elegans*.

2.3. Cell culture and transfection method

HEK-293T and Jurkat-T cells were obtained from the cell culture repository, Central Drug Research Institute, India. HEK-293T cells were cultured in DMEM and Jurkat-T cells were cultured in RPMI. Growth media was supplemented with 10% FBS (Invitrogen). 100 μg/ml penicillin, 100 μg/ml streptomycin and 50 mg/ml gentamycin. Cells were grown using standard cell culture techniques in a humidified atmosphere (95% humidity) at 37 °C and 5% CO_2. HEK-293T cells were seeded at a concentration of 0.1×10^6 cells per well in 24-well culture plate 24 h prior to transfection. Transfection was done using Turbofect (Thermo Fisher Scientific, R0533) as per manufacturer's protocol. Transfection in Jurkat-T cells was done using electroporation. Briefly, 4×10^6 cells were mixed with 20 μg plasmid DNA in 400 μl of total volume of incomplete RPMI media and transferred to 4 mm cuvettes. Electroporation was carried out at 290 V pulse (low-voltage mode) in BTX electroporator. Cells were then gently transferred to respective labeled flasks (T-25) in 5 ml of pre-warmed incomplete. The media was replaced by complete RPMI medium the following day and cells were maintained as described previously.

2.4. Peptide synthesis

The peptides were synthesized using method described elsewhere [9,31]. The nomenclature and sequences corresponding to peptides targeting the C-terminal region of Nef $^{141–207}$ are mentioned in Table 4.

2.5. Mammalian two hybrid assay

The CaMKIIδ gene was amplified using specific primers mentioned in Table 3 from CAMKIIδ clone (ID HSCD00041017) purchased from DNASU plasmid repository. The amplified product was cloned in pBIND vector. To validate interaction of CAMKIIδ with HIV-1 Nef, Nef gene (57–207 a.a) (accession No GQ184335) lacking N-myristoylation domain (1–57 a.a) was cloned in frame of activation domain of pACT vector [9]. The C-terminal truncated sequences of Nef core domain were also cloned in pACT vector using primers mentioned in Table 3.

For mammalian two hybrid assay, HEK-293T cells were seeded in 24-well culture plate as mentioned previously and co-transfected with 0.75 μg plasmid of pACT Nef and pBIND CaMKIIδ with pG5LUC vector (Promega) using Turbofect transfection reagent as per manufacturer's protocol. This model mimics the interaction of Nef with host proteins as would happen in HIV 1 infected cells. The cells were harvested 48 h, post transfection and lysed in 1× Passive Lysis Buffer (PLB) (Promega, E1941)). Relative light units (RLU) of luciferase and Renilla activity was assayed in luminometer (Barthold) using dual-luciferase reporter assay system (DLR; Promega, E1910) to identify the interaction of Nef with CAMKIIδ as well as C-terminal truncated Nef variants with CAMKIIδ.

2.6. Flow cytometry to assess Ca^{2+} influx

HEK-293T cells were seeded in 24-well and transfected with 1 μg pEYFP-N1 plasmid encoding HIV-1 Nef and pEYFP-N1 plasmid (negative control). After 24 h, cells were harvested and washed with phosphate-buffered saline (PBS), followed by incubation with 4 μM of Ca^{2+} binding dye fluo-3/AM (Thermo fisher Scientific, F1242) for 30 min at 37 °C in the dark. The samples were analyzed through flow cytometry using Cell Quest software (Becton Dickinson).

2.7. Immunoprecipitation with CaMKIIδ and p-CAMKII

Jurkat T cells (endogenously expressing CaMKIIδ) transfected with pEYFP-N1 Nef or transfected with pEYFP-N1 plasmid encoding HIV-1 Nef, CAMKIIδ and ASK-1 were cultured, harvested and lysed in I.P. Buffer [20 mM Tris base pH 8, 137 mM NaCl, 10% Glycerol, 1–2% TritonX-100 and 2 mM EDTA]. The precleared solution from this lysate was obtained through centrifugation at 13000 rpm. This extract of endogenous CaMKIIδ expressing cells was incubated with p-CaMKII antibody whereas cell extract of pEYFP-N1 of CaMKIIδ and ASK-1 transfected cells was incubated with CaMKIIδ antibody overnight with shaking at 4 °C. 50 μl protein A agarose beads (Santa Cruz Biotechnology) were mixed with CaMKIIδ/ p-CaMKII antibody cell extract as well as with rabbit IgG cell extract (negative control) for 4 h at 4 °C. Further three extensive washings were given with lysis buffer followed by mixing the sample with 5× sample loading dye to elute the protein complex and subjected to SDS-PAGE and transferred on a PVDF membrane. After two washes with PBS, the membrane was incubated in blocking buffer (5% BSA in PBST) for 1 h at room temperature. Following blocking, membrane was incubated with primary antibodies against Nef, ASK-1, CaMKIIδ/p-CaMKII. The membrane was washed five times with PBST followed by incubation with HRP-conjugated secondary antibodies for 2 h and the peroxidase activity was analyzed with the ECL chemi luminescence substrate system (USA).

2.8. Annexin-V and PI staining

Apoptosis / necrosis index was evaluated using BD Annexin-V/ PI apoptosis detecting kit following manufacturer's protocol. Briefly, Jurkat T cells were transfected with full length Nef cloned in pcDNA3.1 vector and vector alone for 24 h, followed by treatment with 20 μM $CdCl_2$ and 10 μM peptide P3 or P4 for 24 h, respectively. After that, cells were harvested followed by washing twice with PBS and re-suspended in binding buffer at a concentration of 1×10^5 cells/ml. Thereafter, FITC conjugated Annexin-V and PI were added to the 100 μl aliquot of the cell suspension and incubated for 30 min at RT (25 °C) in the dark. After adding 400 μl of 1× binding buffer samples were subjected to flow cytometry in BD FACS Calibur system (USA) to assess early and late apoptosis. Data was analyzed using CellQuest software.

2.9. Computational studies

Protein sequence retrieved from accession number 'ACS68794.1' was modelled with Modeller Version 9.15 [32]. Using methodology reported from our laboratory [33] and Other Protein Structures were retrieved from Protein Data Bank, and were prepared using 'Protein preparation wizard' of DISCOVERY STUDIO-4.1. Their binding pockets were identified using 'Binding site prediction wizard' of Discovery Studio 4.1. Protein-protein docking studies were carried out using ZDOCK module of DISCOVERY STUDIO-4.1[34]. Molecular dynamic studies were performed to check the temporal stability of the protein-protein complex, which were carried out using Gromacs version 5.04. Charm M force field [35] was used in a cubic box with TIP3P water molecules at 10 Å radius. At physiological pH, protein was found to be negatively charged. Thus, to make electrostatically neutral, sodium ions were added replacing solvent molecules. Energy minimization was performed on system using Steepest-descent energy minimization. Isothermal-isochoric equilibration and isotropic-pressure coupling were performed using PME method and Parrinello-Rahman method [36], respectively, using 100,000 steps. Finally, the system was subjected to molecular dynamics simulation for 10 ns for Dimer (ASK-1 and CAMKII) and 15 ns for Trimer complex (Nef, ASK-1 and CAMKII). To identify interacting residues, NON-BONDING interaction monitor module of Discovery Studio 4.1 was used. This module identifies all the non-bonding interactions including hydrogen bonds (classical, non-classical, salt bridges), electrostatic interactions (charge and pi-charge), hydrophobic interactions (Pi-hydrophobic, alkyl-hydrophobic, mixed/ Pi/alkyl-hydrophobic). Image files were generated using UCSF chimera [37].

2.10. Statistical analysis

Numerical data was tested for statistical significance using paired student's *t*-test (two-tailed). Differences were considered significant when $p \le 0.05$(*), and very significant when $p \le 0.01$(**). All data were analyzed by graph pad-prism 5.

3. Results

3.1. Identification and validation of novel interaction of HIV 1 Nef with CaMKIIδ

Using pull down assay with HIV-1 Nef in *C. elegans* model [19], we identified six novel Nef interacting host proteins Table 1 (S.1A & B.). Amongst the proteins identified, UNC-43 (Acc. No. gi71991647) was shortlisted for the present study based on top score values and high percentage peptide coverage. Further sequence-based homology search confirmed Calcium/calmodulin dependent protein kinase II subunit delta (CaMKIIδ) (Acc. No. D6R938) as the corresponding human homolog of UNC-43 Table 2.

To further assess the interaction of HIV-1 Nef with human homolog CaMKIIδ, mammalian two-hybrid assay was performed in HEK-293T cells This model mimics the interaction as would happen in HIV-1 infected cells [9]. Co-transfection with VP16-Nef and GAL4-CaMKIIδ clones in HEK-293T cells for 48 h demonstrated a 43.6-fold increase in Luciferase/Renilla expression when compared to their respective controls (VP16 and GAL4 without inserts) (Fig. 1A). To validate the physical association between Nef and CaMKIIδ, a coimmunoprecipitation assay was also performed. Jurkat T cells were cotransfected with pEYFP-N1 plasmids encoding for HIV-1 Nef and CaMKIIδ. The complexes were immunoprecipitated with anti-CaMKIIδ antibody. Fig. 1B depicts the western blot analysis of the immunoprecipitated lysates confirming the physical interaction of HIV-Nef and CaMKIIδ when compared to IgG control. Altogether, these results confirmed the novel

Table 1
Nef interacting proteins of *C.elegans* identified by MALDI TOF-TOF. The proteins were detected by MALDI TOF-TOF and gene, accession number and protein description as shown in the table. **a**: Percent wise matching of the peptide with the whole protein sequence. **b**: Score is - 10*Log(P), where P is the probability that an observed match is a random event. The value obtained during MALDI TOF-TOF on the basis of matching of the peptide within the protein sequence.

Sr. no	Accession	Gene	Protein name	Coverage[a]	Score[b]
1	gi71991647	UNC-43	Coordinated family member	15	48
2	Q9GTF9	nhr-54	Nuclear hormone receptor family member	12	34
3	P34382	Far-1	Fatty-acid and retinol-binding protein	23	34
4	Q21570	Nphp-1	Nephrocystin-1-like protein	13	33
5	Q21988	Amx-1	Amine oxidase family member	10	31
6	Q9XXK1	H28O16.1	ATP synthase subunit alpha	10	36

interaction of human CaMKIIδ protein with HIV-1 Nef.

3.2. Molecular characterization and validation of HIV-1 Nef protein domains interacting with CAMKIIδ

HIV-1 Nef does not have any enzymatic activity and functions as an adaptor protein bringing together different host proteins [6]. Interaction with host proteins is mainly attributed to the core domain structure of Nef consisting of 57–207 amino acids [38]. To better explore the Nef-CaMKIIδ interaction, Nef mutants harboring 33 (Nef $^{57–174}$) and 66 (Nef $^{57–141}$) amino acids deletions at the C-terminal were prepared (Fig. 1C). The truncations were made to eliminate the known functional domains of the protein. Through mammalian-two hybrid assay in HEK-293T cells, we found that Nef $^{57–174}$ and Nef $^{57–141}$ exhibited only 9.15 and 6.20-fold increase in Luciferase/Renilla expression compared to reference core domain of Nef $^{57–207}$ showing 43.6-fold increase in Luciferase/Renilla expression and pACT - pBIND (negative control) vector control, respectively (Fig. 1D). Analyzing the effect of Nef mutants, we observed a deletion of 33 and 66 amino acids from C-terminal of Nef significantly abrogated the interaction affinity of HIV-1 Nef with CaMKIIδ. The data suggests C-terminal of Nef $^{141–207}$ to be crucial for the association with CaMKIIδ. Based on these findings, we next sought to analyze the minimal region in the C terminal of Nef mediating physical interaction with CaMKIIδ. We designed five different peptide inhibitors (P1, P2, P3 and P4) corresponding to different regions of the C terminal of HIV-1 Nef to disrupt the interaction between Nef and CaMKIIδ through competitive binding (Table 4). We also designed peptide P5 targeting Nef$^{73–78}$, a known domain of Nef interaction with MAPK (Hck) Src family kinase [39], to check the involvement of this motif in CaMKIIδ interaction.

The peptide inhibitors were screened in HEK-293T cells through mammalian two hybrid assay post transfection with VP16-Nef $^{57–207}$and GAL4-CaMKIIδ constructs along with pGLUC5. Treatment of 10 μM of P1, P2, P3, P4 and P5 for 24 h resulted in 21.80, 19.20, 5.37, 4.43 and 37.6-fold luciferase/Renilla expression, respectively (Fig. 1E). In our results, treatment with the peptide P3 and P4 demonstrated significant impairment in the Nef-CaMKIIδ interaction. The inhibition of Nef-CaMKIIδ interaction in presence of Peptide P3 and P4 was also confirmed through co-immunoprecipitation assay. Jurkat T cells were co-transfected with pEYFP-N1plasmids encoding Nef and CaMKIIδ for 24 h followed by treatment with 10 μM P3 and P4 separately for 24 h. The complex was immunoprecipitated with CaMKIIδ and IgG (negative control). Western blot of the immunoprecipitated complex with anti-CaMKIIδ and anti-Nef antibodies confirm that no interaction was found in presence of P3 and P4 between CAMKIIδ and Nef (Fig. 1F). Collectively, the data indicates that the physical interaction of CAMKIIδ with HIV-1 Nef is mediated at the peptide P3 (Nef$^{152–159}$) and P4 (Nef$^{171–178}$) regions within the C-terminal of Nef.

3.3. HIV-1 Nef physically associates with CAMKIIδ and ASK-1 to form Nef - CAMKIIδ - ASK-1 heterotrimeric complex

The Peptide P3 used in the present study surprisingly corresponds to the same domain in Nef responsible for Nef-ASK-1 interaction [9]. Earlier studies have shown that ASK-1 is present downstream to CAMKIIδ [40]. CAMKIIδ has also been shown to directly phosphorylate ASK-1 upon Ca^{2+} induction [40,41]. Expression of HIV-1 Nef in Jurkat T cells and other cell lines have demonstrated increased intracellular Ca^{2+} levels [26]. In our results, we observed a modest but significant higher intracellular calcium levels in Nef-transfected HEK-293T cells compared with identically processed cells transfected with the control vector (S.2A & B). These findings led us to delineate the structural relationship of Nef, CAMKIIδ and ASK-1.

We performed *in silico* studies to check our hypothesis. We observed that all three proteins *viz.* Nef, ASK-1 and CAMKII interact with one another and possibly form a heterotrimeric complex. To decipher the possible mode of protein-protein interaction, we performed docking studies using *Z*-DOCK. We carried out 3 sets of studies to predict which protein could have highest possibility of interaction with another subunit, to form a dimer. Table 5A summarizes the docking results of protein-protein interactions using all possible combination of proteins to a dimer. From the *in silico* results, it was predicted that there might be a strong possibility of dimer formation between ASK-1– CaMKII (Fig. 2A & B), while the possibility of formation of dimer between ASK-1– Nef and CAMKII – Nef might be less (Table 5A). Nevertheless, the possibility of formation of other dimers could not be ruled out. For set-1 studies as depicted in Fig. 2A & B, docking studies indicated that the largest cluster of docked posed between protein ASK-1 having PDB-id '4BF2' [42]. The protein CaMKII having PDB-id '3SOA' [43]. For the lowest energy pose of top most cluster, dimer had *Z*-Dock score of '66.43' which was energy minimized and subjected to simulation studies using Gromacs (Table 5A). The dimer was observed to be stable having flat peak in the RMSD plot for 10 ns MD run (Fig. 2B). The conformation which was generated after simulating the dimeric system was then analyzed to identify interacting residues which were involved in non-bonding interactions. The residues of CAMKII important for non-bonded interaction with ASK-1, as identified by computational studies, were Arg^{52}, Trp^{170}, Phe^{171}, Phe^{173}, Gly^{175}, Leu^{185}, Arg^{186}, Lys^{187}, Asp^{188}, Gln^{218}, His^{219}, Tyr^{222}, Gln^{223}, Lys^{226}, Leu^{308}, Leu^{408} and Ile^{413}. Similarly, residues of ASK-1 important for non-bonded interaction with CAMKII were Gln^{798}, Lys^{827}, Ala^{830}, Gly^{831}, Ile^{832}, Asn^{833}, Pro^{834}, Thr^{836}, Glu^{849}, Asp^{852}, Lys^{853}, Arg^{856}, Gly^{857}, Tyr^{858}, Lys^{860}, Met^{896}, Phe^{897}, Asp^{922} and Asp^{924}. The Nef protein was modelled with template having PDB-id '2XI1' [38]. The dimeric complex of CAMKII – ASK-1 was further docked with Nef protein using ZDOCK module having ZDOCK score '74.00' (Table 5B). The best pose of highest ranked cluster was further subjected to molecular dynamic simulation to stabilize the trimeric complex (Fig. 2C). The trimeric system was found to be stable after simulation studies as indicated by the RMSD plot (Fig. 2D). Visual inspection of the trimeric model does indicate that the fragment of Nef from '**152–159**' occurs near with ASK-1 chain, while fragment '**171–178**' occurs near to CAMKII chain (Fig. 2E). It was observed that the residues of Nef which were important for interaction with ASK-1 protein were Lys^{105}, Arg^{135}, Glu^{156}, Glu^{161}, Asn^{163}, Cys^{163}, Leu^{166} and His^{167}. Of these, **Glu^{156}** exhibited highly stable hydrogen bond with 1.4 Å. Similarly, residues of Nef-1 which were important for interaction

Table 2
Blast search of *C. elegans* protein with human protein database. The *C.elegans* proteins were aligned with the human protein database in NCBI. The BLAST search tool was used and identified their human homolog as shown in the table. **a:** Homology of *C.elegans* protein with human protein in term of amino acid identity. **b:** Score is a numerical value that describes the overall quality of an alignment. Higher numbers correspond to higher similarity.

Sr. no	*C. elegans* protein	*C. elegans* accession	*H. sapiens* protein	*H. sapiens* accession	Identity[a]	Score[b]
1.	Coordinated family member	gi71991647	Calcium/calmodulin-dependent protein kinase type II subunit delta	D6R938	71.9	1814
2.	Nuclear hormone receptor family member	Q9GTF9	Steroidogenic factor 1 nuclear receptor	Q5T6F6	45.6	188
3.	Fatty-acid and retinol-binding protein	P34382	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase beta-4	Q151472	33.7	78
4.	Nephrocystin-1-like protein	Q21570	isoform 3 of nephrocystin 1	O152592	22.6	378
5.	Amine oxidase family member	Q21988	1-phosphatidylinositol 4,5 bisphosphate phosphodiesterase beta-4	Q8NB78	34.7	1006
6.	ATP synthase subunit alpha	Q9XXK1	ATP synthase subunit alpha, mitochondrial	V9HW26	84.1	2169

Table 3
Primer sequences for amplifying CaMKIIδ, Nef and truncated Nef.

Primer name	Sequence (5′……3′)
FP CAMKIIδ (pBIND)	GTCGACCCATGGCTTCGACCACAACCT
RP CAMKIIδ (pBIND)	GCGGCCTACAACTTGATGGGTACTGT
FP CAMKIIδ (pEYFP-N1)	GAATTCATGGCTTCGACCACAACCTGCA
RP CAMKIIδ (pEYFP-N1)	GGATCCTTCAACTTGATGGGTACTGTTGGTGA
FP Nef 400 (pACT)	CATGGATCCGTGGAGCACTTACAAGCAGCA
RP Nef 400 (pACT)	CATGGATCCTCAGCAGTCTTTGTAATACTCC
RP Nef 300 (pACT)	CATTCTAGACTTGAAGCACCATCCAAAAGT
RP Nef 200 (pACT)	CATTCTAGAGAAGCTGAGATCTACTGCC

Table 4
Table demonstrating peptide sequences with respect to the amino acid regions in Nef protein.

Peptide nomenclature	Targeted amino acids region within Nef	Sequence
P1	$Nef^{129–138}$	TPGPGVRYP
P2	$Nef^{160–168}$	GENNCLLH
P3	$Nef^{152–159}$	DEVGEANN
P4	$Nef^{171–178}$	LHGMEDPE
P5	$Nef^{70–80}$	PVTPQVPLRP

Table 5A
The table summarizes the different set of proteins used to study the protein-protein interaction possibilities for different dimeric complexes along the *Z*-dock score obtained from the protein-protein interaction of different proteins with one another using Z-DOCK module of Discovery Studio-4.1.

Name of protein	Nef	ASK-1	CaMKII	Z-DOCK SCORE
Set-1 studies		+	+	66.43
Set-2 studies	+		+	23.56
Set-3 studies	+	+		49.05

Table 5B
The table summarizes the different set of proteins used to study the protein-protein interaction possibilities for different heterotrimeric complexes along the Z-dock score obtained from the protein-protein interaction of different proteins with one another using Z-DOCK module of Discovery Studio-4.1.

Name of protein dimer	Nef	ASK-1	CaMKII	Z-DOCK SCORE
CaMKII – ASK-1	+			74.00
NEF – CaMKII		+		55.00
NEF – ASK-1			+	24.33

with CAMKII were Tyr^{128}, Pro^{130}, Pro^{132}, Ser^{170}, His^{173}, Glu^{198}, Leu^{199} and His^{200}. Corresponding residues of ASK-1 and CAMKII which were found to be interacting with Nef-1 were 'Lys^{688}, Glu^{713}, Asp^{715}, Gly^{748}, Arg^{767}, Lys^{805}, Asp^{807}, Phe^{839}, Gln^{844}, Lys^{878}' and 'Arg^{296}, Ala^{309}, Arg^{311}, Asn^{312}, Phe^{313}, Ser^{314}, Lys^{317}, Asn^{386}, Ala^{400}, Ile^{402}' respectively. Although many residues in segment '160–170' were found to be interacting with the ASK-1 and CAMKII but large fluctuations in this region do indicate that the interactions were not stable unlike segment '152–155' and '171–178' (Fig. 2F). Interestingly, it was observed that the residues namely Thr^{383} of CAMKII and Thr^{842} of ASK-1 had 15.754 Å distance between their side chains which may be suitable for assisting these residues for binding ATP or ADP molecules for phosphorylation. Other hydroxyl containing residues namely Ser^{419} and Thr^{405} of CAMKII also occur near Thr^{383} and there may be possibility of interaction of these residues during phosphorylation. Interestingly, the Nef protein in heterotrimeric complex binds to the same cavity where residues namely Thr^{383}, Ser^{419}, Thr^{405} of CAMKII and Thr^{842} of ASK-1 occurs making the site inaccessible for phosphorylation and thereby abolishing kinase activity of ASK-1 on binding with CAMKII (Fig. 2G). Our results demonstrated that all three proteins *viz.* Nef, ASK-1 and

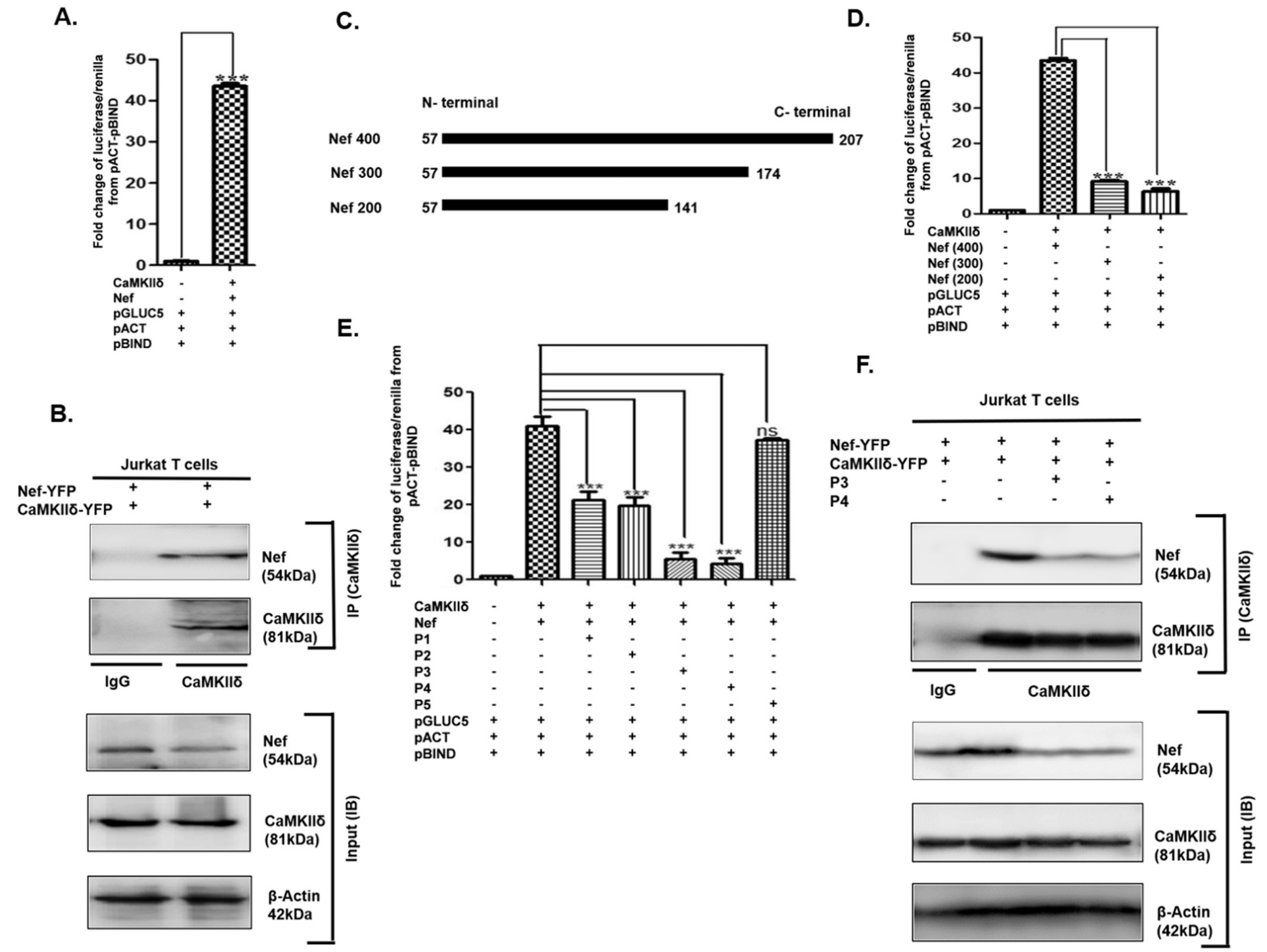

Fig. 1. Molecular characterization of the Nef-CaMKIIδ interaction.

A. Mammalian-two hybrid assay to confirm Nef-CaMKIIδ interaction**.** HEK-293T cells were co-transfected with pACT-Nef and pBIND-CaMKIIδ along with pG5LUC vector. Nef-CaMKIIδ interaction is measured by fold increase in luciferase/renilla expression over pACT-pBIND negative control vector.

B. pEYFP-N1 plasmids encoding Nef and CaMKIIδ were co-transfected in Jurkat T cells and after 48 h of transfection, co-immunoprecipitation was performed with anti-CaMKIIδ antibody. Western blot analysis of immunoprecipitated lysates, performed with the anti-CaMKIIδ and anti-Nef antibodies, shows that Nef interacts with CaMKIIδ.

C. Representative image of the truncated Nef sequences *viz.* Nef 400 (57-207aa), Nef 300 (57–174 aa) and Nef 200 (57–141 aa). The nomenclature was made based on number of nucleotides.

D. Mammalian two-hybrid assay in HEK-293T cells confirmed that C-terminal of Nef ranging from 174 to 207aa was majorly responsible for Nef-CaMKIIδ interaction; the other domains Nef 300 and Nef 200 exhibited relatively less (yet statistically significant) interaction with CaMKIIδ.

E. Mammalian two-hybrid assay in HEK-293T cells as described in panel (A) showing the efficacy of competitive peptide inhibitors P1,P2,P3,P4 and P5 (10μM each for 24 h) in destabilizing the physical association of Nef and CaMKIIδ.

F. Treatment with 10uM of peptides P3 and P4 for 24 h post 24 h of transfection as done in panel (B) significantly reduced Nef - CaMKIIδ interaction confirmed through co-immunoprecipitation in Jurkat T cells.

(*** indicates *p* value ≤ 0.0001 and ns for non-significant). All experiments were done three times and found similar results.

CaMKII interact with one another and possibly form a heterotrimeric complex.

The suspected Nef-CaMKIIδ-ASK-1 interaction was also checked in Jurkat T cells upon transfection with vectors encoding all three proteins. Upon IP using CaMKIIδ antibody, the Nef-CaMKIIδ-ASK-1 heterotrimeric complex was confirmed (Fig. 2H). The results suggest that Nef mediated CAMKIIδ-ASK-1 interaction provides a platform for binding of Nef to both the proteins.

3.4. Calcium induction enhance the stability of the heterotrimeric complex association

The regulation of CaMKII - ASK-1 complex in presence of HIV-1 Nef mediated $[Ca^{2+}]_I$ is not known. To study the same, we first investigated whether the rise in cytosolic $[Ca^{2+}]_I$ lead to physical interaction of endogenous pCaMKII with ASK-1 in Jurkat T cells. We used already reported cadmium treatment [44] and Nef expression to activate CaMKII and assessed its interaction with ASK-1 through immunoprecipitation with pCaMKII antibody. We used 20 μM $CdCl_2$ treatment for 24 h to induce cadmium [Cd (2+)] mediated phosphorylation of CaMKII through increasing Ca^{2+} concentration in Jurkat T cells and checked the viability of the cells at the given dose by screening range of $CdCl_2$ concentration treatments (S. 3A-E). As shown in Fig. 3, expression of Nef resulted into interaction of Nef-pCaMKII and ASK-1 trimeric complex when confirmed through western blotting following immunoprecipitation. Further the treatment of Cd (2+) along with Nef

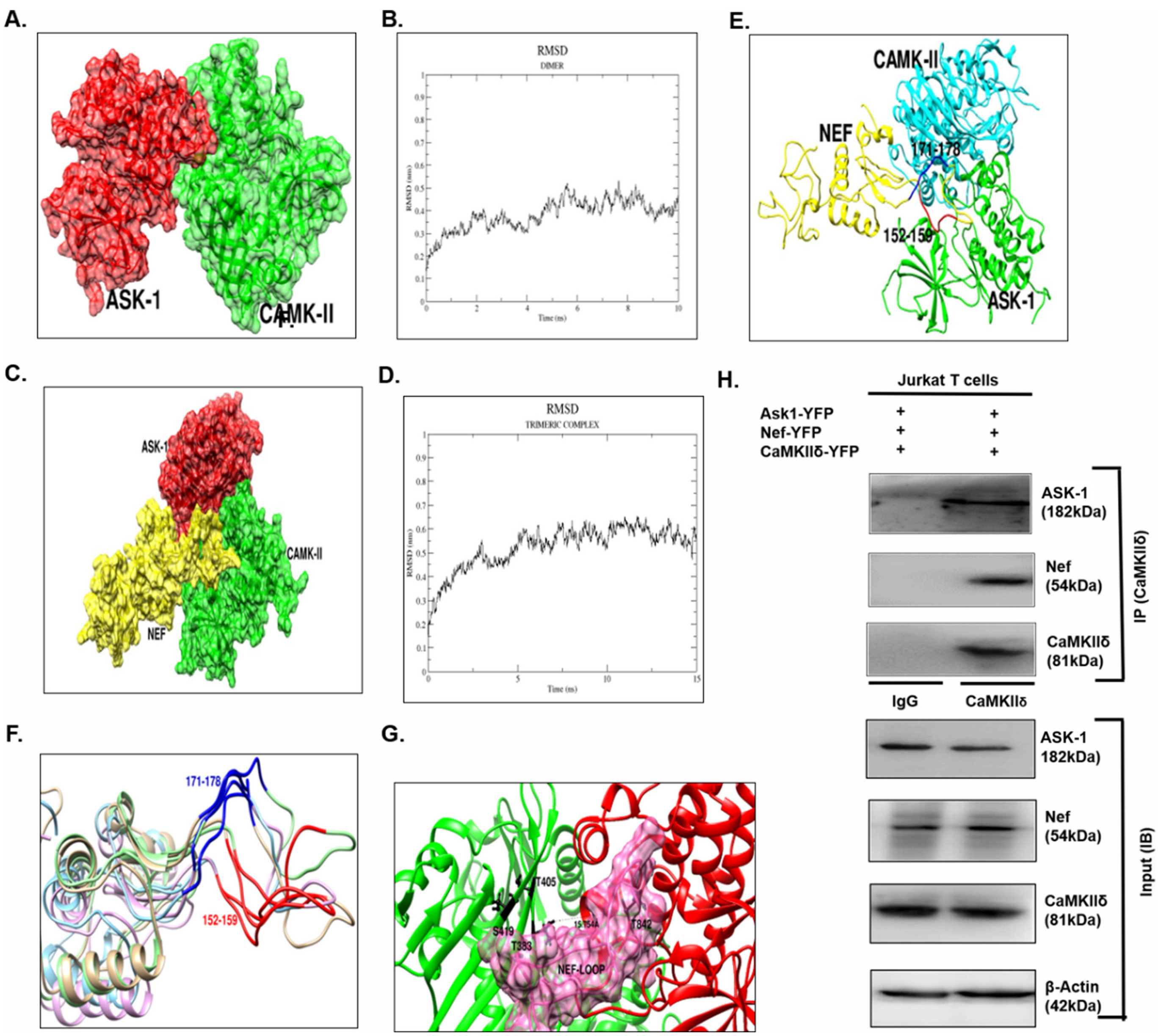

Fig 2. CaMKII formed a stable dimer with ASK-1 and led the formation of a Trimeric complex with Nef and ASK-1.
A. The images indicate the surface view model of a dimeric protein complex between ASK-1 – CaMKII proteins.
B. The image indicates the RMSD plot the dimeric of the CaMKII-ASK-1 complex after the initial 5 ns the complex was observed to be stable.
C. The trimeric protein complex formed between Nef, ASK-1 and CaMKII proteins, respectively
D. The image indicates the RMSD plot of the trimeric complex of CaMKII-ASK-1-Nef. After the initial 5 ns the complex was observed to be stable.
E. The image indicates the segments of Nef protein, which occurs in close proximity to ASK-1 protein (**152–159**) and CaMKII protein (**171–179**) in the docked trimeric complex.
F. The image indicates overlapped Nef protein conformations, extracted after 5, 10 and 15 ns along with initial docked conformation. The red region indicates the fragment '152–159'which is found to be interacting with ASK-1 protein while blue region indicates the fragment '171–178′ which is interacting with CaMKII protein. These red and blue fragments were found to be less fluctuating and stable as compared to the region '160–170.
G. The image indicates the possible domain site of ASK-1 and CaMKII for interaction with Nef protein loop region making a trimeric complex with both proteins and alter the signalling.
H. Jurkat T cells were co-transfected with pEYFP-N1 plasmids encoding ASK-1 and CaMKIIδ and Nef. Co-immunoprecipitation was performed with anti-CaMKIIδ antibody. Western blot analysis of immunoprecipitated lysates, performed with the anti-CaMKIIδ, anti-ASK-1 antibodies and anti-Nef antibodies, shows that Nef-ASK-1 and CaMKIIδ forms a heterotrimeric complex. All the experiments were done three times and similar results were found. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

expression was found to intensify the heterotrimeric complex when compared to the interaction intensity of Nef alone. Interestingly, treatment with either of the competitive peptide inhibitors P3 or P4 leads to reduction in the binding intensity of Nef from pCAMKII-ASK-1 complex in presence of Cd(2+) mediated $[Ca^{2+}]_I$ in Jurkat T cells (Fig. 3). Collectively, the data suggests that the interaction of pCaMKII-ASK-1 is required for the interaction of HIV-Nef during $[Ca^{2+}]_I$ and blocking of Nef binding domains in either CaMKII or ASK-1individually destabilizes the binding of Nef to the pCaMKII-ASK-1 complex.

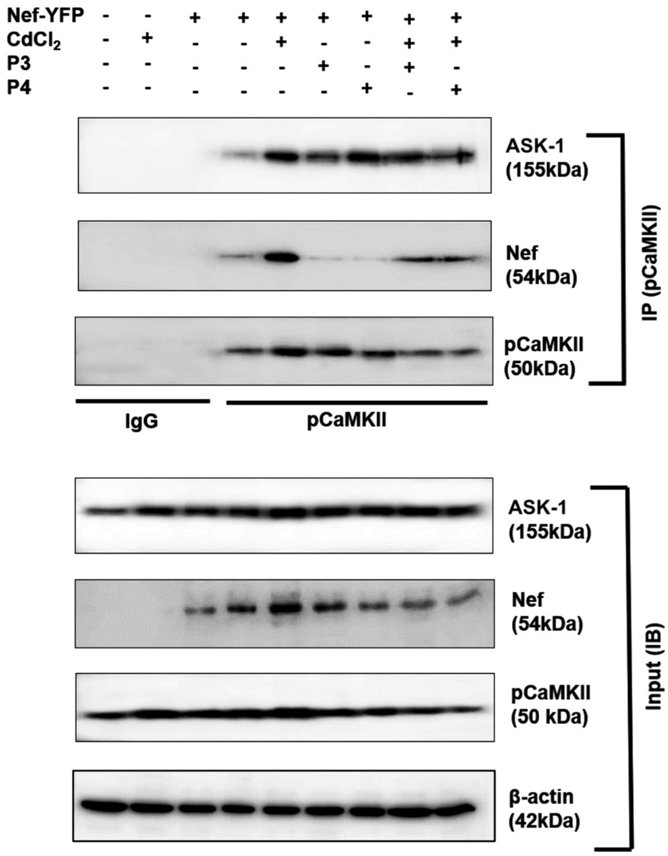

Fig. 3. Calcium stimulation induced association of heterotrimeric complex. Jurkat T cell line was transfected with Nef and vector control followed by treatment with 20 μM $CdCl_2$. Nef-expressing cells were treated with 10uM of peptide 3 and 4 precisely after the 24 h of transfection. Cells were harvested after 48 h of transfection and protein lysate were incubated with anti-pCaMKII antibody and immunoblotted with anti-ASK-1, Nef and pCaMKII antibody.

3.5. HIV-1 Nef alleviates CaMKII-ASK-1 mediated p38 phosphorylation and apoptosis

The interaction of pCaMKII with death signalling molecule ASK-1 during Ca^{2+} signalling has been reportedly implicated in apoptosis induction through activation of downstream p38 MAP kinase signalling pathway [40,45]. Also, Cd(2+) treatment mediated apoptosis in mesangial cells have been previously shown to be dependent on CAMKII phosphorylation, where specific inhibition of CAMKII but not Erk demonstrated suppression of the apoptosis [46]. Furthermore, p38 activation is an early and specific regulatory event for the Cd(2+) provoked apoptosis in promonocytic cells [47]. We first evaluated p38 phosphorylation status in presence of Nef and Cd(2+) mediated $[Ca^{2+}]_I$ in HEK-293T and Jurkat T cells. We found that alone 20 μM of Cd(2+) treatment displayed a significant p38 phosphorylation, which was reduced in presence of Nef in both the cell lines. However, the treatment with peptides P3 and P4 individually restored the p38 phosphorylation levels comparative to Cd(2+) treated cells when confirmed through western blotting (Fig. 4A). Furthermore, apoptosis by 20 μM of Cd(2+) treatment in Jurkat T cells was also found to get reduced in presence of Nef, which was restored upon addition of peptide inhibitors P3 or P4, confirmed through AnnexinV/FITC based flow cytometry (Fig. 4B & C). Additionally, treatment with P3 or P4 also restored the apoptosis in Nef transfected HEK-293T cells (Fig. 4B & C). Taken together the results suggest the role of Nef in impairing apoptosis mediated through pCAMKIIδ-ASK-1-p38 axis. Also, the peptide inhibitors P3 and P4, by blocking binding sites of CAMKIIδ and ASK-1 demonstrates the ability to restore pCAMKIIδ-ASK-1-p38 mediated apoptosis. Our study demonstrates that Nef interacts with CaMKIIδ to modulate its downstream signalling resulting into inhibition of apoptosis.

4. Discussion

In this study we identified a novel HIV-1 Nef interacting host protein, Calcium/calmodulin-dependent protein kinase type II subunit delta (CaMKIIδ) employing previously established *C.elegans* host model for Nef pathogenesis [19,48]. Ca^{2+}/calmodulin-dependent serine/threonine protein kinases (CaMK) are one of the central transducers of Ca^{2+} signalling in different cells. [49]. CaMK subfamily comprises of CaMKI, CaMKII, and CaMKIV members [41]. CaMKII is a multimeric ubiquitous enzyme encoded by four different genes in mammals known as CaMKII α, β, ϒ and δ, each of which produces mRNA that can be alternatively spliced, giving rise to approximately 30 different proteins [50]. These genes have a high degree of sequence homology but show differential tissue expression. CaMKIIα and β are mostly expressed in neuronal tissue whereas γ and δ are expressed throughout the body [50]. Structural analysis of all the isoforms of CAMKII reveals an overall similar organization comprising of an N-terminal catalytic domain, followed by a regulatory domain that contains an autoinhibitory region and a CaM-binding site, and a C-terminal association domain, which bring together all the subunits to assemble into holoenzymes [51]. Due to the complex autoinhibitory structural arrangement of CAMKII subunits like other CaMK, CaMKII primarily exists in an inactive state as its autoinhibitory domain blocks catalytic activity. As the intracellular Ca^{2+} influx $[Ca^{2+}]_I$ rises, Ca^{2+} saturated CaM binds to the CaM-binding subunit of CAMKII, which gets autophosphorylated (at Thr^{286} in CaMKIIα and Thr^{287} in the other isoforms) by an adjacent activated CaMKII subunit, thereby fully stimulating each subunit of the CaMKII holoenzyme and generating Ca^{2+-}independent activity [51]. Binding of Ca^{2+}/CaM also facilitates better ATP gripping, which the active site uses as a phosphate source, for increasing the activity of the enzyme [52]. Hence, activation of CAMKII signalling cascade is dependent on the intracellular Ca^{2+} levels.

It has been shown that pharmacological agents that increase intracellular Ca^{2+} rise enhance HIV-1 protein expression whereas Ca^{2+} blocking drugs inhibits viral replication in HIV-1 infected T lymphoblastoid cells [25]. This study indicates that viral-induced mobilization of Ca^{2+} from intracellular storage pools or from increasing extracellular Ca^{2+} influx regulating pathways may represent a key component of HIV-1 replication [27]. Four HIV-1 proteins Tat, membrane glycoprotein gp120, Vpr and Nef have been shown to increase intracellular Ca^{2+} which suggest that Ca^{2+} signalling dependent cellular pathways are playing important role in HIV-1 pathogenesis [25]. The known function induced by Nef-mediated Ca^{2+} increase is the activation of NFAT, a transcriptional factor responding to the low-amplitude intracellular Ca^{2+} oscillation, to promote viral gene transcription and replication [28,29]. However, the increase in Ca^{2+} level induced by Nef that activates CaMKIIδ and mediates its direct binding with Nef is not known. In this study, first we identified the direct binding of HIV-1 Nef −C terminal with CaMKIIδ and then identified minimal domain of Nef (171-LHGMEDPE-178) at the −C terminal crucial for establishing physical interaction with CaMKIIδ. Interestingly, while screening competitive peptide inhibitors targeting different domains of Nef $^{141–207}$ (C terminal) through mammalian two hybrid assay as well as immunoprecipitation assay, we found that apart from peptide P4 ($Nef^{171–178}$), peptide P3 targeting (DEVGEANN) targeting Nef $^{152–159}$ also exhibited significant perturbation in the binding of Nef with CaMKIIδ. Peptide P3 was previously identified domain by our group essential for mediating Nef – ASK-1 interaction by which Nef negatively modulates ASK-1 function in infected cells to avoid FasL/ TNF-α induced apoptosis [9].

In *C.elegans*, UNC-43, homolog of human CaMKII has been shown to act on its downstream target nsy-1, human homolog of ASK-1 to

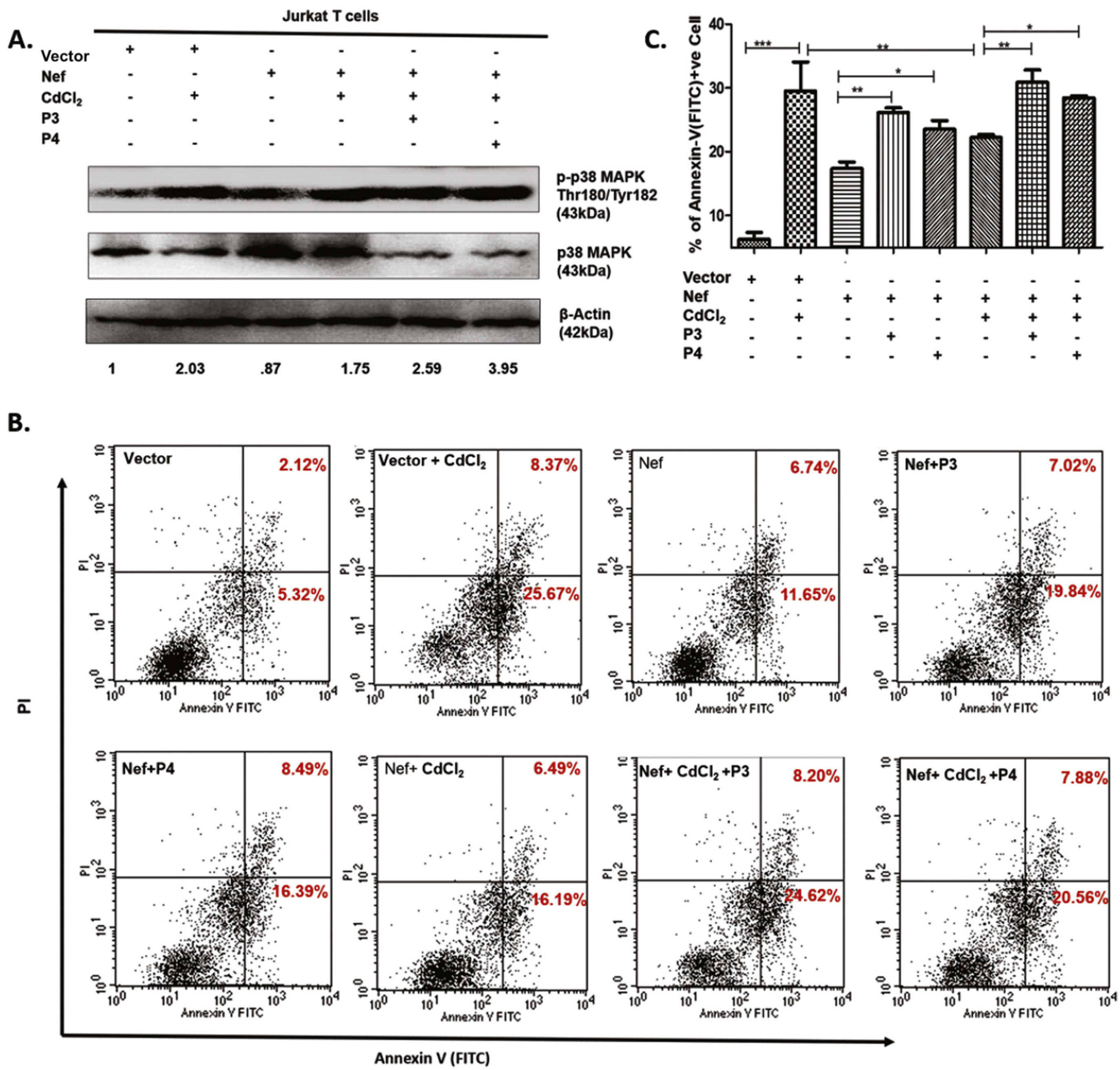

Fig. 4. Nef interferes with the pCaMKII-ASK-1 induced p38 phosphorylation and apoptosis in presence of Cd(+) mediated $[Ca^{2+}]_I$.
A. Jurkat T cells were transfected with Nef and vector control and treated with 20 μM $CdCl_2$ for 24 h. Batches of same set of cells were also treated with 10uM of peptide 3 and 4 precisely after the 24 h of transfection. Total and p-p38 levels were analyzed through western blotting after 48 h of transfection. Integrated density was determined by densitometry analysis using Image J software. All experiments were performed three times and found similar results.
B. Annexin-V/PI labelling followed by flow cytometric analysis showing early and late apoptosis in Nef and vector (pcDNA3.1) transfected Jurkat T cells in presence and absence of $CdCl_2$ treatment. Nef transfected cells were also treated with peptides P3 and P4 alone and in presence of $CdCl_2$ treatment.
C. Bar graph represents the sum of early and late apoptosis of Jurkat T cells as shown in panel (B).
(* indicates p value ≤ 0.05, ** indicates p value ≤ 0.001, *** indicates p value ≤ 0.0001). All experiments were done three times and found the same results.

accomplish a lateral signalling decision required for asymmetric olfactory neuron fates in AWC cells [53]. Additionally, CAMKII have been reported to directly associate with ASK-1 to stimulate indirect phosphorylation at Thr^{845} site of ASK-1 in HEK-293T cells, which is essential for ASK-1 activation [40]. However, it has been also well elucidated that CAMKII(δ_3) mediated activation of ASK-1 by direct binding is specific to Ca^{2+} induction and does not takes place during H_2O_2 induction [41]. In our experiments, we also found pCAMKIIδ-ASK-1 interaction in presence of Cd(2+) treatment which is a known stimulator of Ca^{2+} mediated CAMKII activation [44] in Jurkat T cells upon IP with pCAMKIIδ. Overall, the above findings raised the possibility of Nef mediated increased $[Ca^{2+}]_I$ with direct phosphorylation of CAMKIIδ and further physical association of pCAMKIIδ with ASK-1. Also, the binding sites in Nef with CAMKIIδ identified in the study and with ASK-1 previously [21], are situated very close to each other on the C-terminal of Nef ($Nef^{152–159}$ for ASK-1 and Nef $^{171–178}$ for CAMKIIδ) pointed an indication towards Nef-CAMKIIδ-ASK-1 heterotrimeric complex formation. Based on the *in silico* docking results using known structures of proteins the *Z*-score value indicated CAMKIIδ-ASK-1 as the most favourable complex compared to Nef-CaMKII and Nef-ASK-1.

Further, superimposition of Nef on CaMKII-ASK-1 complex predicted trimeric complex. The amino acid of the side chain of CaMKII and ASK-1 found to be close enough to form a stable complex and maintain a structure so that the loop region of Nef fits in the cleft of dimer protein. These results suggest that ASK-1-CAMKIIδ association act as the crucial intermediate for Nef interaction. We also found interaction of the three proteins in both overexpressed and endogenous, Nef transfected forms in Jurkat T cells. In Jurkat T cells, increased $[Ca^{2+}]_I$ upon recombinant Nef expression has been already reported [23]. We also found that the treatment with any competitive peptide inhibitors against CaMKIIδ or ASK-1 binding Nef domain destabilized interaction of Nef with pCaMKIIδ-ASK-1 complex completely in presence of Cd(2+) treatment. Evidences suggest that the increase in cytoplasmic $[Ca^{2+}]_I$ during viral infection and Nef expression promote viral replication. The present findings indicate that Nef induced $[Ca^{2+}]_I$ also activate Ca^{2+}/CaM binding protein CAMKIIδ to directly phosphorylate its downstream target ASK-1 for its further activation. Perhaps, Nef form a hetero-trimeric complex to block the kinase activity of pCaMKIIδ and ASK-1, hence, perturbing pCaMKIIδ-ASK-1 mediated cellular signalling that may endanger viral persistence within the infected cells.

ASK-1 is a mitogen-activated protein (MAP) kinase kinase family member, which is known to activate and in response to a variety of cytotoxic stresses, including TNF, Fas and reactive oxygen species (ROS). ASK-1 is a crucial constituent in cytokine- and stress-induced apoptosis [21,40]. ASK-1 executes apoptosis through JNK1 and p38 kinase activation [9]. The study by Takeda *et al* identified CaMKII–ASK-1–p38 MAP kinase cascade as a novel Ca^{2+} signal mediator [40]. They demonstrated that inhibiting CAMKII expression in HeLa cells significantly impaired the activation of ASK-1 and p38. Also, in absence of ASK-1 in MEF cells, CAMKII failed to activate p38, however, p38 become active in presence of ASK-1 and CAMKII. The study confirmed that ASK-1 was indispensable for CaMKII-induced p38 activation during Ca^{2+} signalling but not in ROS mediated signalling [40]. In our earlier report, we identified the dynamics of Nef and ASK-1 interaction, where expression of Nef in HEK-293T cells, markedly potentiated Ser^{967} phosphorylation of ASK-1, thereby making it catalytically inactive which abrogated ASK-1 mediated apoptosis [9]. Further, treatment with competitive peptide inhibitor P3 targeting $Nef^{152–159}$ destabilized Nef-ASK-1 interaction and reversed the inhibitory effect of HIV-1 Nef expression on ASK-1 mediated apoptosis [9]. In our investigation, we found Cd(2+) treatment mediated $[Ca^{2+}]_I$ resulted in increase in p38 phosphorylation and apoptosis, through CAMKIIδ mediated ASK-1 activation in Jurkat T cells. Furthermore, expression of HIV-1 Nef inhibited p38 activation and apoptosis. Additionally, treatment with peptide P3 or P4 resulted into p38 phosphorylation in presence of Nef and rescued $[Ca^{2+}]_I$ mediated apoptosis.

Overall in this study, we identified novel Nef and CAMKIIδ interaction and delineated CAMKIIδ binding motif on the C terminal of Nef ($Nef^{171–179}$). Till date, Nef mediated increase in cytosolic calcium concentration has been associated with T cell activation and viral replication. We are the first to report the regulatory function of Nef *in vitro*, where Nef directly activate CAMKIIδ through elevating $[Ca^{2+}]_I$ to inhibit downstream ASK-1 and p38 mediated apoptosis probably to provide a replicative niche to the virus.

5. Conclusion

HIV-1 Nef has evolved to modulate Ca^{2+} signalling for viral replication as well as inhibition of apoptosis at the same time. The present study shows a novel interaction between Nef and CAMKIIδ to inhibit CAMKIIδ-ASK-1 mediated p38MAPK activation leading towards apoptosis. We have delineated the Nef minimal domain involved in interaction with CAMKIIδ and using competitive peptide inhibitor disengaged Nef from CAMKIIδ-ASK-1 complex, which was able to restore p38MAPK activation and apoptosis. Targeting Nef-CAMKIIδ interaction, therefore could be a promising approach for development of novel strategies to interfere with HIV-1 replication within T cells.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.03.039.

Competing interests

The authors declare that they have no competing interests.

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