

The di-leucine motif in the cytoplasmic tail of CD4 is not required for binding to human immunodeficiency virus type 1 Nef, but is critical for CD4 down-modulation

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The human immunodeficiency virus type 1 (HIV-1) *nef* gene encodes a 205 residue, myristoylated phosphoprotein that has been shown to play a critical role in the replication and pathogenesis of the virus. One of the most studied functions of the Nef protein is the down-modulation of cell surface CD4. Nef has been reported to interact with both the cytoplasmic tail of CD4 and proteins that are components of the endocytic machinery, thereby enhancing the endocytosis of CD4 through clathrin-coated pits. A di-leucine motif in the cytoplasmic tail of CD4 (residues 413/414) was reported to be essential both for Nef mediated down-modulation and for Nef binding. In order to further characterize the involvement of this di-leucine motif in CD4 down-modulation we generated a CD4 mutant in which the leucines were substituted by alanines, termed CD4(LL-AA). We demonstrate here that, contrary to previous data obtained with the cytoplasmic tail of CD4 alone, full-length CD4(LL-AA) bound to Nef both *in vivo*, in recombinant baculovirus-infected Sf9 cells, and *in vitro*. In contrast the di-leucine motif was required for both Nef-mediated and phorbol ester-induced CD4 down-modulation, suggesting that the essential requirement for the di-leucine motif in CD4 down-modulation reflects the fact that this motif is needed for the interactions of CD4 with the endocytic machinery, not for the interaction with Nef. We have also exploited the observation that CD4(LL-AA) is refractory to Nef-mediated down-modulation to provide the first experimental evidence for a physical interaction between Nef and CD4 in intact mammalian cells.

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INTRODUCTION

The *nef* genes of human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV) encode a 205–260 amino acid myristoylated phosphoprotein which substantially increases viral load in the infected host and accelerates clinical progression to AIDS. The vital role played by Nef in viral pathogenesis has been demonstrated in a number of systems: rhesus macaques infected with SIV (Kestler *et al.*, 1991), a SCID-hu mouse model of HIV-1 infection (Aldrovandi & Zack, 1996), the Sydney cohort of long-term non-progressors (Deacon *et al.*, 1995) and, most recently, a transgenic mouse in which Nef alone was expressed in CD4⁺ cells (Hanna *et al.*, 1998).

Studies *in vitro* have shown Nef to be a functionally pleiotropic protein, influencing viral infectivity and cellular signalling pathways, as well as modulating the surface expression of a number of cellular proteins (for reviews see Fackler & Baur, 2002; Arora *et al.*, 2002). The molecular mechanisms by which Nef exerts these effects are complex and only partly resolved. Nef has no known enzymatic functions but is

thought to effect its many and diverse influences, over both HIV-1 replication and the cellular environment, through an extensive range of interacting partners.

One well-documented and highly conserved functional role of Nef is the ability to down-modulate CD4 from the cell surface (Garcia & Miller, 1991). CD4, a type I integral membrane protein, is required for T-cell activation and also serves as the primary receptor for HIV and SIV. It has been suggested that this function of Nef could promote virus release, impair CD4⁺ helper T-cell function, alter T-cell receptor signalling and prevent superinfection (Arora *et al.*, 2002). Furthermore, down-modulation of CD4 correlates closely with enhanced virus replication in both peripheral blood mononuclear cells (PBMC) and in human lymphoid tissue *ex vivo* (Glushakova *et al.*, 2001; Stoddart *et al.*, 2003). Nef does not alter CD4 expression levels or transport through the exocytic pathway but acts to accelerate its internalization from the cell surface. Myristoylation of Nef is needed for membrane localization and is essential for down-modulation of CD4; in addition, the cytoplasmic tail

of CD4 is both necessary and sufficient for this Nef-mediated effect (Anderson *et al.*, 1994).

Endocytosis of CD4 has been most extensively studied in non-lymphoid cells stably expressing human CD4 in the absence of p56^{lck} (Pelchen-Matthews *et al.*, 1993). These studies have shown that protein kinase C (PKC)-mediated phosphorylation of serine residues within the cytoplasmic tail of CD4 [induced by phorbol 12-myristate 13-acetate (PMA) activation of PKC], greatly accelerates the rate of endocytosis. A di-leucine motif (Leu-413/414) within the cytoplasmic tail is essential for endocytosis, but only functions when the serine residues are phosphorylated (Pitcher *et al.*, 1999). It has been proposed that adaptor complexes, which couple CD4 to clathrin lattices thus initiating clathrin-dependent endocytosis, bind to the di-leucine motif, and this binding is partly dependent on the CD4 phosphorylation state.

In the presence of Nef there is an enhanced localization of CD4 to clathrin-coated-pits (Foti *et al.*, 1997) and a colocalization of CD4 with members of the AP-2 adaptor complex. Nef interacts with the μ -chain of AP-2 (Piguet *et al.*, 1998), which has led to the suggestion that Nef acts as an adaptor protein, binding both to components of the cellular endocytic machinery and, either directly or via an as yet unknown co-factor, to CD4. There are currently no data demonstrating such an interaction in mammalian cells and it has been hypothesized that the Nef-CD4 interaction is either weak, or exists only transiently and is disrupted upon CD4 internalization. The existence of this complex is supported, however, by studies in non-mammalian systems: the first evidence for a Nef-CD4 complex came from studies in insect cells co-infected with recombinant baculoviruses expressing Nef (as an N-terminal fusion with GST) and CD4 – the interaction required both Nef myristoylation and the cytoplasmic tail of CD4 (Harris & Neil, 1994). In addition the interaction has been described in both the yeast two-hybrid system (Rossi *et al.*, 1996), and *in vitro* assays (NMR, fluorescence spectroscopy) using purified recombinant Nef and peptides derived from the cytoplasmic tail of CD4 (Grzesiek *et al.*, 1996; Preusser *et al.*, 2001). Following on from studies that showed that the CD4 di-leucine motif was required for Nef-mediated down-modulation, the yeast two-hybrid and *in vitro* studies demonstrated that this motif was also required for Nef binding. It has been proposed that a di-leucine motif in a C-terminal loop of Nef acts as an endocytosis signal – recruiting a Nef-CD4 complex to clathrin-coated pits (Aiken *et al.*, 1994).

In this study we have reassessed the role of the CD4 di-leucine motif for both Nef binding and Nef-mediated down-modulation. In order to attempt to address some of the inconsistencies in the published literature, which may result from the use of fragments of CD4 in a non-physiological context, we have analysed the role of the di-leucine motif in the context of full-length, native CD4. Using both the previously described insect cell-based *in vivo* binding assay, and an ELISA assay, we show that the CD4

di-leucine motif is dispensable for Nef binding. In contrast, using HeLa cells stably expressing a CD4 mutant lacking the di-leucine motif, we show that this motif is indeed essential for CD4 down-modulation, mediated either by Nef or PMA treatment. Finally, by exploiting the inability of CD4(LL-AA) to undergo internalization, we present the first experimental evidence for a Nef-CD4 complex in intact mammalian cells.

METHODS

DNA manipulations and plasmids. A mutant of CD4 in which the two leucines (residues 413 and 414) were substituted by alanine residues – hereafter termed CD4(LL-AA), was constructed by PCR (primer sequences available on request). This PCR product was subsequently cloned, as a *Bam*HI fragment, into both the baculovirus transfer vector pAcCL29 (Livingstone & Jones, 1989) and the mammalian expression vector pSG5 (Green *et al.*, 1988). Construction of recombinant baculoviruses expressing NefGST, wild-type CD4 and a CD4 cytoplasmic tail truncation mutant (CD4stop394) has been described previously (Harris & Coates, 1993; Harris & Neil, 1994). For mammalian expression of NefGST, Nef(G2S)GST and myrGST (GST preceded by the myristoylation sequence from HIV-1 Gag – Met-Gly-Ala-Arg-Ala-Ser), the appropriate DNA fragments were subcloned from pAcCL29 derivatives (Harris & Coates, 1993) into pSG5. The expression vectors pCG-NL4.3-IRES-GFP and pCG-NL4.3STOP-IRES-GFP were obtained from Professor Frank Kirchhoff (University of Ulm, Germany) (Carl *et al.*, 2001).

Cell culture. *Spodoptera frugiperda* (Sf9) insect cells were maintained in TC-100 medium (Gibco-Invitrogen) supplemented with 10% foetal calf serum (FCS), 100 IU penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹. HeLa cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% FCS, 2 mM glutamine, 100 IU penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹. HeLa cell lines expressing either wild-type human CD4 or a truncation mutant lacking the majority of the cytoplasmic domain (CD4stop399) were obtained from Mark Marsh (University College, London, UK) (Pitcher *et al.*, 1999). HeLa cells expressing CD4(LL-AA) were generated by co-transfecting pSG5CD4(LL-AA) with an expression vector conferring resistance to neomycin, pSG2-Neo, in a ratio of 10:1. Resistant colonies were selected in media supplemented with 1 mg G418 ml⁻¹ and screened by flow cytometry, described below.

***In vivo* interaction assays.** Sf9 cells (5 × 10⁶) were seeded into a 25 cm² flask and subsequently infected with recombinant baculoviruses at 5 p.f.u. per cell. Cells were harvested at 48 h post-infection and lysed in 250 µl of Glasgow Lysis Buffer (GLB: 10 mM PIPES/NaOH pH 7.2, 120 mM KCl, 30 mM NaCl, 5 mM MgCl₂, 1% Triton X-100 and 10% glycerol) supplemented with protease inhibitors (aprotinin, 2 µg ml⁻¹; leupeptin, 1 µg ml⁻¹; pepstatin A, 1 µg ml⁻¹; AEBSEF, 0.2 mM). For HeLa experiments, cells were transfected with the appropriate pSG5 constructs by lipofection (Lipofectamine, Invitrogen) and harvested into GLB at 48 h post-transfection. For the binding assay 50 µl of the GLB lysates was incubated with 10 µl (packed volume) of glutathione-agarose beads for 2 h at 4 °C. Beads were washed once with GLB/0.5M KCl and a further three times with GLB. Bound proteins were analysed by 12% SDS-PAGE followed by immunoblotting with either sheep polyclonal sera raised to Nef or CD4. Blots were visualized with ECL reagents (Amersham).

Flow cytometry. Infected Sf9 cells (10⁶) were harvested at 24 h post-infection, washed once with PBS, once again with PBS/1% FCS and then incubated with a 1:40 dilution of a phycoerythrin (PE)-conjugated monoclonal anti-CD4 (CalTag) in PBS/1% FCS. After a 30 min incubation at 4 °C cells were washed three times with PBS/

1% FCS and analysed on a Becton Dickinson FACSCalibur with Cell Quest software. HeLa cell lines expressing full-length CD4 (HeLaCD4), or CD4 mutants – HeLaCD4stop399 and HeLaCD4(LL-AA) – were transfected by lipofection with bicistronic vectors expressing either wild-type Nef (strain NL4.3), or a mutant containing multiple in-frame stop codons, and GFP (pCG-NL4.3-IRES-GFP or pCG-NL4.3STOP-IRES-GFP) (Carl *et al.*, 2001). As controls (black lines) the three cell lines were transfected with pEGFP-N1 (Clontech); the negative control (dotted lines) represents control HeLa cells transfected with pEGFP-N1. Cells were harvested 48 h post-transfection and stained as outlined above. The levels of cell-surface CD4 were assayed on the GFP-positive cell populations. PMA treatment (10 ng ml⁻¹) was carried out 15 min before harvest.

ELISA protein interaction assay. C-terminally His-tagged Nef (strain BH10) was expressed and purified by standard techniques. Serial dilutions of purified Nef-His₆ were incubated in 96-well plates (Greiner Bio-one PS-microplate) in 50 µl of PBS/0.1% Tween (PBT) overnight at 4 °C. Plates were washed in PBT and blocked in PBT containing 5% non-fat dried milk (PBTM) for 2 h at room temperature. Plates were washed in PBT and cytosolic extracts from either Sf9 cells infected with AcCD4, AcCD4(LL-AA) or AcCD4stop394, or HeLa cells expressing CD4, CD4(LL-AA) or CD4stop399, diluted in

GLB supplemented with protease inhibitors (50 µl per well) were added for 2 h (Sf9) or overnight (HeLa) at 4 °C. After three washes with PBT, CD4 was detected using the CD4 monoclonal antibody Q4120 (obtained from the MRC AIDS Reagent Repository, NIBSC, UK) (1 : 1000 in PBTM) for 1 h followed by a horseradish peroxidase-conjugated anti-mouse antibody (Sigma, 1 : 500 in PBTM) for a further hour. Bound antibody was visualized using *o*-phenylenediamine (Dako) and quantified at 490 nm with a reference filter at 630 nm using an MRX microplate reader (Dynex).

RESULTS

The di-leucine motif in the cytoplasmic tail of CD4 is not required for Nef binding in insect cells

We have previously shown that CD4 forms a stable complex with Nef in Sf9 insect cells co-infected with recombinant baculoviruses expressing full-length human CD4 and an N-terminal fusion of Nef with GST (NefGST) (Harris & Neil, 1994). This interaction was dependent on Nef myristoylation, as a non-myristoylated Nef mutant failed to bind to

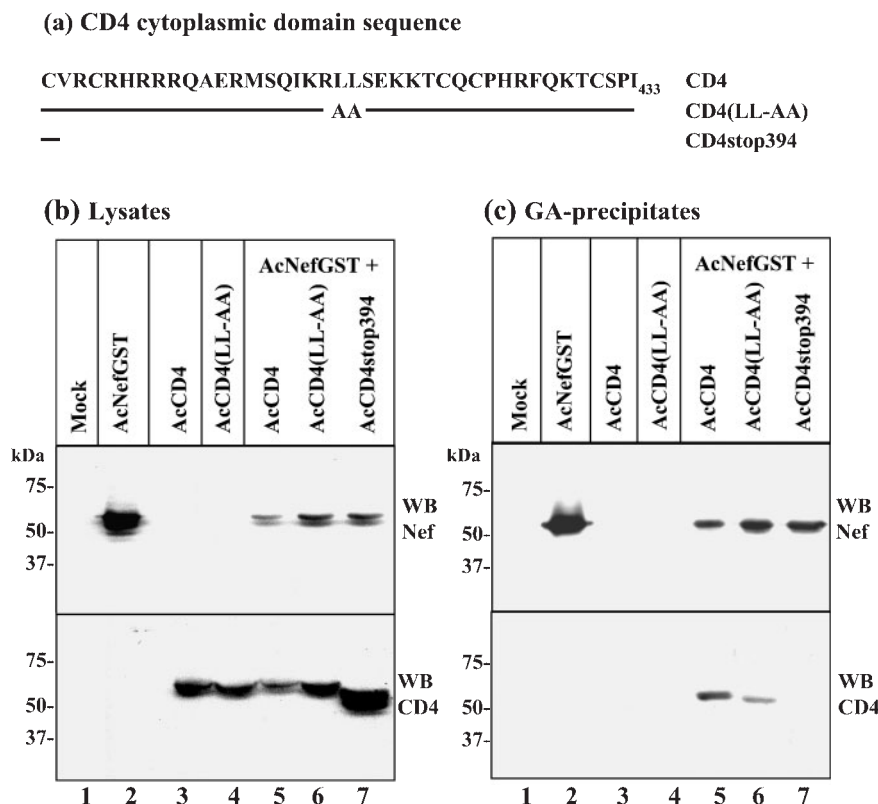


Fig. 1. Nef interacts with a CD4(LL-AA) mutant *in vivo*. (a) Schematic of the amino acid sequence of the cytoplasmic tail of human CD4. Residues 394 and 395 are proposed to be embedded within the membrane. The position of the di-leucine motif (residues 413 and 414) is shown, together with the truncation point for the CD4stop394 mutant. (b) Expression of NefGST and CD4 in Sf9 cells. Cells were infected with the indicated baculoviruses and harvested into GLB 48 h post-infection. Aliquots of lysates were analysed by immunoblotting with polyclonal sheep sera raised to either bacterially expressed GSTNef (upper panel) or CHO-derived recombinant CD4 (obtained from the MRC AIDS Reagent Repository, NIBSC) (lower panel). (c) Lysates from infected cells were precipitated with glutathione-agarose beads and bound protein was analysed by immunoblotting as described in (b).

CD4; in addition, binding was not observed when NefGST was coexpressed with a mutant of CD4 that lacked the entire cytoplasmic tail (CD4stop394). More recently, it has been shown that a di-leucine motif in the cytoplasmic tail of CD4 was required not only for Nef-mediated down-modulation (Aiken *et al.*, 1994), but also for the interaction with Nef both in the yeast two-hybrid system (Rossi *et al.*, 1996) and *in vitro* (Grzesiek *et al.*, 1996; Preusser *et al.*, 2001). As these more recent interaction studies were performed with peptides derived from the cytoplasmic tail of CD4 and non-myristoylated Nef, we considered it important to investigate the potential role of the di-leucine motif in the context of the native CD4 and myristoylated Nef proteins within intact cells. We therefore generated a recombinant baculovirus expressing full-length human CD4 in which the di-leucine motif had been substituted by alanines [AcCD4(LL-AA)] and compared the phenotype of this mutant to wild-type CD4 and CD4stop394. The sequence of the CD4 cytoplasmic tail and the position of the two mutations is depicted in Fig. 1(a). To determine whether CD4(LL-AA) was able to bind Nef, Sf9 cells were either singly infected, or co-infected with the indicated

recombinant baculoviruses (Fig. 1b). The expected pattern of protein expression was verified by immunoblotting of lysates with either anti-Nef or anti-CD4 antisera. Fig. 1(b) (lower panel) shows that AcCD4(LL-AA) expressed a protein product that was reactive with a polyclonal anti-CD4 antiserum and, interestingly, migrated slightly faster than wild-type CD4. These lysates were then precipitated with glutathione-agarose beads and precipitated protein was analysed by immunoblotting for the presence of either Nef or CD4. As shown in Fig. 1(c), NefGST coprecipitated both wild-type CD4 and CD4(LL-AA) (lanes 5 and 6), and as expected from previous data the CD4stop394 truncation failed to bind to NefGST (lane 7).

To confirm that CD4(LL-AA) was appropriately processed and expressed on the cell surface we performed a flow cytometric analysis on baculovirus-infected Sf9 cells. Fig. 2(c-e) demonstrates that all three CD4 proteins were expressed on the cell surface at comparable levels. Fig. 2(f-h) also shows that NefGST was unable to down-modulate the cell surface expression of any of these CD4 proteins; the slight reduction in the height of the peaks (e.g. compare Fig. 2c with 2f) has

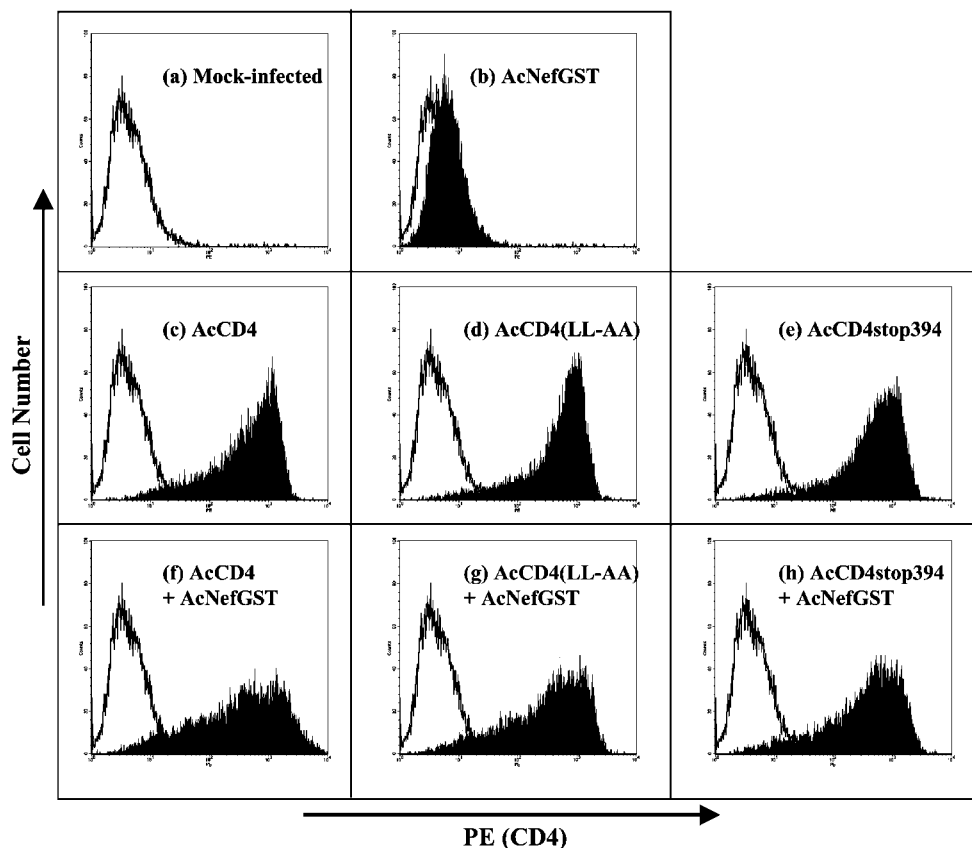


Fig. 2. Nef does not down-modulate cell surface CD4 in Sf9 insect cells. Sf9 cells were infected with the indicated recombinant baculoviruses and harvested 24 h post-infection. Cells were labelled with a PE-conjugated monoclonal antibody to CD4 (CalTag) and analysed by flow cytometry. The unshaded histograms in each panel correspond to the histogram in panel (a) and show the background labelling of uninfected cells.

been observed previously (Harris & Neil, 1994) and probably reflects lower levels of expression from each virus in the co-infected cells. The Nef allele used in these experiments (BH10) had been shown to be competent for CD4 down-modulation in HeLa cells (Cooke *et al.*, 1997). Taken together with the previous observations that NefGST is myristoylated (Harris & Coates, 1993) and membrane associated (data not shown), we conclude that in the context of the native CD4 and Nef proteins, in the appropriate membrane localization, the CD4 di-leucine motif is dispensable for the interaction between Nef and CD4.

Nef binds CD4(LL-AA) *in vitro*

Although our previous data had demonstrated that Nef-CD4 binding *in vivo* exhibited an absolute requirement for Nef myristoylation, studies from other laboratories indicated that non-myristoylated Nef could bind to CD4 *in vitro*, suggesting that the role of the myristate residue was not structural but merely acted to localize Nef at the plasma membrane. To further investigate the binding of Nef to CD4 we therefore established an ELISA assay in which purified recombinant Nef was used to capture CD4 from lysates of baculovirus-infected Sf9 cells. Nef (strain BH10) was expressed in *E. coli* as a non-myristoylated protein with a C-terminal His₆ tag and purified by standard techniques (purification and characterization of this protein will be published elsewhere). Fig. 3(a) shows that Nef-His₆ was able to capture either wild-type CD4 or CD4(LL-AA) from lysates of infected Sf9 cells. Very little binding to CD4stop394 was observed; indeed the signal obtained with CD4stop394 was comparable to background. To confirm that Nef was also able to bind mammalian-derived CD4 *in vitro* we generated a stable HeLa cell line expressing CD4(LL-AA) and performed the same assay with lysates from these cells and HeLa cells expressing wild-type CD4 and a mutant lacking the cytoplasmic domain (CD4stop399). Fig. 3(b) shows that similar results were obtained with this mammalian-derived CD4. The stronger signal obtained from HeLa CD4(LL-AA) in comparison to wild-type CD4 reflects the higher level of expression of the mutant, rather than any difference in affinity for Nef. This is confirmed by Fig. 3(c) – an immunoblot analysis of overall levels of CD4 expression in both baculovirus-infected Sf9 cells and HeLa cell lines – and also by Fig. 5(a) (lower panel).

CD4(LL-AA) is refractory to both PMA- and Nef-mediated down-modulation

Our data clearly showed that Nef was able to bind to CD4(LL-AA) both *in vivo* and *in vitro*; however, as CD4 is not down-modulated in insect cells either by Nef or PMA treatment it was not possible to correlate Nef binding to CD4 down-modulation. To address this issue we analysed cell surface CD4 expression in the three HeLa cell lines by flow cytometry. All three lines exhibited a similar pattern of surface CD4 expression (Fig. 4a–c, black lines). As expected PMA treatment of cells expressing wild-type CD4 (Fig. 4a, red line), resulted in an approximate 3-fold decrease in the

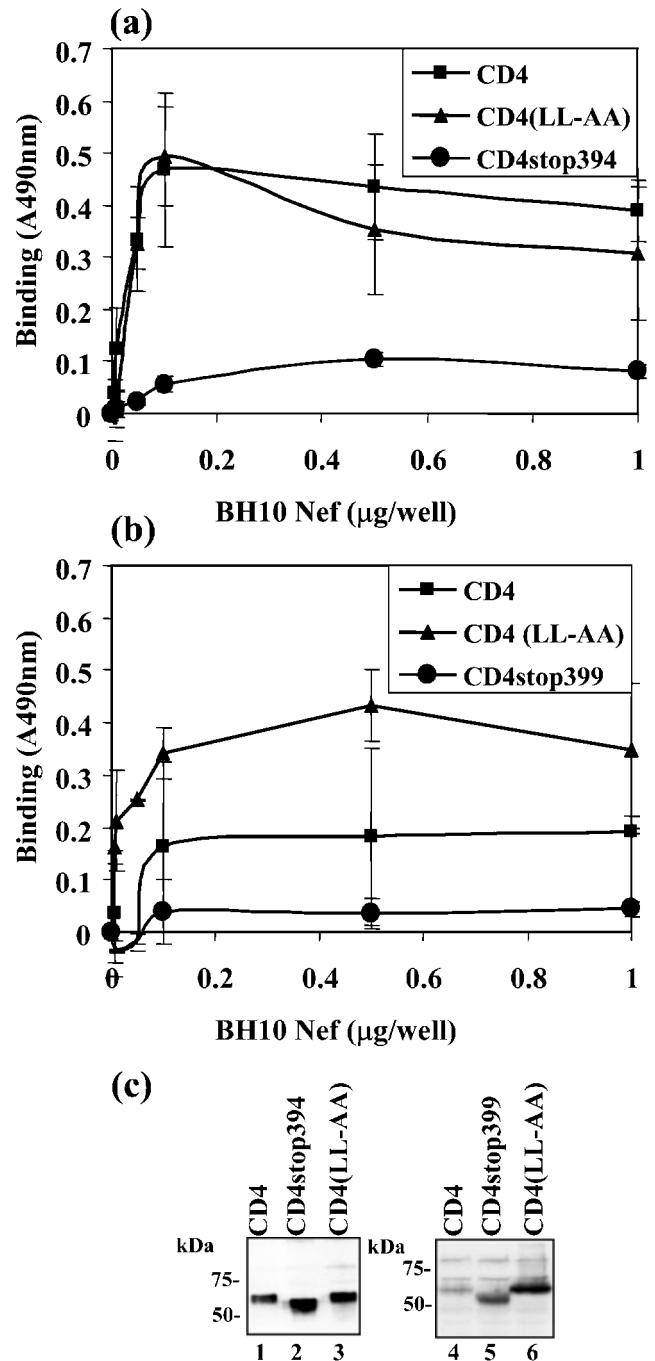


Fig. 3. Nef interacts with a CD4(LL-AA) mutant *in vitro*. (a) Lysates from Sf9 cells infected with the indicated recombinant baculoviruses were captured on ELISA plates coated with the indicated amounts of non-myristoylated, prokaryotically expressed Nef-His₆ (strain BH10). Bound CD4 was detected with the monoclonal antibody Q4120 (obtained from the MRC AIDS Reagent Repository, NIBSC) followed by an HRP conjugate. Results show the combined data from three experiments. (b) As (a) except lysates were prepared from stable HeLa cell lines expressing the indicated CD4 derivatives. (c) Aliquots of lysates were analysed by immunoblotting with polyclonal sheep sera raised to CHO-derived recombinant CD4 (obtained from the MRC AIDS Reagent Repository, NIBSC). Lanes 1–3, lysates from recombinant baculovirus infected Sf9 cells; lanes 4–6, lysates from stable HeLa cell lines.

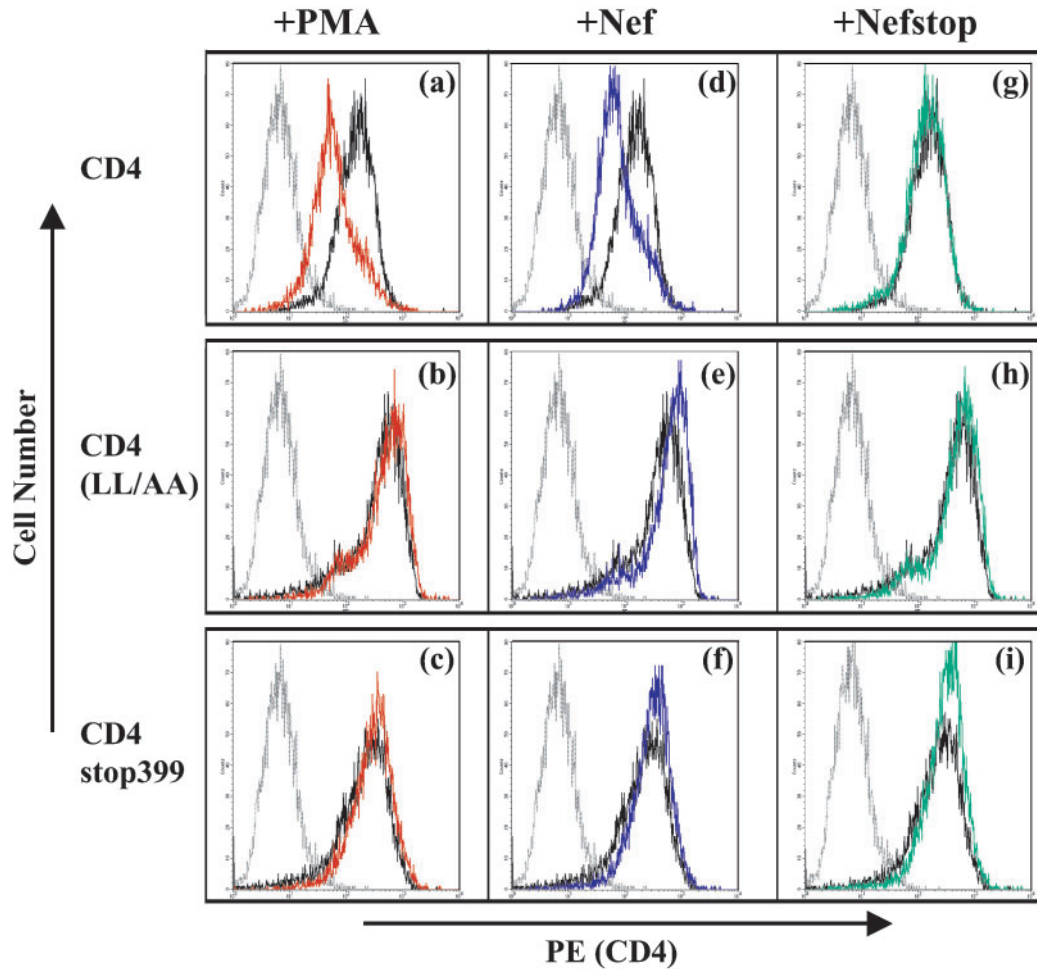


Fig. 4. CD4(LL-AA) is refractory to both Nef- and PMA-mediated down-modulation in HeLa cells. HeLa cells stably expressing either wild-type CD4 (panels a, d and g), CD4(LL-AA) (panels b, e and h) or CD4stop399 (panels c, f and i), were labelled with a PE-conjugated monoclonal antibody (CaTag) to CD4 and analysed by flow cytometry. The grey dotted histogram in each panel shows the background labelling of GFP-positive cells from HeLa cells stably transfected with pSG2.Neo alone (not expressing CD4), and transiently transfected with pEGFP-N1. The black histograms show labelling of GFP-positive cells from each CD4 expressing cell line transiently transfected with pEGFP-N1. The red histograms in panels (a)–(c) show the labelling of GFP-positive cells following transient transfection with pEGFP-N1 and treatment for 15 min with PMA; the blue histograms in panels (d)–(f) show labelling of GFP-positive cells following transfection with the pCG-NL4.3-IRES-GFP plasmid; and the green histograms in panels (g)–(i) show labelling of GFP-positive cells following transfection with the pCG-NL4.3STOP-IRES-GFP plasmid.

levels of cell surface CD4, consistent with published data showing that activation of PKC results in phosphorylation of serine residues in the cytoplasmic tail of CD4, promoting rapid internalization. In contrast the surface levels of CD4stop399 and CD4(LL-AA) were unaffected by PMA (Fig. 4b, c).

If, as previously suggested, an endocytosis signal within Nef was able to mediate CD4 down-modulation, the ability of Nef to bind to CD4(LL-AA) would imply that Nef would also be able to down-modulate CD4(LL-AA). To test this we utilized a bicistronic vector (pCG-NL4.3-IRES-GFP)

containing both the NL4.3 allele of Nef and EGFP separated by the encephalomyocarditis virus IRES. The three HeLa cell lines were transfected with this construct and surface levels of CD4 on the GFP-positive (Nef expressing) cells compared to cells expressing GFP alone (pEGFP-N1 transfected: black lines) by flow cytometry. As expected Nef was able to effect a 3-fold down-modulation of wild-type CD4 (Fig. 4d) and had no effect on surface levels of CD4stop399 (Fig. 4f). Similarly Nef was unable to down-modulate CD4(LL-AA) (Fig. 4e), demonstrating that binding of Nef to CD4 is not sufficient to mediate endocytosis. Control cells transfected with a bicistronic vector containing a mutant Nef with

multiple in-frame stop codons did not exhibit any CD4 down-modulation (Fig. 4g–i).

Nef forms a stable complex with CD4(LL-AA) in mammalian cells

We and others have previously hypothesized that the failure to detect a Nef–CD4 complex in mammalian cells might be due to the transient nature of the complex – upon CD4 internalization and degradation in lysosomes the complex would be disrupted (Harris & Neil, 1994; Rossi *et al.*, 1996). Because CD4(LL-AA) was able to bind Nef both in insect cells and *in vitro*, and was also refractory to Nef-mediated endocytosis, we investigated whether a stable complex between Nef and CD4(LL-AA) could be detected in mammalian cells. HeLa CD4(LL-AA) cells were therefore transfected with pSG5 vectors expressing NefGST, a non-myristoylated derivative [Nef(G2S)GST] or myristoylated GST (myrGST). Lysates were incubated with glutathione–agarose beads and precipitated protein was analysed by immunoblotting for the presence of either Nef or CD4. Fig. 5(b) clearly shows that NefGST was able to precipitate CD4(LL-AA) (lane 8), but failed to form stable complexes with either wild-type CD4 or CD4stop399 (lanes 4, 6). As expected neither Nef-(G2S)GST nor myrGST bound to CD4(LL-AA) (lanes 9, 10). Appropriate expression of either CD4 or the GST fusions was verified by immunoblotting of lysates with antibodies to either GSTNef or CD4 (Fig. 5a, upper and lower panels respectively). As described above, wild-type CD4 is clearly expressed at a lower level than both the CD4stop399 and CD4(LL-AA) mutants; however, the ability to detect a complex with NefGST is not a consequence of

the higher level of expression of CD4(LL-AA), as over-exposure of the immunoblot failed to reveal a signal for wild-type CD4 in lane 4 (Fig. 5b, lower panel) (data not shown). We conclude that a CD4 mutant that is refractory to endocytosis is capable of forming a stable complex with Nef in mammalian cells – the data presented in Fig. 5 provide the first experimental evidence for such interaction.

DISCUSSION

The data presented in this paper clearly demonstrate that in the context of full-length, native CD4 the di-leucine motif at residues 413 and 414 is not required for a direct interaction with Nef. At first glance this result would seem to be contradictory to other published studies that have looked at Nef–CD4 binding; however, a more detailed comparison of the data suggests that in fact these discrepancies could be explained by experimental differences. Importantly, none of the *in vitro* studies have examined the effect of the LL-AA mutation in the context of either the complete cytoplasmic tail of CD4 or the native (full-length) protein. NMR studies have shown that mutation of the di-leucine motif in the context of a synthetic peptide corresponding to residues 403–419 (Grzesiek *et al.*, 1996) or 407–419 (Preusser *et al.*, 2001) abolishes binding to Nef. One of these studies also showed that the addition of residues 420–433 (to the end of the cytoplasmic tail) decreased the K_d from 1.4 μ M to 0.87 μ M (Preusser *et al.*, 2001) and likewise the presence of residues 403–406 increased the affinity of binding twofold. These data imply that, in the context of residues 403–419, the di-leucine motif is indeed critical for Nef binding; however, in the context of the complete cytoplasmic tail

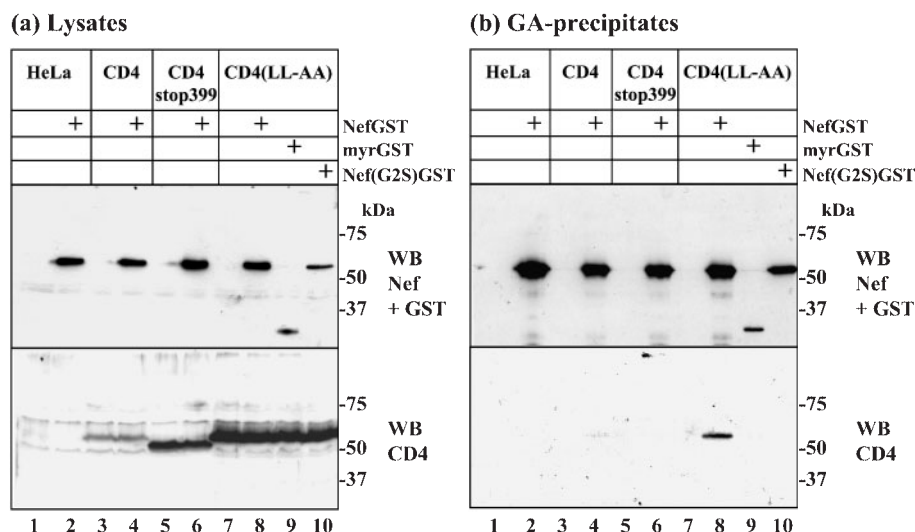


Fig. 5. CD4(LL-AA) forms a stable complex with Nef in HeLa cells. The indicated cell lines were transfected with pSG5 plasmids expressing either NefGST (lanes 2, 4, 6 and 8), myrGST (lane 9) or Nef(G2S)GST (lane 10). Cells were harvested into GLB at 48 h post-transfection. (a) Aliquots of lysates were analysed by immunoblotting with polyclonal sheep sera raised to either bacterially expressed GSTNef (detects both Nef and GST; upper panel) or CHO-derived recombinant CD4 (obtained from the MRC AIDS Reagent Repository, NIBSC; lower panel). (b) Lysates from transfected cells were precipitated with glutathione–agarose beads and bound protein was analysed by immunoblotting as described in (a).

additional contacts might occur that stabilize the interaction. Alternatively, the presence of sequences from 394–402 and 420–433 might contribute to the structure of the cytoplasmic tail to facilitate Nef binding – this suggestion is supported by the observation of a direct correlation between the amount of alpha-helical content in peptides derived from the cytoplasmic tail, and the binding affinity for Nef (Preusser *et al.*, 2002). Further support comes from comparison of two structural studies on the cytoplasmic tail of CD4: the solution structure of a peptide spanning residues 403–419 was only helical between residues 403–412 (Willbold & Rosch, 1996), whereas structural analysis of a peptide spanning the complete cytoplasmic tail was helical between 402–417 (Wray *et al.*, 1998). In addition, our own recent data have demonstrated that mutation of three cysteines between residues 420–433 abrogates Nef binding in insect cells (M. Bentham and others, unpublished), implying that the carboxy-terminal (membrane-distal) part of the CD4 cytoplasmic tail contributes to the interaction with Nef. It is also formally possible that sequences in the transmembrane or extracellular domains could influence the structure of the cytoplasmic tail. In this regard it is interesting to note that binding of antibodies specific for the CDR3-like region of the extracellular domain of CD4 transmits signals via the cytoplasmic domain that inhibit HIV- replication, whereas antibodies specific to other extracellular regions fail to elicit such responses (Benkirane *et al.*, 1995). These observations imply that the extracellular domain of CD4 can regulate intracellular events involving the cytoplasmic tail, and therefore may also influence the structure of that part of the protein.

Our data confirm the previous observation that the di-leucine motif in CD4 is essential for both PMA- (Pitcher *et al.*, 1999), and Nef- (Aiken *et al.*, 1994) mediated down-modulation. Taken together with the demonstration of constitutive internalization of a chimeric molecule in which the cytoplasmic tail of CD4 was replaced by Nef (Mangasarian *et al.*, 1997), a widely accepted interpretation is that Nef binds to the di-leucine motif and then directly associates with the endocytotic machinery to mediate internalization of the Nef–CD4 complex. Our data imply that this interpretation will have to be reassessed – we propose that Nef binds to the cytoplasmic tail of CD4 and facilitates the interaction between the CD4 di-leucine motif and adaptor proteins. This could occur either by the association between Nef and clathrin-coated pits to juxtapose CD4 and the endocytotic machinery, or by the induction of a conformational change in the cytoplasmic tail to expose the di-leucine motif. A number of previous observations lend support to this hypothesis: firstly the binding of Nef to components of clathrin-coated pits (Greenberg *et al.*, 1997; Lu *et al.*, 1998); secondly the changes in secondary structure of CD4 peptides upon Nef binding as measured by CD spectra (Preusser *et al.*, 2002); and lastly the fact that Nef-mediated CD4 down-modulation is independent of serine phosphorylation in the cytoplasmic tail (Garcia & Miller, 1991). Alternatively, the presence of the

CD4 di-leucine motif might induce a conformational change in Nef, thus facilitating binding of Nef to endocytotic proteins. This hypothesis is supported by the observation that although a CD4–Nef chimera (with Nef replacing the cytoplasmic tail) was constitutively endocytosed, this molecule was unable to mediate endocytosis of a chimeric protein consisting of CD8 extracellular and transmembrane domains fused to the CD4 cytoplasmic tail containing the LL-AA mutation (Mangasarian *et al.*, 1997). It is important to note that the structure and function of Nef may be dramatically influenced by the presence of a transmembrane domain at the N terminus instead of the myristate group as, in comparison to native myristoylated Nef, a CD4–Nef chimera will be unable to dynamically associate with the membrane and may be subject to abnormal structural constraints. Further experiments are clearly needed to fully characterize the mechanism of Nef-mediated down-modulation.

The existence of a complex between Nef and CD4 in mammalian cells has been frequently alluded to, and indeed has become part of the accepted dogma used to explain the mechanism of Nef-mediated CD4 down-modulation. However, to date there has been no experimental evidence in support of this hypothesis. The data presented in Fig. 5 thus provide the first proof that Nef can physically associate with CD4 in a mammalian context. The observation that CD4(LL-AA) binds to Nef in HeLa cells also supports the conjecture that the complex is normally transient and is disrupted during endocytosis. It raises the possibility that a complex between Nef and wild-type CD4 might be stabilized (and therefore identified) in cells treated with pharmacological inhibitors of endocytosis, or transfected with dominant-negative mutants of endocytotic proteins (e.g. dynamin). Such experiments are currently in progress in our laboratory and we expect will shed further light on the mechanism of Nef-mediated CD4 down-modulation.

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