Identification and cloning of a novel cellular protein Naf1, Nef-associated factor 1, that increases cell surface CD4 expression

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Abstract The *nef* gene of human and simian immunodeficiency virus is a key factor in acquired immunodeficiency syndrome pathogenesis and virus replication. Several Nef-induced phenomena, including the down-regulation of CD4 molecule, have been previously reported. In this study, we have identified and cloned a novel cellular protein Naf1 (Nef-associated factor 1), which associated with Nef in the yeast two-hybrid system and pull-down assay. The *Naf1* gene generates two isoforms (Naf1α and β) containing four coiled-coil structures. The *Naf1* mRNA is ubiquitously expressed in human tissues with strong expression in peripheral blood lymphocytes and spleen. Naf1 overexpression increased cell surface CD4 expression. Nef suppressed this Naf1-induced augmentation of CD4 expression, providing a novel mode of Nef action in CD4 down-regulation.

Key words: Human immunodeficiency virus-1; Nef; Naf1; CD4 expression

1. Introduction

Progression of acquired immunodeficiency syndrome (AIDS) is closely related to human and simian immunodeficiency virus (HIV and SIV) replication [1]. In human, *nef*-defective HIV-1 can be isolated from some cases of long-term non-progressors (reviewed in [2]). In monkey, Nef is essential for high-titer replication of SIV, and *nef*-deleted SIV does not induce AIDS in most cases [2,3]. In humanized mouse models, *nef*-defective HIV-1 replicates less efficiently and shows less profound CD4+ cell depletion than wild-type HIV-1 [2–4]. Moreover, it has been reported that Nef augments HIV replication in T cells and macrophages in vitro [2–4]. Nef associates with several cellular tyrosine kinases [5–7] and serine/threonine kinases [8,9]. It has been thought that the interaction between Nef and these kinases may alter the level of host cellular activation, thus augmenting virion infectivity [2,3,8,9].

On the other hand, Nef induces down-regulation of cell surface expression levels of CD4 [2,10] and major histocompatibility complex (MHC) class I [11,12] molecules by accelerating cellular endocytosis through clathrin-coated pits (CCPs) [13–15]. This process is dependent on a dileucine motif within the cytoplasmic tail of CD4 and is thought to be initiated by the interaction of Nef with CD4 at the plasma membrane [16]. Recently it was found that Nef colocalizes with the AP-2 complex, which recruits transmembrane proteins to CCPs, at the plasma membrane and in the region of the Golgi complex [14]. Furthermore, recent reports have demonstrated that the Nef-mediated endocytosis occurs through direct interaction between Nef and the μ chain of the AP-2 complex [13,15]. This interaction serves not only to promote formation of CCPs but also to increase the association of CD4 with the CCPs [17]. Moreover, Nef also suppressed cell surface CD4 expression levels via additional routes, i.e. inhibition of transport of CD4 from Golgi to plasma membrane and inhibition of CD4 recycling from endosomes [13,14,18]. Mutational analysis of Nef showed that augmentation of viral replication and CD4 down-regulation are separable functions of Nef [19]. The physiological role of CD4 down-regulation remains controversial although possible functional implications in HIV replication and pathogenesis have been proposed.

As described above, several Nef-mediated phenomena have been reported. However, there remain many questions about their molecular mechanisms. Accordingly, we have tried to identify cellular factors which interact with HIV-1 Nef. Here we describe a novel Nef binding protein Naf1 (Nef-associated factor 1) and discuss its modulatory roles in cell surface CD4 expression. Our results might provide a novel mode of Nef action in CD4 down-regulation.

2. Materials and methods

2.1. Plasmids

To construct pAS2-nef$_{JR-CSF}$ and pAS2-nef$_{NL4-3}$, *nef* amplified from infectious HIV-1 clones [20,21] by polymerase chain reaction (PCR) were inserted in frame into the pAS2 vector (Clontech, Palo Alto, CA). The *nef*$_{JR-CSF}$ was also inserted into pQE32 (Qiagen, Chatsworth, CA) and pRc/CMV (Invitrogen, Carlsbad, CA) vectors, resulting in pQE-Nef and pCMV-Nef, respectively. To construct pCMV-Naf1HA, hemagglutinin (HA) epitope-tagged *Naf1*β was generated by PCR and inserted into pRc/CMV. The pCEP/CD4 vector was donated by Dr. A. Yamashita. The pBCMGS/CD4 and the pCD19 vectors have been described previously [22,23]. pNL4-3 and pNL4-3Δnef, which encodes *nef*-minus HIV-1$_{NL4-3}$, were kindly provided by Dr. A. Adachi [21].

2.2. Yeast two-hybrid screening

To identify cellular factors that interact with HIV-1 Nef, the Matchmaker Two-Hybrid System (Clontech) was used. pAS2-nef$_{JR-CSF}$ was used as bait in screening of the phytohemagglutinin (PHA)-stimulated human leukocyte cDNA library (library size 3×10^6) that was fused to the GAL4 DNA activation domain vector pGAD10 (Clontech). Positive yeast clones were selected by auxotrophy for histidine and expression of β-galactosidase. Plasmids were harvested from the positive clones and re-examined for their ability to bind to Nef in the cotransformation assay. cDNA inserts were characterized by DNA sequencing.

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2.3. cDNA cloning

The 5′- and 3′-rapid amplification of cDNA ends (RACE) methods were performed as described [24] using poly(A)$^+$ RNA extracted from human skeletal muscle (Clontech). The COILS version 2.0 program was used to calculate the probability of coiled-coil conformation (28 window width, MTK matrix and weighting of positions a and d) [25].

2.4. Genomic analysis

Cosmids isolated from a chromosome-specific library (Los Alamos National Laboratory, Los Alamos, NM) were digested with *Sau*3AI, *Alu*I, *Pst*I or *Sst*I and shotgun-cloned into M13. Recombinant plaques were screened with restriction fragments of the *Naf1* cDNA. Sequence data generated from the positive clones were compared with the cDNA sequence and intron-exon boundaries identified by comparison with the published consensus sequences [26].

2.5. Northern blot analysis

Northern blot analysis was performed with Multiple Tissue Northern (MTN) Blot I and II (Clontech), according to the manufacturer's instructions, using a probe corresponding to nucleotides 510–1346 of the *Naf1* cDNA which had been radiolabeled with [^{32}P]α-deoxy-CTP (NEN Life Science Products, Boston, MA; specific activity of the probe was 2×10^8 cpm/μg). The membranes were analyzed using a bioimage analyzer BAStation (Fujifilm, Tokyo, Japan).

2.6. Cell culture

Human embryonic kidney cell line 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

2.7. In vitro pull-down assay

The bacteria M15 (pREP4) transformed with pQE-Nef were induced with 1 mM isopropyl thiogalactoside (IPTG) at 30°C for 2 h. The lysates were prepared according to the manufacturer's instruction (Qiagen). pCMV-Naf1HA was transfected with SuperFect (Qiagen) into 293T cells, which were plated at a cell density of 2×10^6 cells per 10 cm dish 1 day before transfection. Two days later, cell lysates were extracted with RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium lauryl sulfate (SDS), 1 mM sodium orthovanadate, 2 mM PMSF and 1 μg/ml aprotinin in phosphate buffered saline (PBS)) at 4°C for 30 min, and were cleared by centrifugation. The bacterial lysates were mixed with the Ni-NTA (nitrilotriacetic acid) resin (Qiagen) suspended in the binding buffer (0.05% Tween 20 and 10% Block Ace (Dai-Nippon Co., Osaka, Japan) in PBS) and incubated at 4°C for 1 h with rotation. The Ni-NTA resin was washed with the washing buffer (0.3 M NaCl, 10% glycerol, 10% Block Ace and 1% Tween 20 in phosphate buffer, pH 6.0) and treated with Block Ace, to reduce the background, at 4°C for 1 h with rotation. The resin was then washed with the washing buffer, mixed with the 293T cell lysates and incubated at 4°C for 1 h with rotation. Following washing, the complexes were treated with 0.5 M imidazole (Qiagen) for 10 min at room temperature. The supernatants were subjected to Western blot analysis, using either an anti-Nef antibody (ABI, Columbia, MD) or an anti-HA antibody (12CA5; Boehringer Mannheim, Indianapolis, IN).

2.8. Flow cytometry

Cell surface CD4 levels were examined by a modification of a previously reported method [16]. In brief, the vectors were cotransfected with FuGENE (Boehringer Mannheim) into 293T cells, which were plated at a cell density of 1×10^5 per six well dish 1 day before transfection. The next day, the cells were harvested and stained with phycoerythrin (PE)-conjugated anti-human CD4 antibody (MT310; Dako, Copenhagen, Denmark) and fluorescein isothiocyanate (FITC)-conjugated anti-human CD19 antibody (HD37; Dako), and were subsequently analyzed by FACS Calibur (Becton Dickinson, Mountain View, CA).

3. Results

3.1. Identification of a novel Nef-associated factor 1, Naf1

To identify cellular proteins that interact with HIV-1 Nef, we used the yeast two-hybrid system. The full-length Nef from HIV-1$_{JR-CSF}$, which is a macrophage-tropic strain, was fused to the GAL4 DNA binding domain. A PHA-stimulated human leukocyte cDNA library fused to the GAL4 activation domain was screened. Three His$^+$ and LacZ$^+$ transformants were obtained out of 1.4×10^7 clones screened and the sequences of these clones were analyzed using BLAST sequence similarity search. Two of the three clones were found to encode a *src*-family tyrosine kinase Hck, which has been reported to associate with Nef through its SH3 (*src* homology 3) region [27,28], and an η subunit of chaperonin containing T-complex protein (CCTη). The third clone contained part of the open reading frame (ORF) of a novel cellular protein, because it showed no significant homologies to sequences in GenBank except sequences submitted as EST and cDNA fragment with putative ORF. This clone also associated with Nef from a different HIV-1 strain, T cell-tropic HIV-1$_{NL4-3}$, in the yeast two-hybrid system (Fig. 1). We termed this novel cellular protein Naf1 (Nef-associated factor 1) and analyzed it further.

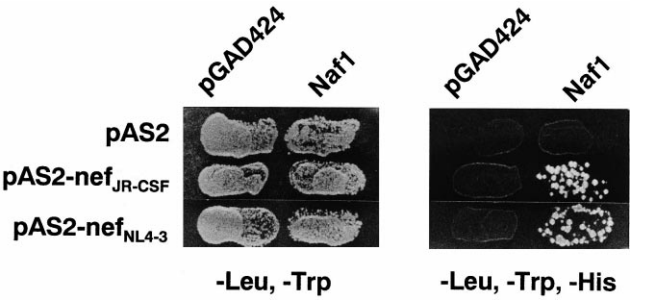

Fig. 1. Association of Naf1 with Nef from HIV-1$_{JR-CSF}$ and HIV-1$_{NL4-3}$ in the yeast two-hybrid system. Histidine auxotrophy of the yeast transformants was analyzed. The CG1945 yeast strain was cotransformed with GAL4 DNA binding domain vectors (pAS2, pAS2-nef$_{JR-CSF}$ and pAS2-nef$_{NL4-3}$) and GAL4 activation domain vectors (pGAD424 and Naf1). The transformants were seeded on control medium lacking Leu and Trp (left panel) and medium lacking Leu, Trp and His (right panel). The only transformants in which Nef interacted with Naf1 could form colonies in the medium lacking Leu, Trp and His, and had increased β-galactosidase activity (data not shown).

3.2. Cloning of the full-length cDNA and the gene for Naf1

To isolate full-length *Naf1* cDNA, the RACE technique was used. *Naf1* cDNA fragments containing 5′ and 3′ ends were isolated, resulting in assembly of the entire *Naf1* cDNA (Fig. 2). Analysis of the isolated full-length cDNA clones revealed that Naf1 has two isoforms (Naf1α and β) with different C-termini (Fig. 2). The molecular weight calculated from the deduced amino acid sequences of Naf1α/β is approximately 72 kDa. Both isoforms contain a proline-rich region within the C-terminal 100 amino acids, in which 24% of amino acids are prolines. A data base search revealed that the full-length *Naf1* cDNA had weak homology with some proteins containing coiled-coil structures. Using the COILS algorithm [25], Naf1α/β were predicted to contain four coiled-coil structures (Fig. 2). The region through which Naf1 binds to Nef is located within amino acids 94–412 (Fig. 2). We also elucidated the genomic structure of the gene encoding *Naf1*. This gene consists of 18 exons; the first exon is untranslated and exons 2–18 contain the coding sequence (Fig. 2). The genomic structure indicated that *Naf1*α/β are produced by alternative splicing; *Naf1*β mRNA is synthesized when a splicing acceptor site within exon 18 is utilized (Fig. 2). The *Naf1*

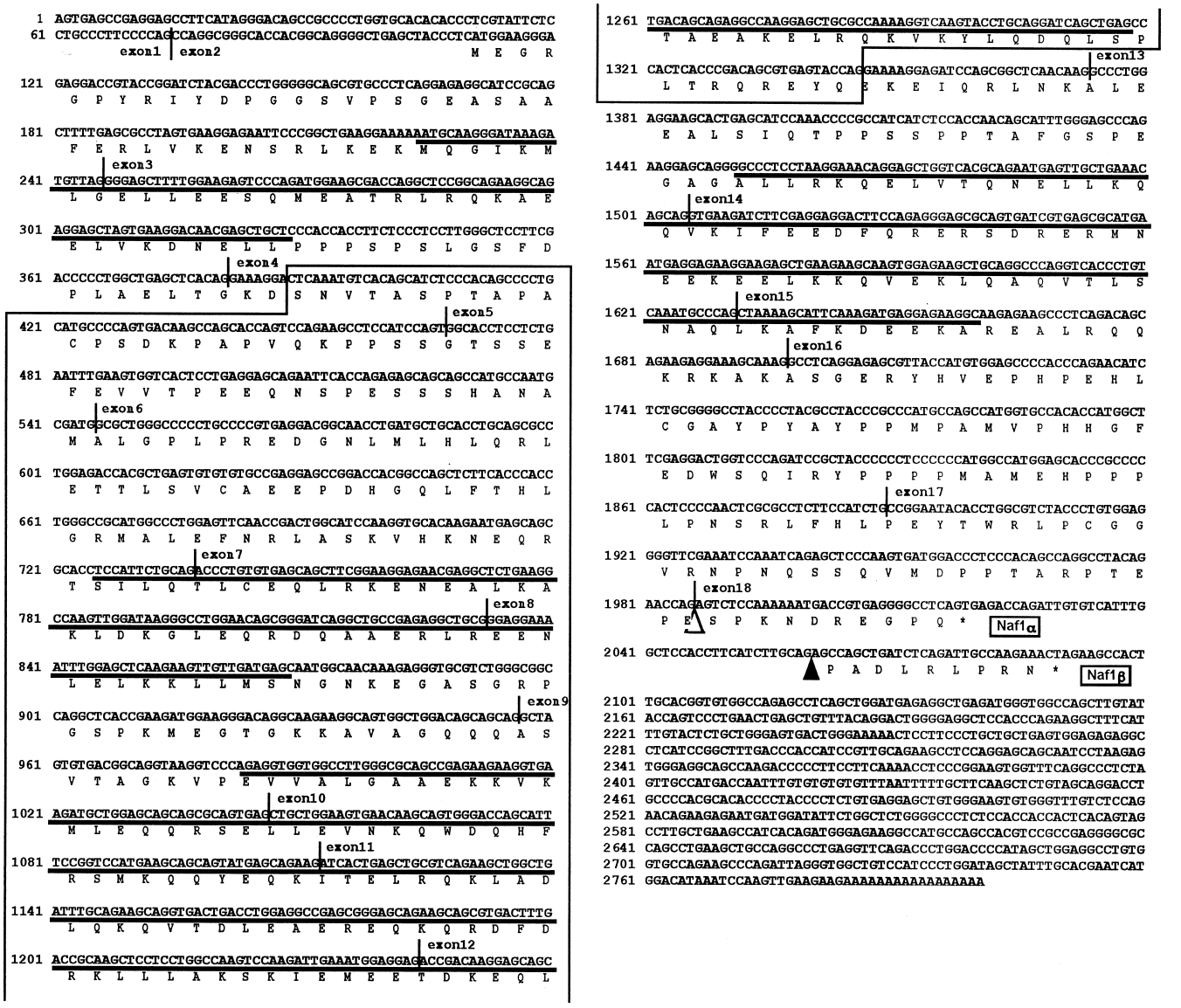

Fig. 2. The nucleotide and amino acid sequences of full-length Naf1α/β. Full-length *Naf1*α/β was characterized by cDNAs obtained by yeast two-hybrid screening and the 5′- and 3′-RACE methods. Probability of coiled-coil conformation was calculated as described in Section 2. The four underlines show putative coiled-coil structures. Alternative splicing produced *Naf1*α/β. The arrowheads show alternative splicing sites at 1987 nt (*Naf1*α, open arrowhead) and 2061 nt (*Naf1*β, solid arrowhead). The Nef interacting region is enclosed. The bars indicate borders of exons.

gene is located on human chromosome 5q32–33.1 (data not shown).

3.3. *Naf1 expression in human tissues*

The expression of *Naf1* mRNA in human tissues was examined by Northern blot analysis. This revealed that the 2.8 kb *Naf1* mRNA is ubiquitously expressed with strong expression in peripheral blood lymphocytes, spleen and skeletal muscle and weak expression in brain (Fig. 3). The mRNA was also detected in various human hematopoietic cell lines, for example, MOLT-4, Jurkat and HL60 (data not shown).

3.4. *Association of full-length Naf1 and Nef*

The yeast two-hybrid study demonstrated that a part of Naf1 associated with Nef from two different HIV-1 strains. Next, we examined whether the full-length Naf1 associates with Nef by pull-down assay. Initially, we produced Nef fusion protein (Nef-6×His) containing six consecutive histidine residues in its N-terminus in bacteria (Fig. 4, left panel). Because Ni-NTA resin binds to hexahistidine, the bacterial extract containing the Nef-6×His and the control extract were mixed with the Ni-NTA resin. On the other hand, HA-tagged Naf1β (Naf1-HA) was produced in human 293T cells (Fig. 4, middle panel). Then, the cell extracts were incubated with Ni-NTA resin that had been pretreated with bacterial extracts. Proteins bound to the resins were eluted using imidazole, and Western blot analysis was performed using anti-HA antibody. As shown in the right panel of Fig. 4, Naf1-HA was pulled down together with Nef-6×His-bound resin but only background level was associated with unbound resin, indicating that the full-length Naf1 specifically associates with Nef in the presence of human cellular extracts.

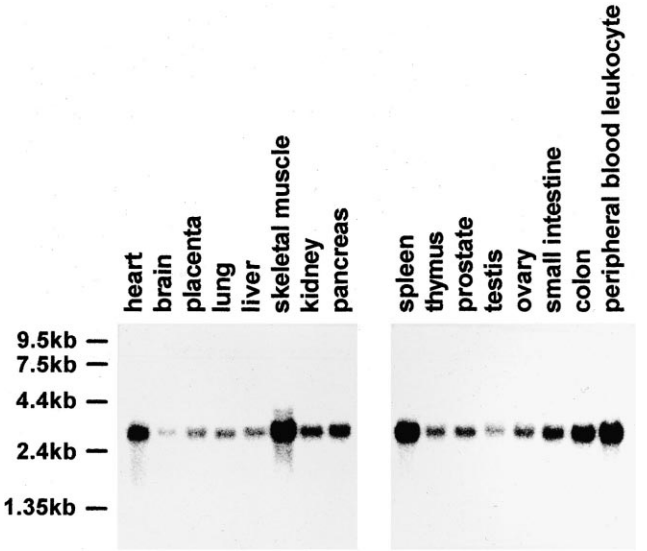

Fig. 3. *Naf1* mRNA expression in human tissues. Northern blot analysis was performed with using Multiple Tissue Northern (MTN) Blot I and II (Clontech) containing 2 μg of human poly(A)$^+$ RNA in each lane, according to the manufacturer's instructions. The used probe was Nef interacting region of the *Naf1* cDNA that had been radiolabeled. Names of the tissues are shown at the top of the panel.

3.5. Augmentation of cell surface CD4 expression by Naf1

We studied whether Naf1 affects cell surface CD4 expression. For this purpose, we chose a well-established model, often used to examine the turnover of CD4 molecule such as Nef-mediated CD4 down-regulation [28]. The CD4 expression vector pBCMGS/CD4 was cotransfected into 293T cells with increasing amounts of Naf1-HA expression vector, and cell surface CD4 expression was examined by flow cytometric analysis. To evaluate the transfection efficiency, the CD19 expression plasmid was also cotransfected. The cell surface CD19 expression level was not affected by Naf1 overexpression (data not shown). As shown in Fig. 5A, the cell surface CD4 expression level was increased by Naf1-HA expression to a maximum of 2.5-fold in a dose-dependent manner. We confirmed that the Naf1 protein level was linearly increased when the amounts of transfected plasmids increased (data not shown). We next transfected pBCMGS/CD4 at various doses. The cell surface CD4 level linearly increased with the amounts of transfected plasmids (Fig. 5B). The Naf1-induced enhancement of CD4 expression occurred within a broad range of CD4 expression levels (Fig. 5B). Similar results were obtained when another CD4 expression vector, pCEP/CD4, was transfected (data not shown). Thus, Naf1-HA expression could increase cell surface CD4 expression.

Next, we examined whether Nef alters Naf1-induced augmentation of CD4 expression. When Nef expression plasmid was transfected, CD4 levels were reduced by 60% (Fig. 5C), which is consistent with the level of down-regulation described in previous reports using the same system [16]. In the presence of Naf1, Nef still decreased CD4 expression levels by 40%, although the absolute CD4 levels were higher compared to those in the absence of Naf1 (Fig. 5C). In addition, the vectors of HIV-1 infectious clones, pNL4-3 and pNL4-3Δnef, were transfected instead of Nef expression vector. As shown in Fig. 5D, when Nef was co-expressed with Naf1 in the context of full-length HIV-1, Naf1-induced CD4 augmentation was again inhibited but absolute CD4 levels were higher than those of Naf1-untransfected cells. These results indicated that the ratio of Nef and Naf1 could affect the cell surface CD4 expression levels. In addition, HIV-1 production was analyzed by an HIV-1 p24^{gag} enzyme-linked immunosorbent assay (ELISA) (Lumipulse f, Fujirebio, Tokyo). Virus production was similar despite different levels of cell surface CD4 expression (data not shown).

4. Discussion

In this study, we identified and cloned the novel cellular protein Naf1. This Naf1 protein associated with HIV-1 Nef in a yeast two-hybrid system and in a pull-down assay using transfected human cell lysates. Moreover, we showed that Naf1 overexpression increased cell surface CD4 levels and that Nef overexpression inhibited this Naf1-induced CD4 augmentation. Similar results in cell surface CD4 levels were observed when Nef was expressed in the context of an infectious HIV-1 clone.

Nef promotes CD4 down-regulation via multiple pathways, i.e. enhancement of CD4 endocytosis [3], inhibition of its transport from Golgi to plasma membrane [18] and inhibition of its recycling [13]. Recently, it was proposed that Nef-induced CD4 endocytosis is mediated by the complex formation containing Nef, CD4, AP-2 and V-ATPase [13,29]. It still remains unclear which CD4 turnover process step is affected by Naf1. Considering that Naf1 has four extended coiled-coil structures, it is supposed that Naf1 interacts with cellular and/or viral proteins to regulate the turnover of the CD4 molecule. From this viewpoint, it is noteworthy that many coiled-coil proteins such as SNAREs [30,31] bind each other between

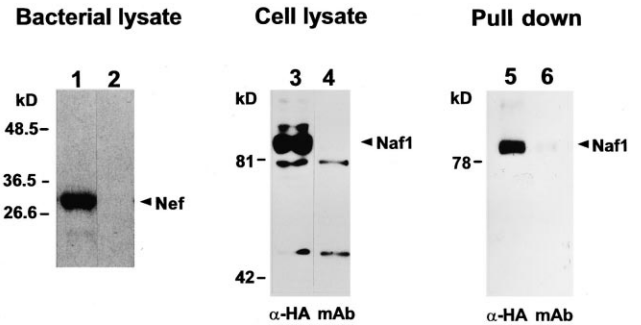

Fig. 4. In vitro pull-down assay of Nef and Naf1. Left panel: Nef-6×His fusion protein. Bacterial lysates were collected from IPTG-induced (lane 1) and uninduced (lane 2) bacteria. The proteins purified by Ni-NTA resin were resolved by 15% SDS-PAGE. The gel was stained with Coomassie brilliant blue (CBB) (lanes 1 and 2). The Nef-6×His was also confirmed by Western blot analysis with anti-Nef antibody (data not shown). The sizes of a prestained marker (Bio-Rad, Hercules, CA) are shown. Middle panel: Naf1-HA protein. The human embryonic kidney cell line 293T cells were transfected with pCMV-Naf1HA (lane 3) and pRc/CMV (lane 4). The lysates were resolved by 8% SDS-PAGE and subjected to Western blot analysis with an anti-HA antibody. The sizes of kaleidoscope marker (Bio-Rad) are shown. Right panel: Ni-NTA resin pull-down assay. The 293T cell lysate containing Naf1-HA was divided and mixed with Ni-NTA resin pretreated with the lysates from IPTG-induced (lane 5) and uninduced (lane 6) bacteria. The proteins bound to the resin were washed three times with washing buffer, pulled down and analyzed by Western blot analysis with anti-HA antibody. As shown in lane 5, Naf1-HA was pulled down with Nef specifically. The sizes of kaleidoscope marker are shown. These experiments were repeated three times with similar results.

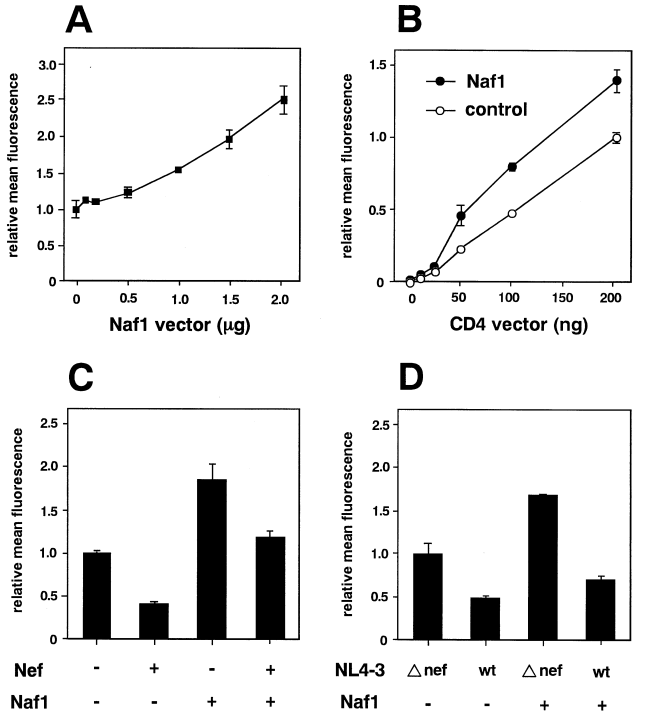

Fig. 5. Modulation of cell surface CD4 levels by Naf1 and Nef. 293T cells were cotransfected with several expression vectors, and the cell surface CD4 levels were analyzed by flow cytometry. The mean ± S.D. of geometric means of CD4 expression levels was calculated in the CD19-positive population. These values were then calibrated by transfection efficiency and are shown here. Transfection efficiency was evaluated by the percentage of CD19-positive cells. A: Augmentation of CD4 level by Naf1 in a dose-dependent manner. 293T cells were cotransfected with a given quantity of pBCMGS/CD4 (50 ng), pCD19 (0.1 μg) and increasing amounts of pCMV-Naf1HA (0–2.0 μg). The empty vector pRc/CMV (0–2.0 μg) was added to adjust the total amounts of transfected DNA to 2.0 μg. The CD4 fluorescence is shown as 1.0 when pCMV-Naf1HA was not cotransfected with pBCMGS/CD4 and pCD19. B: Augmentation of a broad range of CD4 levels by Naf1. 293T cells were cotransfected with various quantities of pBCMGS/CD4 (0–200 ng), pCD19 (0.1 μg) and a given quantity (1.8 μg) of pCMV-Naf1HA (●) or control pRc/CMV (○). The CD4 fluorescence is shown as 1.0 when 200 ng of pBCMGS/CD4 and 1.8 μg of pRc/CMV were cotransfected with pCD19. C and D: Inhibition of Naf1-induced CD4 augmentation by Nef. In C, 293T cells were cotransfected with pBCMGS/CD4 (50 ng), pCD19 (0.1 μg), pCMV-Naf1HA (0.9 μg), pCMV-Nef (0.9 μg) and control vectors. The CD4 fluorescence is shown as 1.0 when only the control vector pRc/CMV was cotransfected with pBCMGS/CD4 and pCD19. In D, 293T cells were cotransfected with pBCMGS/CD4 (50 ng), pCD19 (0.1 μg), pCMV-Naf1HA (0.9 μg), pNL4-3 or pNL4-3Δnef (0.9 μg). The CD4 fluorescence is shown as 1.0 when pNL4-3Δnef and the control vector pRc/CMV were cotransfected with pBCMGS/CD4 and pCD19.

vesicular and plasma membranes, thereby regulating vesicle trafficking, sorting and fusion events. When overexpressed, these coiled-coil-containing proteins often accumulate in specific membrane compartments, change the morphology of these compartments and affect the rate of vesicle trafficking, sorting and fusion. Similar to these proteins, immunostaining of Naf1-HA-transfected cells showed punctate staining within cytoplasm which appeared to be the enlarged membrane compartments (M. Fukushi, unpublished data), implicating its possible involvement in membrane trafficking and sorting events.

Naf1 binds to Nef in the yeast two-hybrid system and in the in vitro pull-down assay using mammalian cell lysates. Because of a lack of anti-Naf1 antibodies against endogenous Naf1, we performed immuno-pull-down assays with Naf1-HA and Nef-cotransfected cell lysates. However, detection of interaction in mammalian cells was unsuccessful. One of the reasons for the inability to detect their interaction in vivo is Naf1 accumulation in restricted cellular compartments as described above. Generation of antibodies to detect endogenous Naf1 is necessary for further clarification of this point.

Given Naf1's coiled-coil structure, cellular localization and ability to augment the CD4 molecule, we envisage that Naf1 may positively modulate cell surface CD4 expression presumably during the trafficking and sorting processes, and Nef may inhibit Naf1 function through a physical association. From this point of view, Nef may down-regulate cell surface CD4 levels by two distinct molecular mechanisms: (i) activation of cellular pathways which down-regulate CD4 such as activation of CCP-mediated endocytosis, and (ii) inhibition of cellular pathways which up-regulate CD4 such as inhibition of Naf1 action. Further studies are required to determine how and at which step Naf1 could affect cell surface CD4 expression. When these aspects of Naf1 action are made clear, we may obtain a new twist in the mechanism of Nef-induced CD4 down-regulation.

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