The role of myristoylation in the interactions between human immunodeficiency virus type 1 Nef and cellular proteins

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Introduction

The nef gene of the primate lentiviruses encodes a myristoylated polypeptide of 206 (human immunodeficiency virus type 1 (HIV-1)) to 265 (Simian immunodeficiency virus) amino acids with an apparent molecular mass of between 27 and 35 kDa [1]. Early studies defined nef as a negative regulator of viral replication; however, there is now a growing consensus that Nef can be considered an essential regulatory protein critical for achieving high viral load and disease progression. A number of functions in vitro have been ascribed to Nef but it is unclear at present how each of these functions contributes to the requirement for Nef in vivo. What is clear is that myristoylation plays an important role in most of these functions and it also follows that Nef myristoylation is critical for the life cycle of the virus in vivo. This conclusion is borne out by the conservation of the myristoylation signal in both laboratory and primary isolates of Nef, despite the high level of variability in the rest of the gene.

Myristoylation has a profound effect on the subcellular localization of Nef: transient transfection studies in Cos cells demonstrated that the association of Nef with cytoplasmic membrane structures required myristoylation; however, myristoylated Nef was also found in the cytosol [2,3]. Non-myristoylated Nef was found in the cytosolic fraction and also in the nucleus. Furthermore myristoylation was shown either to enhance [4], or to be absolutely required for [3], the binding of Nef to cytoskeletal elements. Interestingly, cytoskeleton binding also required a proline-rich motif implicated in binding...
to SH3 domains, raising the possibility that Nef might interact with cytoskeletal SH3-containing proteins [3].

Nef expression has been shown by many groups to down-regulate cell surface expression of the viral receptor glycoprotein CD4 [5–8]. The precise biochemical mechanisms underlying this process remain to be elucidated but it has been shown to result from an increase in the rates of endocytosis and lysosomal degradation of CD4 [8,9]. Nef myristoylation was shown to be required for the increase in endocytosis [8]; however, the proline-rich motif was not required [10].

Nef has also been demonstrated to enhance the infectivity of HIV-1 in primary lymphocyte cultures by increasing the capacity of viral particles to infect these cells productively [11,12]. This effect was independent of the effects of Nef on CD4 and concomitant effects on envelope expression, because it was shown to occur in Cos cells transfected with HIV-1 proviral clones and with envelope-defective HIV-1 pseudotyped with amphotropic (murine leukaemia virus) envelope [13]. Again, myristoylation was required for this function of Nef [11], as was the proline-rich domain [10].

One consequence of Nef myristoylation that has not been addressed as yet is the effects of acylation on the structure of the protein. One published study used NMR to obtain structural information about Escherichia coli expressed non-myristoylated Nef protein [14]. The data indicated that the molecule could be divided into two distinct domains, an N-terminal 'membrane anchor' domain that lacked any well-defined structure, and a compactly folded hydrophobic core domain starting between amino acids 40 and 66 and extending to the C-terminus. The two domains could be physically separated by cleavage in vitro with recombinant HIV-1 protease between residues 67 and 58, and it has been suggested that this cleavage might play a role in the regulation of Nef function in vivo [15]. It is interesting to speculate that myristoylation might confer some structure on the N-terminal domain, perhaps even interacting directly with hydrophobic regions in the core domain, as has been shown for the myristoylated catalytic subunit of cyclic-AMP-dependent protein kinase [16]. This might also explain the distribution of myristoylated Nef between membrane and cytosol: two conformationally distinct forms of Nef might exist in which the myristoyl group is either anchored into the lipid bilayer or bound to hydrophobic regions of the protein itself. The conformational switch could be generated by an additional modification such as phosphorylation. In this context a number of studies have demonstrated phosphorylation of Nef by members of the protein kinase C family in vitro and in vivo [5,17]. Alternatively, as is the case for myristoylated alanine-rich C kinase substrate (MARCKS), phosphorylation might simply displace Nef from the membrane by electrostatic repulsion [18].

A key question to be addressed when examining the role of myristoylation in Nef function is whether myristoylation merely targets the protein to membrane structures or whether acylation is more directly involved in the interactions between Nef and cellular processes. Myristoylation has been shown to play a role in stabilizing protein–protein interactions in the capsids of picornaviruses and is required for the interactions of G-protein α subunits with their βγ subunits [19]. It was therefore conceivable that myristoylation of Nef was required for its interactions with cellular proteins. As a first step in addressing this question we generated fusion proteins containing Nef as an N-terminal fusion with glutathione-S-transferase (GST) and expressed these proteins in the baculovirus expression system [20]. The resulting Nef–GST fusion proteins were myristoylated and proved to be useful reagents to identify cellular proteins that interacted with Nef in vivo. These reagents were also used to demonstrate a direct myristoylation-dependent interaction between Nef and the cytoplasmic tail of CD4 in vitro. These reagents could be divided into two distinct proteins that bound to Nef-GST in vitro as the Src-family tyrosine kinase p56Lck and β-COP, a major coat component of non-clathrin-coated vesicles.

**Methods**

**Baculovirus expression**

A cDNA for murine Lck was subcloned from pSM.Lck (a gift from Dr. Mark Marsh, University College, London) into the baculovirus transfer vector pAcCl29 and a recombinant baculovirus (AcLck) expressing Lck generated by standard procedures. Expression was verified by immunoblotting with a rabbit serum raised to a peptide corresponding to amino acids 478–509 of murine Lck (a gift from Dr. Mark Marsh). Labelling with [3H]myristic acid or [3H]palmitic acid was carried out in TC100/1% dialysed foetal calf serum/1% DMSO from 24 to 40 h post infection as described [20]. GST fusion proteins were expressed and purified as previously described [20].
**Immunoblotting**

Samples were separated by SDS-PAGE and transferred to PVDF membrane (Millpore Immobilon P) with the use of a semi-dry blotter. Membranes were blocked with 10% (w/v) dried milk in Tris-buffered saline/0.1% Tween 20 (TBT-T) for 10 min at room temperature. Antibodies were added in TBT-T containing 5% (w/v) dried milk and 20% (w/v) serum corresponding to the derivation of the secondary antibody. Mouse monoclonal antibodies to phosphotyrosine (Santa Cruz PY69) and β-COP (M3A5, a gift from Dr. Mark Marsh) were detected with sheep anti-(mouse horseradish peroxidase) (HRP); rabbit anti-Lck was detected with goat anti-(rabbit HRP). Visualization was by Enhanced chemoluminescence (Amersham).

**Nef binding assays in vitro**

Assays were performed as previously described [20] except that lysates were made from Sf9 cells infected with AcLck at 48 h post infection (for Lck binding) and unlabelled SupT1 cells (for β-COP binding). Assays contained lysate from 10^8 Sf9 cells or 10^7 SupT1 cells.

**Results**

Baculovirus-expressed Lck is enzymically active and authentically acylated

Polypeptides of 55 or 57 kDa from a metabolically labelled extract of Jurkat T-cells were previously observed as binding to immobilized Nef-GST in vitro. Lck was not identified as one of these proteins by immunoblotting; however, this was possibly due to low abundance. In the light of the documented effects of Nef on T-cell signal transduction, a baculovirus recombinant expressing murine Lck was generated to investigate whether Lck could bind to Nef in vitro. To determine whether Lck expressed in Sf9 cells retained the autophosphorylating activity of the mammalian enzyme, lysates from Sf9 cells infected with AcLck were incubated at 37°C for 15 min in the presence of ATP (10 μM) and analysed by immunoblotting with antibodies to Lck (Figure 1a) or phosphotyrosine (Figure 1b). In the presence of ATP, Lck was tyrosine autophosphorylated as expected. The absence of any other phosphotyrosine-containing polypeptides in extracts from control cells (expressing β-galactosidase) strongly suggests that this is not the result of phosphorylation by a cellular tyrosine kinase. Lck is modified by the addition of both an N-terminal myristate residue and palmitate residues on cysteines at positions 3 and 5 [22]. Figure 1c demonstrates that Lck is authentically modified by both myristoylation and palmitoylation when expressed in Sf9 cells.

Lck and β-COP bind to Nef in vitro

To determine whether baculovirus-expressed Lck could bind to Nef in vitro, a lysate from Sf9 cells infected with AcLck was incubated with myristoylated Nef-GST, a non-myristoylated derivative (Nef(m-)GST) or myristoylated GST (myrGST) immobilized on glutathione agarose beads as described previously and bound proteins were analysed by immunoblotting with anti-Lck antiserum. Figure 2a shows clearly that Lck bound to Nef–GST essentially quantitatively. This binding was myristoylation-dependent, as a Nef-GST derivative in which the myristoylation signal had been disrupted by a Gly–Ser change failed to bind Lck. It was also dependent on Nef amino acid sequences because GST alone with an N-terminal myristoylation signal failed to bind significant amounts of Lck.

A recent study with the yeast two-hybrid system described a physical interaction between Nef and β-COP [23]. The binding of β-COP to
Figure 2
Binding of Lck and β-COP to Nef in vitro

(a) Lysate from 10⁶ Sf9 cells infected with AcLck was incubated with immobilized fusion proteins as indicated. Bound proteins were resolved by SDS PAGE and immunoblotted with anti-Lck serum. Lane 1 contained total lysate from 10⁶ AcLck infected cells.

(b) Lysate from 10⁷ SupT1 cells was incubated with immobilized fusion proteins as indicated. Bound proteins were resolved by SDS PAGE and immunoblotted with a monoclonal antibody to β-COP. Lane 1 contained total lysate from 10⁶ SupT1 cells.

Nef-GST in vitro was therefore examined by immunoblotting of bound proteins with a monoclonal antibody (M3A5) to β-COP after incubation of a cytosplasmic lysate of SupT1 cells with immobilized fusion proteins as described above. Figure 2b shows that M3A5 detected a protein of 110 kDa, corresponding to β-COP, that bound to myristoylated Nef-GST but failed to bind to either Nef(m-)GST or myrGST. M3A5 also detected a band of 55 kDa that probably represents a proteolytic degradation product of β-COP because it increased in intensity after repeated freeze-thawing of extracts. This polypeptide also bound to Nef-GST. This result confirms and extends the previous study, demonstrating that β-COP binding to Nef in vitro is dependent on Nef myristoylation.

Discussion

This report demonstrates that a baculovirus-derived myristoylated Nef-GST fusion protein interacts in vitro with both the Src-family tyrosine kinase p56Lck and β-COP. The interaction between Nef and these two proteins was dependent on Nef myristoylation as a non-myristoylated derivative failed to bind. Under the assay conditions employed, essentially all the Lck was retained by Nef-GST (Figure 2a, lanes 1 and 2); however, approx. 5–10% of the β-COP was retained (Figure 2b, lanes 1 and 2).

At first glance the demonstration of a direct interaction between Nef and Lck contradicts a recent study of the interaction of a proline-rich motif (PXXP) in Nef with SH3 domains of the Src family kinases Hck and Lyn but not Lck [10]. However, that study used a filter-binding assay in which a bacterially expressed GST-PXXP fusion protein was probed with biotinylated GST–SH3 fusion proteins, whereas the data described here were obtained by using intact proteins in a native configuration. It is plausible that one role of myristoylation may be to allow Nef to adopt a structure in which the proline-rich motif is exposed. The role of the proline-rich motif in the myristoylation-dependent interaction of Nef with Lck in vitro is currently being assessed. Although a myristoylated GST protein bound a low level of Lck (Figure 2a, lane 4), this result was not due to a non-specific interaction of the acyl groups on the two proteins, because efficient binding required Nef amino acid sequences.

An interaction between Nef and β-COP was first identified by using the yeast two-hybrid system. This study identified a cDNA encoding the C-terminal 303 amino acids of β-COP that bound to bacterially expressed GST–Nef in vitro [23]. This result does not rule out the possibility that binding of Nef to intact β-COP requires, or is enhanced by, Nef myristoylation. In this regard, long exposures of immunoblots revealed the presence of low levels of β-COP in the Nef(m-)GST lane of Figure 2b (results not shown).

In conclusion, this report identifies Lck and β-COP as two proteins that bind to the HIV-1 Nef protein in vitro in a myristoylation-dependent fashion. Coupled with the previously observed myristoylation dependence of Nef binding to CD4 in vivo, these interactions provide an explanation of why myristoylation of Nef is required for its biochemical functions in viral replication. These results therefore provide an answer to the question posed in the introduction and show that myristoylation of Nef is not merely a mechanism for targeting the protein to membrane structures, but is likely to play a direct functional role by mediating its interactions with cellular proteins. Future studies must assess the contribution and physiological relevance of these interactions to Nef function in vivo.

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24 Accepted 3 March 1995

Demyristoylation of myristoylated alanine-rich C kinase substrate
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Introduction
Covalent modification of proteins by fatty acids has received considerable attention in the last few years and may be considered a new way of regulating protein localization and/or function. Proteins involved in signal transduction like G proteins [1], p60CS tyrosine kinase [2], cyclic-AMP-dependent kinase (PKA) [3] or NO synthase [4] are all modified by the addition of fatty acids, which includes the addition of N-terminal myristic acid, C-terminal isoprenyl groups and thioester-linked palmitic acid. Whereas palmitoylation occurs as a dynamic molecular process, with cycles of palmitoylation and depalmitoylation regulating protein localization, myristoylation and isoprenylation are considered to be irreversible processes. The effect of these fatty acid modifications may be to mediate protein–lipid and protein–protein interactions as well as stabilizing polypeptide structure.

Myristoylated alanine-rich C kinase substrate (MARCKS) is an acidic myristoyl protein often used as a marker for the activation of protein kinase C (PKC) (for reviews see [5,6]). Interaction with calmodulin and actin filaments in vitro [7,8] suggests that MARCKS may be involved in the regulation of interactions between these two proteins, but the cellular mechanisms for such a function remain to be established. The sites of interaction with actin and calmodulin are contained in the so-called phosphorylation site domain (PSD), a conserved basic region of 25 amino acids that also contains a cluster of PKC phosphorylation sites. The main population of MARCKS is membrane-bound in various cell

Abbreviations used: MARCKS, myristoylated alanine-rich C kinase substrate; PKA, cyclic AMP-dependent kinase; PKC, protein kinase C; PSD, phosphorylation site domain.

Received 3 March 1995