Identification of cellular proteins that bind to the human immunodeficiency virus type 1 nef gene product in vitro: a role for myristylation

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The human immunodeficiency virus (HIV) type 1 nef gene product was expressed as an N-terminal fusion protein with glutathione-S-transferase (GST) in the baculovirus system. The resulting nefGST fusion protein was found to be authentically myristylated at the N terminus and could be purified to homogeneity by one-step affinity chromatography on immobilized glutathione. The high affinity of nefGST for glutathione was exploited to develop an assay to identify cellular proteins capable of interacting with nef. Several such proteins were identified in extracts from the Jurkat human T cell line. The interaction between nef-binding proteins and immobilized nefGST could be specifically competed by the addition of soluble nef. Cell fractionation showed that nef-binding proteins were present in both cytosolic and membrane-associated fractions. A non-myristylated derivative failed to bind to the membrane-associated proteins but was able to bind to the cytosolic group, albeit with reduced affinity. In addition, a single protein present in both soluble and membrane-associated fractions exhibited myristylation-independent binding to nef. By analogy with other myristylated proteins such as MARCKS (myristylated alanine-rich C kinase substrate) and the Rous sarcoma virus transforming protein, src, the membrane-associated proteins that bind only to myristylated nef may represent a specific membrane target for nef. The cytosolic proteins that interact with nef may constitute soluble components of an as yet unidentified signal transduction pathway which is the target of nef action in the HIV-1-infected cell.

Introduction

The nef gene of human immunodeficiency virus (HIV) type 1 encodes a polypeptide of between 200 and 205 amino acids, varying between isolates (Harris et al., 1992a). Despite intensive work, the precise function of the nef gene product remains the subject of controversy. Initial studies identified the protein as a repressor of viral replication (Fisher et al., 1986; Luciw et al., 1987; Terwilliger et al., 1986) that acted by down-regulation of transcription from the HIV-1 long terminal repeat (LTR) (Ahmad & Venkatesan, 1988; Niederman et al., 1989; Maitra et al., 1991), but other studies failed to reproduce these results (Kim et al., 1989; Hammes et al., 1989; Bacherlie et al., 1990). There is a significant degree of amino acid sequence variation between nef isolates both in vivo and in vitro (Harris et al., 1992a) and the contradictions regarding nef function may represent the consequences of this variation. This problem was highlighted by a number of recent studies. Terwilliger et al. (1991) demonstrated that the replication of the IIIB provirus was suppressed by the corresponding IIIB nef allele but that replacement of nef in the IIIB provirus by the EL1 nef allele enhanced the growth of the provirus. A number of amino acid residues in the ELI nef gene were subsequently identified as important for this enhancement, including Cys143, Cys170 and Leu182 (Zazapoulos & Haseltine, 1992). Luria et al. (1991) examined the effect of nef on the induction of interleukin 2 receptor (IR2R) mRNA: Jurkat T cells constitutively expressing nef were unable to respond to a wide range of stimuli that normally induced the synthesis of IL2R mRNA. In contrast, cells expressing an alternative nef allele that differed at only three amino acid residues responded normally.

The nef gene product is myristylated at the N terminus (Allan et al., 1985) and this acylation is required for the location of nef on the cytoplasmic face of the plasma membrane in infected cells (Yu & Felsted, 1992). Myristylation of nef enhances HIV-1 replication (Zazapoulos & Haseltine, 1992) and, conversely, suppresses HIV-1 LTR transcription (Yu & Felsted, 1992). Myristylation is thus important for nef function but the precise role of acylation remains to be elucidated.

Limited sequence similarity between nef and the GTP-binding domains of ras and G proteins has been reported (Samuel et al., 1987; Guy et al., 1987). Furthermore, bacterially expressed nef was shown to bind and hydrolyse GTP (Guy et al., 1987). Other studies with both bacterial and baculovirus-expressed nef gene pro-
ducts, however, failed to reproduce these findings, suggesting that the activity was due to a contaminating bacterial enzyme (Kaminchik et al., 1990; Nebreda et al., 1991; Matsuura et al., 1991; Harris et al., 1992a). It has been reported that nef is phosphorylated by protein kinase C, although this observation was made following the expression of nef in BHK21 cells infected with a recombinant vaccinia virus (Guy et al., 1987) and has not been demonstrated for nef expressed in HIV-1-infected human cells. Nef also possesses limited sequence similarity with the human thyrotropin receptor (Burch et al., 1991) and scorpion neuroactive peptides (Werner et al., 1991). All these observations, together with the location of nef on the plasma membrane and its potential ability to modulate IL2R activation, suggest that its role in the viral life cycle involves perturbation of cellular signal transduction pathways. In this context, nef has been reported to down-regulate cell surface expression of CD4 (Guy et al., 1987; Garcia & Miller, 1991) although even this putative function is subject to controversy because Cheng-Mayer et al. (1989) failed to observe this phenomenon.

Unambiguous definition of the biochemical function of nef in the life cycle of HIV will involve the precise identification of the target(s) of nef action. We previously described the expression of both laboratory and primary isolates of HIV-1 nef in Escherichia coli as fusions with glutathione-S-transferase (GST) (Harris et al., 1992a) using the pGEX system (Smith & Johnson, 1988). By exploiting the high affinity of GST for glutathione immobilized on agarose beads to generate nef affinity reagents, these fusion proteins appeared to provide the ideal method for identifying nef-binding proteins. However, although GST-nef fusion proteins linked to glutathione–agarose (GA) beads were able to precipitate specifically a set of cytoplasmic proteins from extracts of human T cell lines (Harris et al., 1992b) binding was inefficient and not reproducible. One possible explanation for this might be that nef was not expressed in its authentic eukaryotic conformation. In the pGEX system proteins are expressed as C-terminal fusions with GST and it is likely that the N terminus of nef needs to be exposed. Moreover, myristylation does not occur in prokaryotes and, by analogy with other myristylated proteins such as poliovirus P1 capsid precursor (Ansardi et al., 1992) and the Rous sarcoma virus transforming protein src (Resh & Ling, 1990), myristylation of nef is potentially important for its interaction with cellular proteins. Nef was therefore expressed in the baculovirus expression system as the N-terminal portion of a GST fusion molecule. Nef expressed in this system was myristylated at the N terminus and could be efficiently purified by one-step affinity chromatography on GA. This new reagent allowed the definition of three classes of nef-binding proteins on the basis of subcellular localization and myristylation dependence.

Methods

Plasmid construction. The coding sequences for GST were isolated by PCR using the primer pair NNGGATCCCTAACGAGGTGTTTTGAGGATCGGT (coding strand) and NNNNATCGAGATCCGACTCTGATGAGGC (non-coding strand) (where N is any nucleotide) and were cloned into pBluescript as a SalI–KpnI fragment. A KpnI–EcoRI– HindIII–BamHI oligonucleotide linker adaptor was ligated into the KpnI site to facilitate further cloning steps. This construct was termed pGST.

The coding sequence of the HIV-1 nef gene (strain BH10) was isolated by PCR using the primer pair NNGGATCCCTAACGATGGGGTGGCAAGTGG (coding strand) and NNNNATCGAGATCCGACTCTGATGAGGC (non-coding strand), and ligated onto the Clal–BamHI fragment of pGST in the presence of BamHI. The ligation product was gel-purified and ligated into the BamHI site of the transfer vector pAcCl29 (Livingstone & Jones, 1989). For GST expression, a BamHI linker-adaptor was ligated to the 3' end of the SalI–BamHI fragment of pGST and this was cloned into pAcCl29. The myristylated GST derivative (myrGST) was constructed by cloning oligonucleotides encoding the myristylation sequence of HIV-1 gag p55 (Met-Asp-Ala-Arg-Ala-Asp) into the SalI site of pGST to generate the plasmid pmGST.

For the production of non-myristylated nef, the coding strand primer NNGGATCCCTAACGAGGTGTTTTGAGGATCGGT was used, replacing the glycine residue at position 2 with a serine. Nef tagged with six histidine residues at the C terminus (nef-6H) was produced by PCR using the coding primer described above and NNGGATCCCTAACGATGGGGTGGCAAGTGG (coding strand) and NNNNATCGAGATCCGACTCTGATGAGGC (non-coding strand) as the non-coding primer. This PCR product was cloned directly into pAcCl29 as a BamHI fragment.

Isolation and propagation of recombinant baculoviruses. Sf9 cells were grown in TC100 medium (Gibco) containing 10% fetal calf serum (FCS) at 27 °C. Cells (1 × 10⁶ per 35 mm dish) were transfected by the calcium phosphate method with 5 to 10 μg of the appropriate pAcCl29 recombinants and 1 μg of AcRPIacZ viral DNA linearized with Rsul (Kitts et al., 1990). After 2 days the medium was harvested, filtered through a 0.22 μm filter unit and plated onto Sf9 cells. White plaques were picked and underwent three rounds of plaque purification prior to expansion.

Purification of recombinant proteins. Sf9 cells were infected with the appropriate recombinant viruses at 10 p.f.u./cell and harvested at 72 h post-infection (p.i.). For the production of GST and GST fusion proteins, infected cells were pelleted, washed twice with ice-cold PBS and lysed in PBS containing 1% Triton X-100, 5 mM-EDTA, 10 mM-iodoacetamide and the protease inhibitors aprotinin (2 μg/ml), PMSF (1 mM) and leupeptin (1 μg/ml). After incubation at 4 °C for 30 min the lysate was clarified by centrifugation at 10000 r.p.m. for 15 min at 4 °C. Clarified supernatant was incubated with GA beads at 4 °C for 2 h and the beads were then washed extensively in 50 mM-Tris–HCl pH 8.0. Fusion protein was eluted by the addition of 50 mM-Tris–HCl pH 8.0 containing 10 mM reduced glutathione. Fractions containing protein were pooled and dialysed overnight against two changes of 50 mM-Tris–HCl pH 8.0 containing 10 mM reduced glutathione. Fractions containing protein were pooled and dialysed against 50 mM-Tris–HCl pH 8.0. Then, 1 mM-PMSF and 5 mM-EDTA were added and purified protein was stored in aliquots at −70 °C.

For the production of purified nef-6H, clarified lysate was incubated with nickel nitritotriacetate acid (Ni-NTA)–agarose, washed with PBS and eluted with 100 mM-citrate buffer pH 6.0. Protein-containing fractions were pooled, dialysed against 20 mM-sodium phosphate buffer pH 7.2 and stored at −70 °C.
**Results**

**Expression and purification of nef affinity reagents**

Myristylated and non-myristylated nef gene products were expressed as N-terminal fusion proteins with GST [nefGST and nef(m-)-GST respectively] using the baculovirus vector system. Myristylated nef was also expressed with a C-terminal tail of six histidine residues to permit purification by metal chelate affinity chromatography. GST was expressed alone and with an additional N-terminal myristylation sequence (Met-Gly-Ala-Arg-His6) to permit purification by metal chelate affinity chromatography on either GA or Ni-NTA–agarose. Fig. 2 (b), (e) and (d) confirm that the proteins purified from infected lysates were recognized on Western blots by the appropriate monoclonal antibodies (MAbs). NefGST and nef(m-)GST fusion proteins were recognized by MAbs to both the N and C termini of nef and also by a MAAb to GST (vpg66). Nef-6H was recognized only by the anti-nef MAbs and bacGST/myrGST were recognized only by vpg66. The ability of nefGST fusion proteins to bind strongly to GA indicates that the GST portion of the fusion protein is in the native state. Interactions between the two parts of the fusion protein which could potentially interfere with the binding of cellular proteins to nef are therefore unlikely, but cannot be ruled out. Interestingly, the N terminus-specific antibody recognizes a dimer of nefGST, nef(m-)GST and nef-6H that is not recognized by the C terminus MAAb or vpg66. This observation suggests that the epitope bound by the C terminus MAAb is involved in dimerization and that the epitope on GST recognized by vpg66 might be obscured in the dimer. The significance of this apparent dimerization of nef is not clear.

To confirm the nefGST, nef-6H and myrGST were myristylated, SF9 cells were infected with the appropriate ...
Fig. 2. Expression and purification of recombinant protein affinity reagents. Infected Sf9 cell lysates (lanes 1, 3, 5, 7 and 9) or purified proteins (lanes 2, 4, 6, 8 and 10) were separated by 12% SDS–PAGE and either stained with Coomassie brilliant blue R-250 (a), or transferred to nitrocellulose and probed with an anti-GST MAb (b), MAbs directed against the N terminus (c) or C terminus (d) of nef. Cells were infected with recombinant viruses expressing the following. Lanes 1 and 2, nefGST; lanes 3 and 4, nefm-)GST; lanes 5 and 6, nef-6H; lanes 7 and 8, myrGST; lanes 9 and 10, bacGST.

recombinant viruses and labelled with either \(^{35}\)S)methionine or \(^{3}H\)myristic acid (Fig. 3). Whereas \(^{35}\)S)methionine was incorporated into all the recombinant proteins (Fig. 3a), only those containing an N-terminal myristylation sequence incorporated \(^{3}H\)myristic acid (Fig. 3b). The presence of myristate at the N terminus renders that protein refractory to N-terminal amino acid sequencing by Edman degradation, so this method was used to determine the proportion of myristylated product. Only 1% of a sample of nef/GST was accessible to cleavage by Edman reagent, indicating that the protein was essentially completely myristylated (data not shown). The correlation between the expected and observed patterns of myristylation indicated that correct initiation of translation was occurring at the first methionine codon in nef. Initiation has been reported at the second methionine (residue 20) in some expression systems (Ahmad & Venkatesan, 1988; Kaminchik et al., 1991). The data presented in Fig. 2 and 3 thus confirm the physical integrity of the proteins used in this study.

**Binding of cellular proteins to nef in vitro**

Lysates were prepared from either unstimulated or phorbol 12-myristate 13-acetate (PMA)-stimulated Jurkat cells as described in Methods. Aliquots of the lysates were sequentially precleared by incubation with GA beads alone followed by *E. coli*-expressed GST bound to GA beads. Cleared lysates were then incubated with baculovirus-expressed bacGST or nefGST bound to GA beads. Following extensive washing of the beads with lysis buffer, bound proteins were eluted with sample buffer and analysed by SDS–PAGE on 6 to 20% linear gradient gels followed by fluorography (Fig. 4). A number of proteins in the Jurkat extracts bound strongly either to GST, glutathione or to agarose beads and thus were retained by bacGST–agarose beads. In the presence of nefGST (lanes 3, 4), an additional subset of proteins, designated p280, p97, p75, p55/57 and p35 according to their apparent *M*~s~, were retained on the beads. A number of less abundant proteins of lower *M*~s~,
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Fig. 3. Baculovirus-expressed nef/GST is myristylated at the N terminus. Autoradiograph of lysates (lanes 1, 2, 3, 5, 7, 9 and 11) and purified fusion proteins (lanes 4, 6, 8, 10 and 12), prepared from infected Sf9 cells labelled with either [35S]methionine (a) or [3H]myristic acid (b) and separated by 12% SDS-PAGE. Cells were infected with recombinant viruses expressing proteins as follows. Lanes 1, Mock-infected; lanes 2, β-galactosidase; lanes 3 and 4, nef/GST; lanes 5 and 6, nef(m-)GST; lanes 7 and 8, nef-6H; lanes 9 and 10, myrGST; lanes 11 and 12, bacGST.

p32, p28 and p26, were also observed. Only one difference could be discerned between unstimulated and stimulated extracts, a protein of 45K, that bound to nef/GST but not bacGST was present only in extracts of PMA-stimulated Jurkat cells (Fig. 4, lane 4).

The subcellular location of these nef-binding proteins was determined by separating a Jurkat cell lysate into cytosolic and membrane-associated protein fractions. These fractions were preclearred as described and incubated with either bacGST– (Fig. 5, lanes 1 to 3) or nefGST–GA beads (Fig 5, lanes 4 to 6). The results show that nef-binding proteins could be separated into three classes. The first is proteins that were exclusively membrane-bound, including p280, p35, p32, p28 and p26, and that were only observed in the total cytoplasmic fraction (lane 4) or the membrane-bound fraction (lane 5). The second class is proteins that were present in the total cytoplasmic fraction (lane 4) and the cytosol fraction (lane 6, p57/55). A number of nef-binding proteins, and in particular a doublet of p75 and a previously unobserved protein of 180K, were present in the cytosolic fraction but not in the total cytoplasmic lysate. This may have been due to differences in extraction procedures or it could be that the absence of proteins such as p280 and p35 permitted the binding of other proteins to nef. Thirdly, only one protein, p97, was present in both cytosolic and membrane-associated fractions (lanes 4 to 6).

The effect of N-terminal myristylation on the binding of nef to cellular proteins was investigated using the non-myristylated nef(m-)GST fusion protein. Precleared Jurkat lysate was incubated with myrGST, bacGST, nef/GST or nef(m-)GST as described. The results are shown in Fig. 6. A number of proteins, p280, p32 and p28, that bound to myristylated nef (lane 3) failed to bind to non-myristylated nef (lane 4). It is unclear whether this is also the case for p35 and p26 because the higher background obtained in the presence of myristylated nef (lane 3) obscures these bands. Proteins p75 and p57/55 (migrating as a single band in Fig. 6) still bound to non-myristylated nef but with markedly lower affinity. The binding of p97 was independent of myristylation: p97 bound equally well to nef/GST or nef(m-)GST.
myristylated GST derivative (myrGST; lane 1), consisting of bacGST with the addition of the N-terminal six amino acids from HIV-1 gag p55 (Met-Gly-Ala-Arg-Ala-Ser), failed to bind to any of the proteins that bound only to myristylated nef. This confirmed that these proteins were specifically binding to myristylated nef, and were not simply interacting with the myristate moiety at the N terminus.

To confirm the specificity of the binding of these cellular proteins to nef, a competition assay was performed (Fig. 7). Increasing amounts of soluble myristylated nef (nef–6H) (lanes 3 to 5) or BSA (lanes 6 to 8) were added to aliquots of precleared Jurkat extract prior to incubation with nefGST–GA beads. Addition of exogenous soluble nef was able to inhibit the binding of p280, p97, p75, and p35 to nefGST–GA beads [compare lane 2 (no competitor) with lane 5 (40 μg nef–6H)], presumably by itself binding to these proteins. Lack of definition of low M₆ proteins resulted in ambiguous visualization of competition for the p26, p28 and p32 proteins. Intriguingly, in the presence of 40 μg nef–6H (lane 5) more p55/57 was bound by nefGST. This result was reproducible and may be explained by the fact that in the presence of high concentrations of soluble nef–6H a small amount was found tightly associated with beads (data not shown). By comparison of the intensity of the bands the relative amounts of p55/57 were clearly higher than those of the other nef-binding proteins and, thus, it would not be as efficiently competed by soluble nef–6H. The presence of nef–6H on the beads, whether by non-specific association or specific dimerization with nefGST as discussed above, would therefore result in the binding of more p55/57. Addition of a non-specific protein (BSA) to the assay had no effect on the binding of these proteins to nefGST–GA beads. These proteins are thus binding to nef amino acid sequences alone and are not
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Fig. 7. Soluble myristylated nef competes with immobilized nef/GST for the binding of cellular proteins. Soluble protein was added to pre-cleared extract from Jurkat cells labelled with [35S]methionine prior to incubation with bacGST-GA beads (lane 1) or nef/GST-GA beads (lanes 2 to 8). Lanes 1 and 2, no soluble protein; lanes 3 to 5, 10, 20 and 40 μg nef-6H; lanes 6 to 8, 10, 20 and 40 μg BSA.

Table 1. Classification of nef-binding proteins from Jurkat T cells

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<th>Localization</th>
<th>Cytosol</th>
<th>Membrane and cytosol</th>
<th>Membrane</th>
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<tr>
<td>Myristylation-dependent</td>
<td>p280, p35, p32, p28, p26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Partially dependent on myristylation</td>
<td>p55/57, p75</td>
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<tr>
<td>Myristylation-independent</td>
<td>p97</td>
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binding to an artefactual combination of nef and GST sequences.

Discussion

In this study, a baculovirus-expressed nef/GST fusion protein was used as an affinity reagent to identify cellular proteins that specifically interact with nef. A number of such proteins, both soluble and membrane-associated, were identified in cytoplasmic extracts of the Jurkat human T cell line. These proteins could be divided into three classes according to their subcellular location and dependence on N-terminal myristylation of nef for binding. This classification is summarized in Table 1.

Prokaryotic GST fusion proteins (expressed in the pGEX system; Smith & Johnson, 1988) have previously been used to identify proteins binding to the retinoblastoma gene product (Kaelin et al., 1991) and the SH2 domain of the actin-binding protein, tensin (Davis et al., 1991). However, as discussed above, GSTnef expressed from pGEX proved to be an unsuitable reagent for the identification of nef-binding proteins. One reason for this is that N-terminal myristylation of nef is required for the interaction with three of the proteins identified in this study. These proteins (p280, p32 and p28), which failed to bind to non-myristylated nef, are all associated with the cytoplasmic membrane, raising the possibility that one or more of them represents a membrane target for nef. Recent data for two other myristylated proteins, the myristylated alanine-rich C kinase substrate MARCKS (Li & Aderem, 1992) and src (Resh & Ling 1990), show that association of myristylated proteins with the membrane is not merely the result of insertion of the fatty acid moiety into the lipid bilayer. Instead, a combination of this fatty acid moiety and amino acid sequences at the N terminus interact with specific receptor molecules on the inner surface of the membrane. In the case of src (Resh & Ling, 1990), a myristylated peptide representing the N-terminal 11 amino acids, but not the corresponding non-myristylated peptide, could be cross-linked to a 32K plasma membrane protein. It is unlikely that this 32K protein is identical to p32 described here because the N-terminal amino acid sequences of nef and src are distinct, and myristyl-peptides other than src did not cross-link to the 32K protein (Resh & Ling, 1990).

Both nef/GST and nef(m−)GST bound to p97 with equal affinity (Fig. 6). p97 was detected in both cytosolic and membrane-bound fractions (Fig. 5). p97 may be only loosely associated with the membrane and readily dislodged by the extraction procedure, or it may be present in both soluble and membrane-bound forms. Interestingly, p97 is the only protein reproducibly bound by pGEX-expressed GSTnef (Harris et al., 1992b; data not shown). These observations suggest that the interaction between nef and p97 is independent of the membrane-bound class of nef-binding proteins and does not involve the N terminus of nef.

A third group of proteins including p55/p57 and possibly p75 bind to non-myristylated nef, but the presence of a myristyl residue increases the affinity of binding. These proteins appear to be exclusively cytosolic although they could be very loosely associated with the membrane and completely dislodged during extraction. The fact that these proteins bind to non-myristylated nef...
in the absence of the membrane-associated proteins again suggests that, with p97, their interaction with nef is independent of this membrane-associated class. It is not clear whether or not they are associated with p97. The interactions of nef with both cytosolic and membrane-bound proteins supports the hypothesis that the mode of nef action is in some way to perturb specific signal transduction pathway(s) in infected cells. By binding independently to both soluble and membrane-bound components of a particular signal transduction pathway nef could effectively prevent the protein-protein interactions required for passage of the signal. Alternatively, nef might alter the signal in some way, perhaps by diverting it down an alternative pathway so as to elicit an inappropriate response from the cell. Both of these actions could contribute to the cytopathogenesis of viral infection. Further speculation must await the identification of these nef-binding proteins, a task that is in progress.

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References


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