BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **238,** 234–239 (1997)
ARTICLE NO. RC977217

A Novel acyl-CoA Thioesterase Enhances Its Enzymatic Activity by Direct Binding with HIV Nef

Hiroyuki Watanabe,*,† Tooru Shiratori,* Hiroki Shoji,*,‡ Shoichiro Miyatake,* Yasushi Okazaki,* Kazuyoshi Ikuta,§ Toshio Sato,† and Takashi Saito*,[1]

**Division of Molecular Genetics, Center for Biomedical Science and †Department of Psychiatry, Chiba University School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260, Japan; ‡Research Center Kyoto, Bayer Yakuhin Ltd., 6-5-1-3 Kunimidai, Kizu-cho, Soraku-gun, Kyoto 619-02, Japan; and §Division of Serology, Institute of Immunological Science, Hokkaido University, North 15 jo, West 17 cho-me, Kita-ku, Sapporo 060, Japan*

Received July 25, 1997

In addition to playing a crucial role in the pathogenesis of AIDS, HIV nef induces down-regulation of CD4 expression and TCR signaling and also regulates the sorting pathway in host T cells. To elucidate the Nef function in HIV progression, we searched for a cellular component which interacts with Nef. A human cDNA encoding a novel acyl-CoA thioesterase (hACTE-III) was isolated as an HIV nef-binding protein by yeast two-hybrid system. hACTE-III is homologous to E. coli thioesterase II but to none of the mammalian thioesterases and therefore belongs to a new type. hACTE-III exhibits enzymatic specificity for a broad range of fatty acyl-CoAs. The hACTE-III-binding region within Nef is localized in the central region (amino acids 109–152). hACTE-III greatly enhances its enzymatic activity upon direct binding to Nef. Considering that either Nef-overexpression or impaired fatty acid regulation induces alteration of subcellular morphology, the augmented hACTE-III function by Nef-binding might induce dysfunction of T cells. © 1997 Academic Press

The Nef protein is a 27-32 kDa myristoylated cytoplasmic protein that is encoded by an open reading frame located at the 3′ end of HIV-1, HIV-2 and SIV, and is expressed at high level early after HIV infection (1). Nef is critical for the pathogenesis of AIDS. Animals infected with SIV viruses carrying large deletions in nef exhibit low viral loads and do not develop AIDS (2). Moreover, studies with long-term progressors of HIV infection also indicate that nef can be a crucial factor for clinical outcome. The efficient control of HIV infection is associated with deletions in nef or functionally defective-Nef protein in several long-term progressors (3-5).

The biological function of Nef in host T cells has also been described (6-15). Nef induces down-regulation of CD4 expression by enhancing endocytosis and their accumulation in endosomes and subsequent degradation (6-8). Although the mechanism for Nef-mediated CD4 degradation is yet unknown, it is suggested that Nef interacts with CD4 (9) and also plays a role in connecting CD4 and sorting machinery in the Golgi apparatus and plasma membrane (10). Nef also down-regulates T cell activation signals, although this appears to depend on the system (11-14). Furthermore, it has recently been described that the expression of Nef induces accumulation of endosomes and lysosomes in T cells (15).

The effect of Nef in HIV-infected T cells is probably mediated by specific interaction with various cellular target proteins. Identification of such cellular component will lead to a clarification of Nef function in HIV pathogenesis. We have tried to identify Nef-binding proteins by yeast two-hybrid system. In the present study, we identified a human novel acyl-CoA thioesterase (hACTE) as a Nef-binding protein. Since this enzyme has no homology to known eukaryotic thioesterases I and II, it was termed thioesterase III. We found that hACTE-III greatly enhances its enzymatic activity by association with Nef. The implication of the specific interaction with hACTE-III for the Nef function in HIV-infected T cells is discussed.

MATERIALS AND METHODS

Yeast two-hybrid screening. A PCR product of HIV nef from pNL432 (provided by Dr. Y. Takebe, Tokyo) by using a 5′ primer (CCGGAATTCATGGGTGGCAA) and a 3′ primer (CGCGGATCC TCAGCAGTTCT) containing a stop codon was subcloned into pGBT 9 (Clontech) to yield GAL 4 DNA-binding domain (GAL4bd) fusion plasmid of nef (pGBT-nef). The pGADGH vector encoding GAL 4 activation domain (GAL4ad) fused to a HeLa cell cDNA library (Clontech) was co-transformed with pGBT-nef into yeast strain HF7c as previously described (16). Positive clones were selected using His (−)

[1] To whom correspondence should be addressed.

0006-291X/97 $25.00

screening and β-gal assay. To eliminate false positives, GAL 4 ad-cDNA plasmid was re-transformed with pGBT-nef or pGBT9. The insert cDNA was sequenced by an automatic sequencer (ABI 373A) and subjected to homology search by BLAST search.

Isolation of a full-length hACTE-III. A full length of hACTE-III cDNA was isolated by screening a human Jurkat cDNA library (Stratagene) with an hACTE-III probe.

Preparation of recombinant Nef and hACTE-III proteins. A full length of HIV nef cDNA and several deletion mutants were prepared in pGEX vectors (Pharmacia) and designated as GST-Nef, GST-Nef 1-107, GST-Nef 1-185, and GST- Nef 103-206 (numbers correspond to amino acid residues). For GST-hACTE-III, a full-length hACTE-III cDNA was cloned into pGEX4T-3. E. coli strain AD202 was used for transformation to obtain the maximum amount of GST-fusion proteins (17). AD202 containing GST-Nef or hACTE III was incubated overnight at 25°C in 0.1 % glucose-containing LB medium with gentle shaking. After induction with 50 μM isopropyl -β-D-thiogalactopyranoside (IPTG), GST-fusion proteins were isolated by using glutathione-sepharose beads as described previously (17).

Northern blot analysis. The hACTE-III probe used for hybridization was a 280 bp cDNA isolated as *Eco*RI-*Sca*I fragment from hACTE-III cDNA. Multiple Tissue Northern Blots (Clontech) were used for various human tissue mRNA and hybridized with the hACTE-III probe labeled with ^{32}P by Megaprime kit (Amersham). The blot was hybridized at 68 °C for 1 hr, washed in a stringent solution (0.1×SSC,0.1%SDS) at 50 °C, and analyzed with an image analyzer (BAS 2000, Fuji Film Inc.).

In vitro binding assay. *In vitro* translation was performed using a TNT coupled reticulocyte lysate system (Promega). hACTE-III cDNA from two-hybrid screening was subcloned into pCITE 4b vector (Novagen) and incubated at 30°C for 1 hr in the presence of ^{35}S-methionine. Luciferase in the kit was also translated as a control. *In vitro* translation products were isolated from glutathione-sepharose beads and incubated with 10μg of GST-fusion proteins for 2 hr at 4°C in 0.5 ml of binding buffer (1% brij, 50mM Tris-HCl (pH 7.5), 150mM NaCl, 5mM EDTA) as previously described (17). After washing 4 times, the bound proteins were eluted by SDS-sample buffer and analyzed by 12% SDS-PAGE.

Measurement of thioesterase activity. The enzymatic activity of hACTE-III was measured in two different systems: (A) a radio-enzyme assay using radioactive acyl-CoA as a substrate and (B) a spectro-photometric assay by detecting the free CoA-SH liberated by the enzyme activity with 5,5′-dithiobis(2-nitrobenzoate) (DTNB) which reacts with free thiol groups.

(A) For radio-enzyme assay, graded concentrations of GST-hACTE III were incubated with 3.0 kBq of [^{14}C]-palmitoyl-CoA (NEN) at 37°C for 1 hr in 25 μl of buffer composed of 50 mM Tris-HCl (pH 7.0) and 1mM CaCl2. The free [^{14}C]-palmitic acid released was extracted by adding 500μl of n-hexane. After shaking and centrifuging, 200μl of the upper oily phase was mixed and then counted in a liquid scintillation counter.

(B) For spectrophotometric assay, especially to determine the substrate specificity, GST-hACTE-III protein (final concentration of 2.4 μM) was incubated with 1mM of various lengths of acyl-CoAs (C2,C4,C8,C10,C14,C16 or C18) at 37°C in 0.1 ml of reaction buffer (50 mM Tris-Hcl (pH 7.0), 0.2 mM $CaCl_2$, 1mM DTNB) as previously described (18). The color developed was read at 415nm with a microplate reader. A standard curve was prepared with free CoA-SH to calculate the amount of liberated CoA-SH in the reaction mixture.

RESULTS

Cloning of a Novel Human acyl-CoA Thioesterase as Nef-Binding Protein

cDNA encoding Nef-binding protein was screened in yeast two-hybrid system by using GAL4bd fused to nef as bait and GAL4ad attached with cDNA library prepared from Hela cells. Several overlapping clones were obtained and the specific binding of these cDNAs with Nef was confirmed in both yeast growth and β-gal assay (data not shown). The full length cDNA contained 1144 bp insert with 30 bp 5′- and 154 bp 3′- flanking sequences. The cDNA contains an open reading frame encoding a 319 amino acid (aa) long protein as shown in Fig. 1. The predicted molecular size was 36 kD. The sequence analysis showed 42% homology to E. coli acyl-CoA thioesterase II (18). However, since we observed no significant homology to mammalian thioesterase I or II (19), cDNA encodes a novel human acyl-CoA thioesterase (hACTE-III) and may represent a new type of eukaryotic thioesterase, which we termed thioesterase III. Northern blot analysis revealed that two different sizes of transcripts, 1.3 and 0.75 kb, were hybridized with the hACTE-III probe, both mRNAs were expressed ubiquitously in all tissues (Fig. 2). Whereas the longer mRNA corresponds to the hACTE-III cDNA we cloned, cDNA corresponding to the shorter mRNA was not obtained from the two-hybrid screening. When RT-PCR was performed with primers corresponding to 5′- and 3′-ends of the coding sequence of hACTE-III,

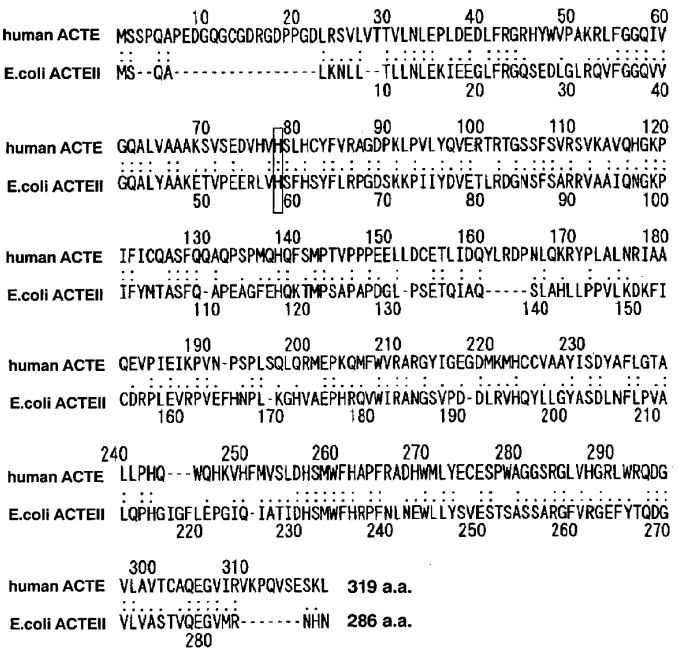

FIG. 1. Amino acid sequence of human Acyl-CoA thioesterase III (human ACTE) and comparison with E. coli Acyl-CoA thioesterase II (E. coli ACTE II). Double dots ":" and single dot "." indicate identical and conserved aa residues, respectively. The human sequence number was deduced from the nucleotide sequence of hACTE-III cDNA. The putative first methionine was decided to be the first in-frame ATG. The boxed His residues (aa 78 for human and aa 58 for E. coli) correspond to the putative active site of the enzyme. The nucleotide sequence of hACTE-III will be submitted to the GenBank Nucleotide Sequence database under the Accession number AF014404.

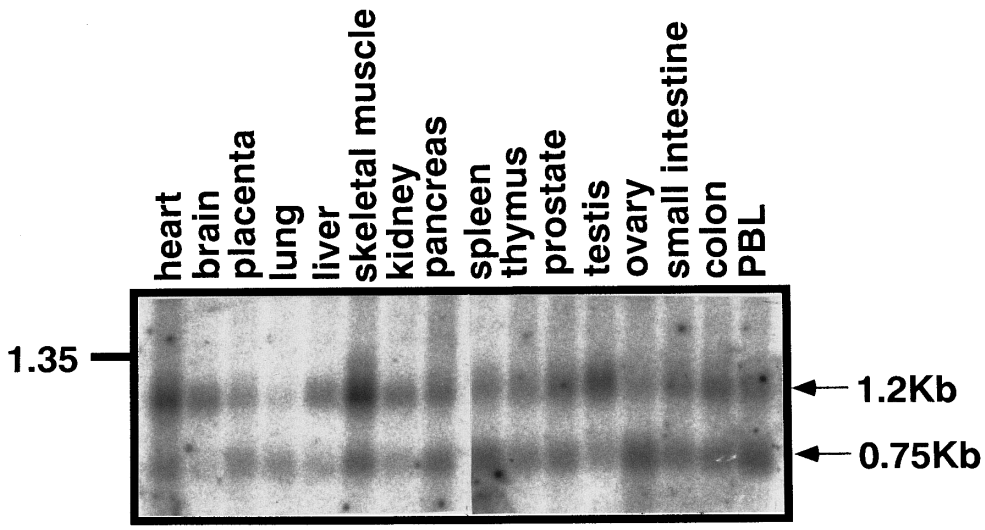

FIG. 2. hACTE III mRNA expression in various human tissues. Multiple Northern blots were hybridized with an hACTE-III probe and analyzed by image analyzer (BAS 2000). Molecular size marker is indicated in kb at the left margin. The size of each of the two hybridized bands is calculated and indicated in the right margin.

1.3 kb but not 0.75 kb product was observed (data not shown), suggesting that the short mRNA may represent a family molecule rather than an alternative-splicing product of hACTE-III.

Central Region of Nef as the Binding Site for hACTE-III

To demonstrate the specific binding between Nef and hACTE *in vitro* and to determine the binding region within Nef, ^{35}S-labeled *in vitro* translated product of hACTE-III or luciferase as a control was mixed with GST-fusion product of Nef or GST alone. As shown in Fig. 3B, lanes 2.3 and 9, specific binding between hACTE-III and Nef was confirmed. The direct binding demonstrated that these proteins are capable of physical interaction without an intermediate protein and that myristoylation of Nef is not required for the interaction with hACTE-III. Then, to determine the specific binding region of Nef, several deletion constructs of nef were prepared (Fig. 3A) and examine their binding capability to hACTE-III. Both Nef (1-152) and Nef (103-206) bound to hACTE-III but Nef (1-109) could not. This result demonstrates that the hACTE-III- binding region of Nef was the central region (aa 109-152).

Enzymatic Activity and Substrate Specificity of Recombinant hACTE-III

Whether hACTE cDNA actually encodes an acyl-CoA thioesterase enzyme was confirmed in two assays: one using radiolabeled palmitoyl-CoA as a substrate (Fig. 4A), and the other by spectrophotomeric assay (Fig. 4B). As shown in Fig. 4A, [^{14}C]-palmitoyl-CoA was catalyzed with hACTE-III in a dose-dependent fashion. To analyze the specificity of the enzyme, a panel of various lengths of fatty acyl-CoAs was used as substrates of hACTE-III (Fig. 4B). The result showed that hACTE-III exhibits broad reactivity to various lengths of substrates from C2 to C18, with a preference for medium-chain acyl-CoAs (C4-C10). This substrate specificity of hACTE-III resembles that of E. Coli ACTE-II (18).

Modulation of Enzymatic Activity of hACTE-III by Direct Association with Nef

We then analyzed the effect of Nef-binding on the enzymatic activity of hACTE-III. Graded doses of GST-Nef or GST alone were added to the enzyme assay for hACTE-III. Unexpectedly, the addition of Nef induced great enhancement of enzymatic activity of hACTE-III in a dose-dependent fashion (Fig. 5). Catalytic activity increased by approximately three-fold by the addition of Nef within the protein concentrations examined.

DISCUSSION

The isolated hACTE-III has no sequence homology to known mammalian thioesterase I and II (19). Whereas ACTE-I has enzymatic specificity for longer fatty acyl-CoA and ACTE-II for medium chain and both are homologous to E. coli ACTE-I, only hACTE-III has homology to E. coli ACTE-II and exhibits broad specificity (18). Thus, hACTE-III belongs to a new subfamily of eukaryotic thioesterases and can be designated as human acyl-CoA thioesterase III. Indeed, we detected a smaller transcript which was hybridized with an hACTE probe (Fig. 2). The preliminary result that this short product was not amplified by PCR using the prim-

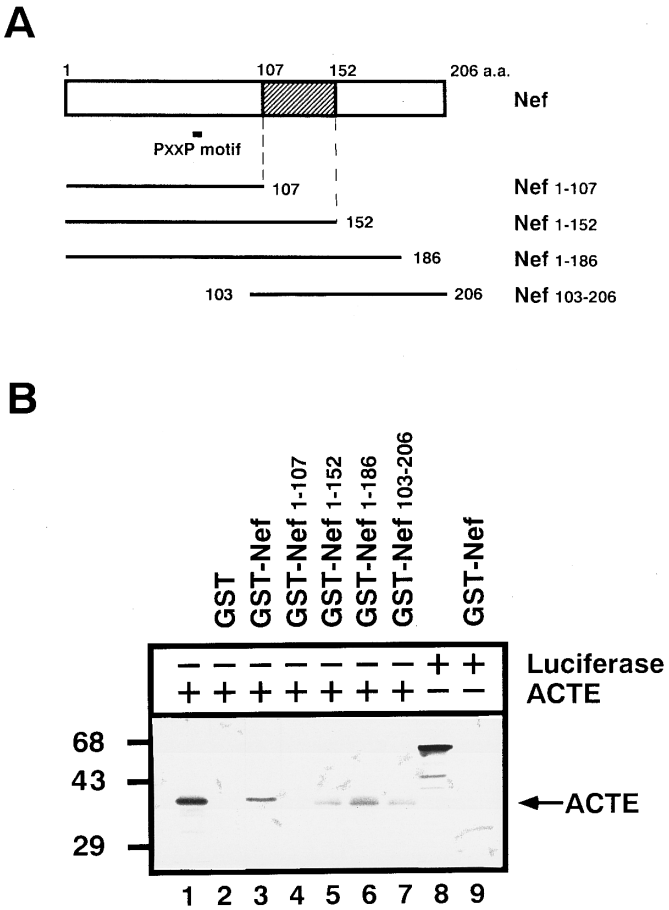

FIG. 3. *In vitro* specific binding to Nef and determination of the hACTE-III-binding region. (A) Preparation of several GST-Nef deletion mutants. The numbers correspond to aa residues. The short heavy line indicates the PXXP motif of Nef which is the target sequence of SH3 of Lck and Hck. The hACTE-III-binding region of Nef is indicated by the hatched block (aa 107-152) from the results of (B). (B) Identification of hACTE-III-binding region of Nef. *In vitro* translated hACTE-III protein and luciferase were incubated with 10 μg of each GST-protein and analyzed by 12% SDS-PAGE. One-hundredth of the total amount of each *in vitro* translation product was applied to lanes 1 and 8 as comparison. Molecular size markers are indicated in the left margin.

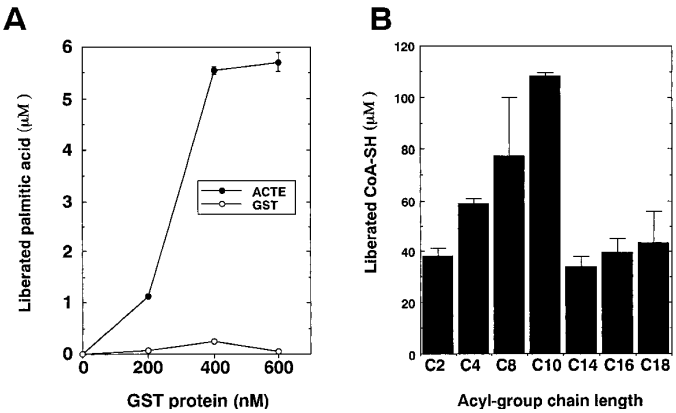

FIG. 4. Thioesterase activity and substrate specificity of hACTE III. (A) Enzymatic activity of GST-hACTE-III (closed circles) and GST alone as a negative control (open circles) in radio-enzyme assay. [^{14}C]-palmitoyl-CoA was used as substrate. Note that various concentrations of hACTE-III (200–600 nM) were used. The results are shown as mean ±SD of triplicates. (B) Substrate specificity of hACTE III by spectrophotometric assay. 2.4 μM of hACTE-III protein was incubated with 7 different lengths of acyl-CoAs (1 mM). The results are shown as mean±SD of triplicates. Note that the difference in concentrations of hACTE-III used in (A) and (B) was due to the different sensitivities of the two assays.

ers corresponding to hACTE-III (data not shown) is consistent with a prediction that this might be a related molecule in this new family. Since hACTE-III is a novel thioesterase, it might possess the specificity as acyl-protein thioesterase which catalyzes the palmitoyl chain of a variety of important proteins such as Fyn/Lyn, Ras and Gα of trimeric G proteins, which associate on plasma membrane with myristoyl and palmitoyl anchors present in N-terminal GCXXSer/Cys conserved motif (20). This possibility will have to be confirmed.

Several Nef-binding proteins were demonstrated previously (11, 13, 23-26). These includes src-family tyrosine kinases, Hck, (13,21) and Lck (22), serin/threonine kinase (11,23,24), PKC theta (25), CD4 (9) and β-COP (26). The src-family tyrosine kinases bound to the proline-rich region of Nef which localizes in the N-terminal half. The region of Nef responsible for mediating *in vivo* function such as CD4 down-regulation and impaired CD3 signaling, was implicated from a study using Nef mutants. Both N- and C-terminal regions are important for CD4 down-regulation, while the blocking

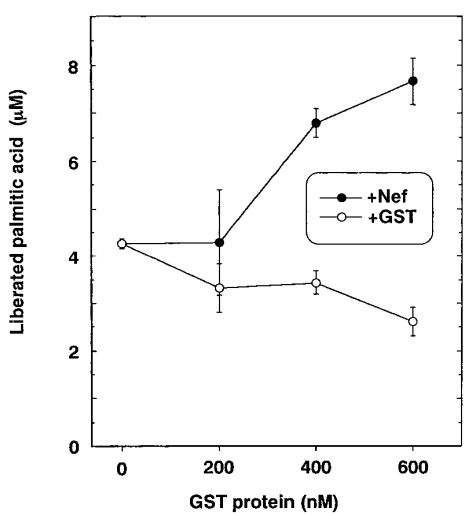

FIG. 5. The effect of Nef binding on the enzymatic activity of hACTE III. Enzymatic activity of hACTE-III (300 nM) was examined by radio-enzyme assay using [^{14}C]-palmitoyl-CoA in which graded doses (0–600 nM) of GST-Nef (closed circles) or GST alone (open circles) were added. GST-Nef alone did not show any enzymatic activity (data not shown). The results are shown as mean±SD of triplicates.

effect of CD3 signaling is mediated through the central region of Nef including the SH3-binding proline-rich sequence (27). The binding region of Nef to hACTE-III is different from these defined regions and does not contain any known motif. Thus hACTE-III-Nef binding is a novel interaction, providing the possibility of a new insight into the role of Nef in cellular function.

Nef appears to have other functions in membrane trafficking (10) and subcellular morphology (15). Nef down-regulates CD4 and MHC class I by physically connecting these receptors with sorting pathways in the Golgi apparatus and plasma membrane (10). It is possible to assume that hACTE-III is involved in this process, based on the fact that hydrolysis of long chain fatty acyl-CoA is crucial for coated vesicle budding (28). The transport of the vesicles is stimulated by long-chain fatty acyl CoA, and the absence of acyl-CoA results in the failure of pinching off coated vesicles from the Golgi network. Furthermore, coated vesicle formation is blocked by a non-hydrolyzable acyl-CoA, suggesting that hydrolysis of long-chain fatty acyl-CoA is required for efficient intracellular sorting. Since hACTE-III hydrolyzes a broad range of acyl-CoAs and Nef enhances the enzymatic activity by specific binding, it is likely to be involved in this process.

Furthermore, the observation that the expression of Nef induced the accumulation of endosomes and lysosomes in T cells suggested additional functions of Nef in subcellular morphology (15). The important role of fatty acid synthesis for the correct organella structure and function has also been shown in yeast; that is, impaired regulation of fatty acid synthesis induced various morphological changes (29). Connecting these two events, it is possible that augmented hACTE-III activity by direct binding with Nef induces these changes of transport and morphology of the subcellular compartments in T cells. This hypothesis can be examined now by testing whether overexpression of hACTE-III in T cells induces alteration of subcellular morphology or function.

A very similar work to our present study was recently reported by Liu et al. (30) during preparation of this manuscript. They also cloned hACTE as a Nef-binding protein and suggested that the interaction between Nef and hACTE is important for CD4 down-regulation by demonstrating that a Nef mutant lacking hACTE-binding capability correlated to the failure of CD4 down-regulation. We defined hACTE as type III of eukaryotic thioesterase together with a possible candidate of another member of the family, and determined the hACTE-III-binding region within Nef which is not correlated directly with the region responsible for CD4 down-regulation. The incapability of CD4 down-regulation might be explained by conformational change occurring in concert with the mutations responsible to the failure of hACTE-III-binding in the case of such Nef mutant. Therefore, the functional significance of the Nef-hACTE-III might lie in the regulation of membrane sorting and subcellular morphology rather than CD4 down-regulation.

ACKNOWLEDGMENTS

We thank Dr. Y. Takebe for nef cDNA and helpful advice, Dr. N. Morisaki for discussion, Dr. M. Hayashi and Ms. B. Li for technical help, Ms. M. Sakuma for technical assistance, and Ms. H. Yamaguchi for secretarial assistance. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture and the Ministry of Welfare, Japan.

REFERENCES

1. Trono, D. (1995) *Cell* **82,** 189–192.
2. Kestler, H. W., Ringler, D. J., Mori, K., Pancali, D. L., Sehgal, P. K., Daniel, M. D., and Desrosiers, R. C. (1991) *Cell* **65,** 651–662.
3. Deacon, N. J., Tsykin, A., Solomon, A., Smith, K., Ludford-Menting, M., Hooker, D. J., McPhee, D. A., Greenway, A. L., Ellet, A., Chatfield, C., Lawson, V. A., Crowe, S., Maerz, A., Sonza, S., Learmont, J., Sullivan, J. S., Cunningham, A., Dwyer, D., Dowton, D., and Mills, J. (1995) *Science* **270,** 988–991.
4. Kirchhoff, F., Greenough, T. C., Brettler, D. B., Sullivan, J. L., and Desrosiers, R. C. (1995) *New Engl. J. Med.* **332,** 228–232.
5. Mariani, R., Kirchhoff, F., Greenough, T. C., Sullivan, J. L., Desrosiers, R. C., and Skowronski, J. (1996) *J. Virol.* **70,** 7752–7764.
6. Garcia, J. V., Alfano, J., and Miller, A. D. (1993) *J. Virol.* **67,** 1511–1516.
7. Aiken, C., Konner, J., Landan, N. R., Lenburg, M. E., and Trono, D. (1994) *Cell* **76,** 853–854.
8. Rhee, S. S., and Marsh, J. W. (1994) *J. Virol.* **68,** 5156–5163.
9. Rossi, F., Gallina, A., and Milanesi, G. (1996) *Virology* **217,** 397–403.
10. Mangasarian, A., Foti, M., Aiken, C., Chin, D., Carpentier, J.-L., and Trono, D. (1997) *Immunity* **6,** 67–77.
11. Bauer, A. S., Sass, G., Laffert, B., Willbold, D., Cheng-Mayer, C., and Peterlin, M. (1997) *Immunity* **1,** 373–384.
12. Luria, S., Chambers, I., and Berg, P. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 5326–5330.
13. Collette, Y., Chang, H. L., Cerdan, C., Chambot, H., Algarte, M., Mawas, C., Imbert, J., Burny, A., and Olive, D. (1995) *J. Immunol.* **156,** 360–370.
14. Skowronski, J., Parks, D., and Mariani, R. (1993) *EMBO J.* **12,** 703–713.
15. Sanfridson, A., Hester, S., and Doyle, C. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 873–878.
16. Shiratori, T., Miyatake, S., Ohno, H., Nakaseko, C., Isono, K., Bonifacino, J. S., and Saito, T. (1997) *Immunity* **6,** 583–589.
17. Nakano, H., Yamazaki, T., Ikeda, M., Masai, H., Miyatake, S., and Saito, T. (1994) *Nucleic Acids Res.* **22,** 543–544.
18. Naggert, J., Narasimhan, M. L., DeVeaux, L., Cho, H., Randhawa, Z. I., Cronan, J. E., Jr., Green, B. N., and Smith, S. (1991) *J. Biol. Chem.* **266,** 11044–11050.
19. Smith, S. (1994) *FASEB J.* **8,** 1248–1259.
20. Camp, L. A., Verkruyse, L. A., Afendis, S. J., Slaughter, C. A., and Hofmann, S. L. (1994) *J. Biol. Chem.* **269,** 23212–23219.
21. Saksela, K., Cheng, G., and Baltimore, D. (1995) *EMBO J.* **14,** 484–491.
22. Greenway, A., Azad, A., Mills, J., and McPhee, D. (1995) *J. Viol.* **70,** 6701–6708.

23. Sawai, E. T., Baur, A., Struble, H., Peterlin, B. M., Levy, J. A., and Cheng-Mayer, C. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 1539–1543.

24. Lu, X., Wu, X., Plemenitas, A., Yu, H., Sawai, E. T., Abo, A., and Peterlin, B. M. (1996) *Curr. Biol.* **6,** 1677–1684.

25. Smith, B. L., Krushelnycky, B. W., Mochly-Rosen, D., and Berg, P. (1996) *J. Biol. Chem.* **271,** 16753–16757.

26. Benichou, S., Bomsel, M., Bodeus, M., Durand, H., Doute, M., Letourneur, F., Camonis, J., and Benarous, R. (1994) *J. Biol. Chem.* **269,** 30073–30076.

27. Iafrate, A. J., Bronson, S., and Skowronski, J. (1997) *EMBO J.* **16,** 673–684.

28. Rothman, J. E. (1994) *Nature* **372,** 55–63.

29. Schneiter, R., and Kohlwein, S. (1997) *Cell* **88,** 431–434.

30. Liu, L. X., Margottin, F., Gall, S. L., Schwartz, O., Selig, L., Benarous, R., and Benichou, S. (1997) *J. Biol. Chem.* **272,** 13779–13785.