

Down-regulation of *N*-myristoyl transferase expression in human T-cell line CEM by human immunodeficiency virus type-1 infection

Nobutoki Takamune, Tadahiro Tanaka, Hiroki Takeuchi, Shogo Misumi, Shozo Shoji*

Department of Biochemistry, Faculty of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-Honmachi, Kumamoto 862-0973, Japan

Received 28 May 2001; revised 3 September 2001; accepted 4 September 2001

First published online 17 September 2001

Edited by Hans-Dieter Klenk

Abstract The present study focuses on the expression level of *N*-myristoyl transferase (NMT) in the course of human immunodeficiency virus type-1 (HIV-1) infection. HIV-1 structural proteins were gradually expressed during the process of infection of the human T-cell line CEM, whereas the expression levels of NMT subsequently decreased under the same conditions. In addition, the chronically HIV-1-infected T-cell line CEM/LAV-1 exhibited low expression levels of NMT. We hypothesize that the decrease in the expression level of NMT due to HIV-1 infection may be related to the virus' strategy that leads to its persistent replication. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: *N*-Myristoyl transferase; Myristoylation; Human immunodeficiency virus type-1

1. Introduction

Human immunodeficiency virus type-1 (HIV-1) utilizes various biological and biochemical machineries of the host cell to replicate itself [1,2]. Protein *N*-myristoylation is one of the crucial machineries by which HIV-1 gag [3–5] and Nef [6] exhibit appropriate functions for survival [7–10]. Protein *N*-myristoylation, which was discovered in the catalytic subunit of adenosine 3',5'-phosphate-dependent protein kinase type-II [11,12], involves cotranslational and post-translational acylation of the NH₂-terminal glycine residues of a number of cellular and viral proteins with myristic acid [13], which is catalyzed by *N*-myristoyltransferase (NMT) (EC 2.3.1.9.7) [14]. NMT is essential for the survival of eukaryotes, since disruption of NMT expression caused recessive lethality and developmental defects in yeast [15] and *Drosophila* [16], respectively. Thus, human NMT (hNMT) is considered to be one of the key proteins for both HIV-1 and its host cell. Although the biological state of the host cell is altered by HIV-1 infection, leading to apoptosis [17], and inadequate immunoresponses [18], in which the expression levels of

many-sided host genes change [19–21], there is no such report related to hNMT in HIV-1-infected host cells.

In this study, we focus on the expression of hNMT in the process of HIV-1 infection at the protein level. We prepared anti-NMT monoclonal antibody (mAb) and detected hNMT in acute HIV-1-infected cells and chronically infected cells by Western blot analysis. As a result, the isozymes of hNMT were detected, expression levels of which were subsequently decreased by HIV-1 infection, in contrast to our expectations.

2. Materials and methods

2.1. Materials

The reagents used in the study were obtained from the following sources: hNMT expression plasmid (pBB218) was a kind gift from Dr. J.I. Gordon (Washington University School of Medicine); RPMI 1640 medium was from Nissui Seiyaku Co. (Tokyo, Japan); and HIV-1-positive human plasma was a kind gift from the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan).

2.2. Preparation of anti-NMT mAb

The antigen peptide, the sequence of which was based on residues 388–394 (GIGDGNL) of the cloned hNMT sequence [22], was synthesized and then coupled with multiple antigen peptide (MAP[®], Perkin-Elmer, Applied Biosystems Division, Foster City, CA, USA) using Fmoc chemistry. Female Balb/c mice were immunized intraperitoneally with GIGDGNL-MAP[®] in Freund's adjuvant at 1 week intervals and administered an intravenous boost of GIGDGNL-MAP[®] 3 days prior to splenectomy. Hybridomas were generated by a standard method in which splenocytes were fused with P3U1 cells and selected in hypoxanthine-, aminopterin-, and thymidine-supplemented media. In the screening process, supernatants were tested for reactivity to GIGDGNL-Multi-Pin-Block[®]. Hybridomas that produced the most potent supernatants were then cloned by the limiting dilution method.

2.3. Antibody specificity

The competitors (GIGDG, GIGD, GNLQ, and GDGNLQ) used were synthesized using Fmoc chemistry. Antibody specificity was determined by enzyme-linked immunosorbent assay (ELISA) as previously described [23].

2.4. Expression of recombinant hNMT

Escherichia coli JM101 with hNMT expression plasmid (pBB218) [22] was grown at 37°C in LB medium containing 100 µg/ml ampicillin to an *A*₆₀₀ value of ~0.8 and hNMT was induced with nalidixic acid (50 µg/ml) at 37°C for 3 h. The bacterial cells were harvested by centrifugation at 5000×*g* for 10 min. The bacterial cells were suspended in cold lysis buffer (30 mM MES, 1 mM EDTA-2Na, 50 mM KCl, 50 mM 2-mercaptoethanol, and 1 mM *p*-APMSF) and homogenized in a Dounce homogenizer. The bacterial suspension was subjected to 20 cycles of vortexing with glass beads for 15 s and then allowed to stand on ice for 45 s. The lysate was centrifuged at 14000×*g* for 30 min at 4°C, and the supernatant was subjected to Western blot analysis.

*Corresponding author. Fax: (81)-96-362 7800.

E-mail address: shoji@gpo.kumamoto-u.ac.jp (S. Shoji).

Abbreviations: HIV-1, human immunodeficiency virus type-1; hNMT, human *N*-myristoyl transferase; mAb, monoclonal antibody; MAP, multiple antigen peptide; ELISA, enzyme-linked immunosorbent assay; p.i., post-infection

2.5. Cell culture and HIV-1 preparation

A human T-cell line, CEM, and a chronically HIV-1-infected T-cell line, CEM/LAV-1, were maintained at 37°C in RPMI 1640 medium supplemented with 10% fetal calf serum (100 IU/ml of penicillin and 100 µg/ml of streptomycin) in 5% CO₂ [24].

Preparation of infectious HIV-1 (LAV-1 strain) and titration of infectivity were performed as described [25].

2.6. Acute HIV-1 infection and cell lysis

CEM cells (2×10^5 cells/ml, 10 ml) were infected with HIV-1 at a multiplicity of infection (m.o.i.) of 0.01, incubated for 1 h at 37°C in 5% CO₂, washed once with phosphate-buffered saline (PBS)(–), and resuspended in RPMI 1640 medium. The cells were incubated for 0, 24, 28, 72 or 96 h at 37°C in 5% CO₂, washed twice with PBS(–) and lysed as previously described [7]. Cell viability was determined by the trypan blue dye exclusion method.

2.7. Western blot analysis

The samples were separated by SDS-PAGE [26] with polyacrylamide gel (MULTIGEL 4/20, Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan) and the separated proteins subsequently electroblotted onto a polyvinylidene difluoride membrane (Immobilon, Millipore Corporation, Bedford, MA, USA). Antigens were probed with anti-NMT mAb followed by anti-mouse IgG conjugated with horseradish peroxidase, probed with HIV-1-positive human plasma followed by anti-human IgG conjugated with horseradish peroxidase, or probed with anti-actin IgM followed by anti-mouse IgM conjugated with horseradish peroxidase (Actin (Ab-1) kit, Oncogene Research Products, Boston, MA, USA) and visualized by chemiluminescence (Renaissance®, NEN® Life Science Products, Inc., Boston, MA, USA) or 0.03% 3,3'-diaminobenzidine tetrahydrochloride staining, respectively.

3. Results

The immunotiter of the antibody raised against GIGDGNL-MAPTM, the sequence of which was based on residues 388–394 (GIGDGNL) of the cloned hNMT sequence [22], was determined by ELISA with GIGDGNL-Multi-Pin-BlockTM. Fig. 1A shows the relative percentages of GIGDGNL-Multi-Pin-BlockTM bound to the antibody in the

presence of log dilutions of the competitor peptides (GIGDG, GIGD, GNLQ, GDGNLQ, and GIGDGNL-MAPTM). GIGDGNL-MAPTM-BlockTM displaced strongly the binding of the antibody to GIGDGNL-Multi-Pin, whereas almost no effect was observed with the other competitors. The results suggest that the entire moiety of GIGDGNL is essential for recognition by the antibody.

To investigate whether the antibody could react with hNMT, hNMT-expressed [22] and -unexpressed *E. coli* (JM101) lysates were subjected to Western blot analysis with the antibody. As shown in Fig. 1B, a major band of 57 kDa was specifically detected in the hNMT-expressed *E. coli* but not in the hNMT-unexpressed *E. coli*. The result indicates that the antibody can recognize hNMT in immunoblot analysis.

To investigate the expression of HIV-1 proteins in acute infection, CEM cells were infected with HIV-1, and the viral proteins were detected by Western blot analysis using HIV-1-positive human plasma. HIV-1 Pr55, p41, p24 and p17 were detected weakly at 48 h post-infection (p.i.) (Fig. 2A, lane 3) and strongly at 72 h and 96 h p.i. (Fig. 2A, lanes 4 and 5), the same as CEM/LAV-1 (Fig. 2A, lane 6).

Under the same conditions, the expression of hNMT was examined by Western blot analysis using the prepared anti-NMT mAb. In CEM cells, four immunoreactive bands were detected, which had apparent molecular masses of 46 kDa, 57 kDa, 63 kDa, and 88 kDa (Fig. 2B, lane 1). During the process of infection of HIV-1 in CEM cells, the expression levels of both 46 kDa and 57 kDa proteins were immediately down-regulated within 24 h p.i. (Fig. 2B, lane 2), whereas that of the 63 kDa protein decreased gradually up to 96 h p.i. (Fig. 2B, lanes 1–5). On the other hand, the expression level of the 88 kDa protein was up-regulated from 24 h to 72 h p.i. (Fig. 2B, lanes 2–4), followed by down-regulation at 96 h p.i. (Fig. 2B, lane 5). Western blot analysis with the antibody of the chronically HIV-1-infected T-cell line CEM/LAV-1 revealed the low expression levels of 46 kDa, 57 kDa, 63 kDa, and 88 kDa proteins (Fig. 2B, lane 6). This profile was similar to that of CEM cells at 96 h p.i. (Fig. 2B, lane 5).

To normalize for the same protein contents in the samples, a cytoskeletal protein, actin, was detected under the same conditions by Western blot analysis using anti-actin mAb, the change of expression level of which due to HIV-1 infection has not been reported so far. As shown in Fig. 2C, the amount of actin in samples from HIV-1-uninfected, acutely infected, and chronically infected CEM cells is constant.

4. Discussion

hNMTs, the products of host genes, play an important role in HIV-1 replication, because HIV-1 gag precursor [3–5] and Nef [6] are *N*-myristoylated in HIV-1-infected cells, which is essential for gaining viral infectivity and membrane association [7–10], respectively. Thus, hNMT is indispensable to the replication of HIV-1, which makes us expect that the expression of hNMT may be accelerated or at least maintained upon HIV-1 infection. Therefore, we prepared anti-NMT mAb and detected hNMT in acutely and chronically HIV-1-infected cells.

In uninfected CEM cells, four immunoreactive bands were detected by Western blot analysis using anti-NMT mAb (Fig. 2). Although the 88 kDa protein recognized by the antibody

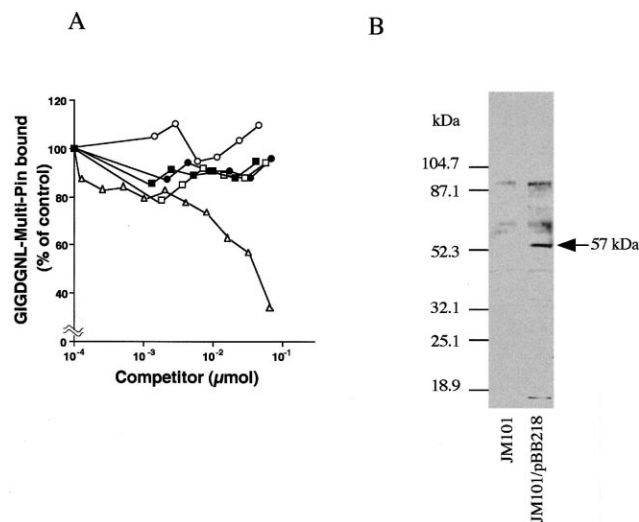


Fig. 1. Antibody specificity. A: Relative percentages of GIGDGNL-Multi-Pin-BlockTM bound to the antibody were determined in the presence or absence of competitors. Competitors used were: GIGDG (○); GIGD (●); GNLQ (□); GDGNLQ (■); GIGDGNL-MAPTM (△). B: hNMT was expressed in *E. coli* JM101 and then lysed. The lysates were subjected to SDS-PAGE (4–20%) followed by Western blot analysis using the antibody. Other experimental details are described in Section 2. Lysates: lane 1, JM101; lane 2, JM101 expressing hNMT. Molecular markers are shown on the left.

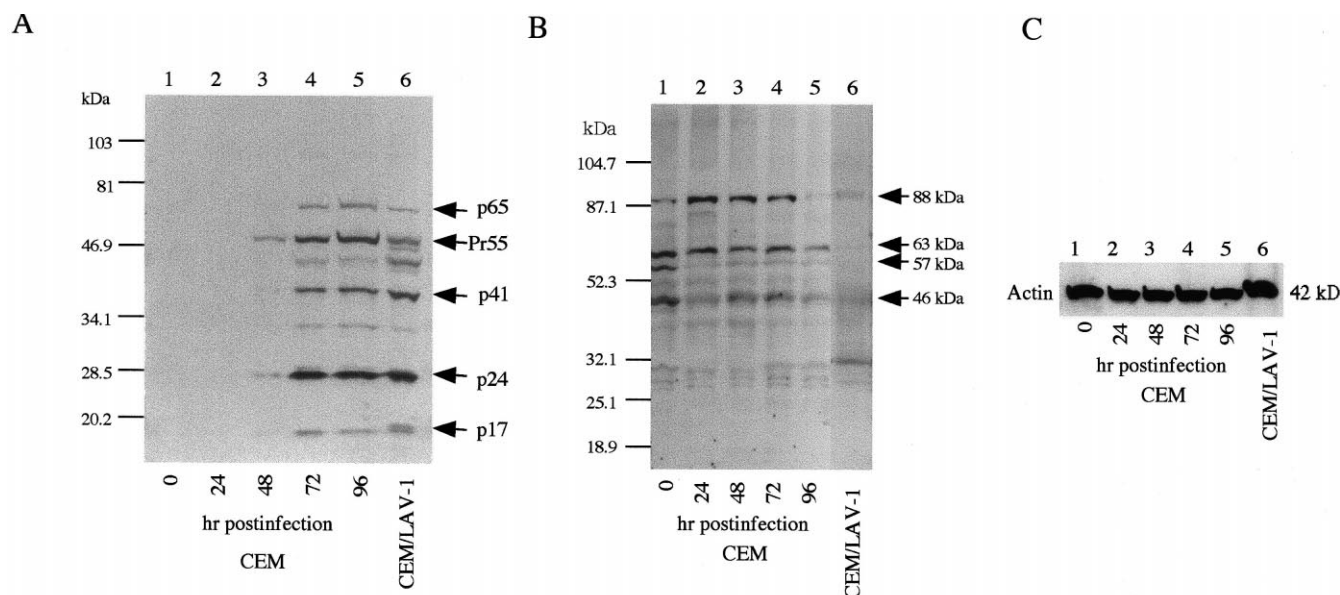


Fig. 2. Expression of HIV-1 structural proteins, hNMT, and actin in acutely and chronically HIV-1-infected CEM. CEM cells infected with HIV-1 at a m.o.i. of 0.01 were incubated for 0, 24, 28, 72 or 96 h at 37°C in 5% CO₂. The cells were lysed and the lysates were subjected to SDS-PAGE (4–20%) followed by Western blot analysis using HIV-1-positive human plasma (A), anti-NMT mAb (B) or anti-actin mAb (C). Other experimental details are described in Section 2. Cell lysates: lane 1, uninfected CEM; lane 2, CEM at 24 h p.i.; lane 3, CEM at 48 h p.i.; lane 4, CEM at 72 h p.i.; lane 5, CEM at 96 h p.i.; lane 6, CEM/LAV-1. Molecular markers are shown on the left.

has not been identified as hNMT so far, the protein that shows a change in expression level upon infection has attracted our attention and is now being investigated in our laboratory to determine its identity. Taking the apparent molecular masses into consideration, the three major protein bands of 46 kDa, 57 kDa, and 63 kDa seem to correspond to NMT_S, NMT_M, and NMT_L, respectively, that were reported by McIlhinney et al. [27]. NMT_L and NMT_M are formed by the initiation of translation from the first and the second methionine residues, respectively. NMT_S is the form resulting from the alternative splicing of mRNA. Alternatively, it may be argued that one of the three major bands is from hNMT2 [28], because it also contains the epitope recognized by the antibody. Although it is still not possible to distinguish hNMT1 from hNMT2, the expression levels of all isozymes of hNMT recognized by the antibody appear to be decreased by HIV-1 infection, in contrast to our previous expectation. The decrease in the hNMT expression level upon HIV-1 infection contrasts the high expression level of the viral proteins in both acutely HIV-1-infected CEM cells and chronically infected CEM/LAV-1 cells, whereas the expression level of actin is constant (Fig. 2). The down-regulation of both 46 kDa and 57 kDa proteins within 24 h p.i. is striking because of the absence of the expression of viral antigens at this time and the small percentage of infected cells. Since it has been confirmed by flow cytometric analysis using anti-gp120 antibody that 93.4% of the total number of cells are attached by virion with gp120 1 h after the inoculation at a m.o.i. of 0.01 (data not shown), the interaction of gp120 with CD4 or CXCR4, which can induce signal transduction [29,30], may be related to the immediate down-regulation of hNMTs.

As the viabilities of CEM cells at 96 h p.i. and CEM/LAV-1 cells are 98–99% (data not shown), the low expression level of hNMT appears to have no effect on cell growth. Moreover, the low expression level of hNMT seems to be sufficient for viral replication, because there are reports that only a rela-

tively small proportion of the total gag molecules need to be myristoylated for efficient virus-like particle budding [31] and that HIV-1 gag is substantially *N*-myristoylated in CEM/LAV-1 cells, from which infective virus is produced [3,4].

What is the biological and virological significance of the lowered expression level of hNMT upon HIV-1 infection? Since NMT catalyzes *N*-myristoylation for inducing particular proteins to exhibit their intrinsic functions, the expression level of NMT may determine the characteristics of a cell.

Ryo et al. reported that 53 cellular genes were differentially expressed upon HIV-1 infection of human T-cell line Molt4 cells [19]. They also indicated that genes with up-regulated expression levels mainly comprised those that accelerated HIV-1 replication, whereas genes with down-regulated expression levels were involved in the defense against apoptosis and the regulation of basic cellular function. We believe that their results are valid. However, since hNMT is clearly important for both HIV-1 replication and basic cellular function, their conclusions seem to be inapplicable to the case of hNMT with HIV-1 infection. Assuming that the virus attempts to take advantage of its efficient replication at all times, a state of persistent infection without cell death is reasonable for viral propagation. Zha et al. recently reported that post-translational and post-proteolytic *N*-myristoylation of BID(p15) occurs when inactive BID(p22) is cleaved off by caspase-8 [32], which serves as a switch that activates BID-induced release of cytochrome *c* and cell death. Furthermore, HIV-1 Tat induces the up-regulation of caspase-8, which results in an increase in sensitivity to apoptosis signals [33]. It follows from the above discussion that if the low expression level of hNMT is insufficient for *N*-myristoylation of BID(p15) in HIV-1-infected cells, induction of apoptosis-mediated caspase-8 will not occur, thereby leading to chronic infection. The decrease in the hNMT expression level may contribute in part to the inadequate immunoresponse in HIV-1-infected individuals, because *N*-myristoylation of src family kinases Lck and Fyn is

required for T-cell receptor-mediated signal transduction that leads to the activation of T-cells [34,35]. Boutin et al. [36] reported that NMT in the cytosolic fraction of CCRF-CEM cells, a human acute lymphoblastic leukemia cell line, most highly recognized a peptide substrate derived from HIV-1 gag from among the many peptide substrates derived from cellular and viral proteins, which are potential substrates for NMT. These data suggest that HIV-1 gag has priority over other potential substrates in the host cell for *N*-myristoylation, even at a low expression of NMT. To confirm this hypothesis, we must investigate whether the reduction of myristoylated proteins of the host cell by HIV-1 infection occurs, which gives a substantial advantage to viral replication.

Further work is under way to clarify the biological significance and the mechanism of the down-regulation of hNMT expression with HIV-1 infection, which may lead to finding a novel target for AIDS therapy.

Acknowledgements: We thank Dr. J.I. Gordon (Washington University School of Medicine) for providing the hNMT expression plasmid (pBB218). This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

- [1] Emerman, E. and Halim, M.H. (1998) *Science* 280, 1880–1884.
- [2] Fauci, A.S. (1996) *Nature* 384, 529–534.
- [3] Shoji, S., Tashiro, A. and Kubota, Y. (1988) *J. Biochem.* 103, 747–749.
- [4] Tashiro, A., Shoji, S. and Kubota, Y. (1989) *Biochem. Biophys. Res. Commun.* 165, 1145–1154.
- [5] Shoji, S., Tashiro, A. and Kubota, Y. (1990) *Ann. N.Y. Acad. Sci.* 616, 97–115.
- [6] Allan, J.S., Coligan, J.E., Lee, T., McLane, M.F., Kanki, P.J., Groopman, J.E. and Essex, M. (1985) *Science* 230, 810–813.
- [7] Furuishi, K., Matsuoka, H., Takama, M., Takahashi, I., Misumi, S. and Shoji, S. (1997) *Biochem. Biophys. Res. Commun.* 237, 504–511.
- [8] Shiraiishi, T., Misumi, S., Takama, M., Takahashi, I. and Shoji, S. (2001) *Biochem. Biophys. Res. Commun.*, in press.
- [9] Kaminchic, J., Bashan, N., Itach, A., Sarver, N., Gorecki, M. and Panet, A. (1991) *J. Virol.* 65, 583–588.
- [10] Yu, G. and Felsted, R.L. (1992) *Virology* 187, 46–55.
- [11] Carr, S.A., Biemann, K., Shoji, S., Parmelee, D.C. and Titani, K. (1982) *Proc. Natl. Acad. Sci. USA* 79, 6128–6131.
- [12] Shoji, S., Ericsson, L.H., Walsh, K.A., Fischer, E.H. and Titani, K. (1983) *Biochemistry* 22, 3702–3709.
- [13] Boutin, J.A. (1997) *Cell. Signal.* 9, 1–35.
- [14] Towler, D.A., Adams, S.P., Eubanks, S.R., Towery, D.S., Jackson-Machelski, E., Glaser, L. and Gordon, J.I. (1987) *Proc. Natl. Acad. Sci. USA* 84, 2708–2712.
- [15] Duronio, R.J., Towler, D.A., Heuckeroth, R.O. and Gordon, J.I. (1989) *Science* 243, 796–800.
- [16] Ntwasa, M., Aapies, S., Schiffmann, D.A. and Gay, N.J. (2001) *Exp. Cell Res.* 262, 134–144.
- [17] Kaplan, D. and Sieg, S. (1998) *J. Virol.* 72, 6279–6282.
- [18] Pantaleo, G. and Fauci, A.S. (1996) *Annu. Rev. Microbiol.* 50, 825–854.
- [19] Ryo, A., Suzuki, Y., Ichiyama, K., Wakatsuki, T., Kondoh, N., Hada, A., Yamamoto, M. and Yamamoto, N. (1999) *FEBS Lett.* 462, 182–186.
- [20] Geiss, G.K., Bumgarner, R.E., An, M.C., Agy, M.B., van't Wout, A.B., Hammersmark, E., Carter, V.S., Upchurch, D., Mullins, J.I. and Katze, M.G. (2000) *Virology* 266, 8–16.
- [21] Scheuring, U.J., Corbeil, J., Mosier, D.E. and Theofilopoulos, A.N. (1998) *AIDS* 12, 563–570.
- [22] Duronio, R.J., Reed, S.I. and Gordon, J.I. (1992) *Proc. Natl. Acad. Sci. USA* 89, 4129–4133.
- [23] Shoji, S., Kida, Y., Takenaka, O., Yoshinaga, T., Funakoshi, T. and Kubota, Y. (1990) *Biochem. Biophys. Res. Commun.* 170, 657–664.
- [24] Harada, S., Koyanagi, Y. and Yamamoto, N. (1985) *Virology* 146, 272–281.
- [25] Takamune, N., Misumi, S. and Shoji, S. (2000) *Biochem. Biophys. Res. Commun.* 272, 351–356.
- [26] Laemmli, U.K. (1970) *Nature* 227, 680–685.
- [27] McIlhinney, R.A.J., Young, K., Egerton, M., Camble, R., White, A. and Soloviev, M. (1998) *Biochem. J.* 333, 491–495.
- [28] Giang, D.K. and Cravatt, B.F. (1998) *J. Biol. Chem.* 272, 6595–6598.
- [29] Popik, W., Hesselgesser, J.E. and Pitha, P.M. (1998) *J. Virol.* 72, 6406–6413.
- [30] Davis, C.B., Dikic, I., Unutmaz, D., Hill, C.M., Arthos, J., Siani, M.A., Thompson, D.A., Schlessinger, J. and Littman, D.R. (1997) *J. Exp. Med.* 186, 1793–1798.
- [31] Morikawa, Y., Hinata, S., Tomoda, H., Goto, T., Nakai, M., Aizawa, C., Tanaka, H. and Omura, S. (1996) *J. Biol. Chem.* 271, 2868–2873.
- [32] Zha, J., Weiler, S., Oh, K.J., Wei, M.C. and Korsmeyer, S.J. (2000) *Science* 290, 1761–1765.
- [33] Bartz, S.R. and Emerman, M. (1999) *J. Virol.* 73, 1956–1963.
- [34] Yasuda, K., Kosugi, A., Hayashi, F., Saitoh, S., Nagafuku, M., Mori, Y., Ogata, M. and Hamaoka, T. (2000) *J. Immunol.* 165, 3226–3231.
- [35] Van't Hof, W. and Resh, M.D. (1999) *J. Cell Biol.* 145, 377–389.
- [36] Boutin, J.A., Ferry, G., Ernould, A.-P., Maes, P., Remond, G. and Vincent, M. (1993) *Eur. J. Biochem.* 214, 853–867.