FIG. 8. ATM phosphorylates Sp1 at Ser-101 but not Ser-56 in vitro. (A) Schematic diagram of GST fusion proteins of truncated Sp1 and identical fragments with one or both of Ser-56 and Ser-101 mutated to alanine. (B) Lysates of 293T cells transfected with plasmid expressing Flag-tagged wild-type ATM (Flag-wt ATM) or kinase-dead ATM (Flag-kd ATM) were immunoprecipitated with anti-Flag antibody. Immunocomplexes of Flag-wt ATM or Flag-kd ATM were resolved on SDS–10% PAGE gel and subjected to immunoblot analysis with anti-ATM antibody. (C) IP-kinase assays. GST-Sp1$_{8-167}$, GST-Sp1$_{8-167}$-S56A, GST-Sp1$_{8-167}$-S101A, and GST-Sp1$_{8-167}$-S56/101A were expressed in *E. coli*, purified, and used as substrates for IP-kinase assays. Immunocomplexes containing Flag-wt ATM (Wt) or Flag-kd ATM (Kd) protein were each incubated with purified GST-Sp1$_{8-167}$, GST-Sp1$_{8-167}$-S56A, GST-Sp1$_{8-167}$-S101A, or GST-Sp1$_{8-167}$-S56/101A as substrates in the presence of [γ-^{32}P]ATP. Samples were resolved by SDS–10% PAGE, followed by autoradiography. The amounts of each GST fusion protein were confirmed by Coomassie brilliant blue (CBB) staining.

DISCUSSION

It was found here that hyperphosphorylation of the transcription factor Sp1 upon HSV-1 infection is mainly due to ATM and/or cellular kinase(s) activated by ATM rather than individual HSV-1 encoded PKs. Hyperphosphorylation of Sp1 upon HSV-1 infection was thus impaired in ATM-deficient or -expression silenced cells. Although at least two sites of Ser-56 and Ser-101 could be phosphorylated upon HSV-1 infection, ATM was here found to directly phosphorylate Sp1 at Ser-101 but not at Ser-56. To our knowledge, this is a first report describing determination of the target site on Sp1 for ATM. Involvement of ATM in Sp1 phosphorylation is also supported by the previous reports that Sp1 and ATM coimmunoprecipitate reciprocally in vivo and that Sp1 directly interacts with the kinase region of ATM in vitro (21).

Hyperphosphorylation of Sp1 is observed also in infection of other herpesviruses such as HCMV and Epstein-Barr virus (EBV). HCMV infection results in increased phosphorylated forms of Sp1, together with an increased level of Sp1 (27, 62),

and the ATM DNA damage checkpoint pathway is activated in response to HCMV infection (18, 38). Also, induction of lytic replication in EBV latently infected cells results in hyperphosphorylation of Sp1 (S. Iwahori, A. Kudoh, and T. Tsurumi, unpublished result) and EBV lytic replication elicits ATM checkpoint signal transduction (33). Thus, ATM-dependent Sp1 hyperphosphorylation might be a common phenomenon during the lytic replication of herpesviruses.

Although phosphorylation of Sp1 at Ser-56 was not detected at all upon infection in ATM-deficient cells, ATM by itself could not phosphorylate Ser-56 on Sp1 in vitro, suggesting that other cellular kinase(s) activated by ATM could be involved in the Ser-56 phosphorylation. The sequence of Ser-56 followed by glutamine is known as putative phosphorylation site for related members of the PI-3-like kinase family such as ATM, ATR, and DNA-PK. Chen et al. have recently suggested that ATM is likely the kinase mediating IR-induced DNA-PKcs phosphorylation required for full activation of DNA-PKcs (7). Sp1 phosphorylation by DNA-PK has already been reported from studies of human immunodeficiency virus type 1 infection (11). However, as shown in Fig. 3C, the level of DNA-PKcs decreases significantly at early stages of HSV-1 infection, the degradation being in a virus-encoded ICP0-dependent manner as reported by others (35, 44). Also, activation of ATR is minimal during HSV-1 infection (37, 53), although the ATR PK activity is dependent upon ATM (29, 42, 58). Furthermore, it has recently been reported that HSV-1 disrupts the ATR-dependent DNA damage response through destruction of the usually tight colocalization of ATR and ATRIP (59). Thus, involvement of ATR and DNA-PK in phosphorylation of Ser-56 on Sp1 upon HSV-1 infection is unlikely. In response to IR, ATM phosphorylates and activates checkpoint kinases, Chk1 and Chk2, that play roles as signal transducers (3). We reported that the activation of Chk1 and Chk2 was induced upon HSV-1 infection (53). However, since the sequence around Ser-56 is distinct from Chk1 and Chk2 target motif (Arg-x-x-Ser) (8, 55), it is also unlikely that activated Chk1 and Chk2 are involved in the phosphorylation of Sp1 at Ser-56.

With respect to the role of Sp1 hyperphosphorylation during HSV-1 infection, Kim and DeLuca have reported purified Sp1 from HSV-1-infected cells at 12 hpi to exhibit reduced transcriptional activity in an in vitro transcription assay, although the DNA-binding activity of Sp1 was unchanged until 8 hpi (31). These researchers suggested that the reduced transcriptional activity of Sp1 may be due to the hyperphosphorylation of Sp1 and contribute to the reduced transcription of immediate-early and early genes with Sp1-binding sites in its promoters at late stages of infection (31). We also observed the reduced Sp1-dependent transcriptional activity upon HSV-1 infection in reporter gene assay (data not shown). As shown in Fig. 9, however, the transcriptional activity from the Sp1 responsive promoter in 293T-ATM shRNA cells upon HSV-1 infection was almost the same as that in 293T-Control vector cells. In addition, there was almost no difference between the expression profile of ICP4, whose promoter possesses several Sp1-binding sites, in 293T-ATM shRNA and that in 293T-Control vector cells throughout HSV-1 infection, although Sp1 hyperphosphorylation was impaired in 293T-ATM shRNA cells (Fig. 3A), a finding corresponding well with our previous report that there is no difference in the yields of HSV type 2 in

(A)

p65F1CAT

(D)

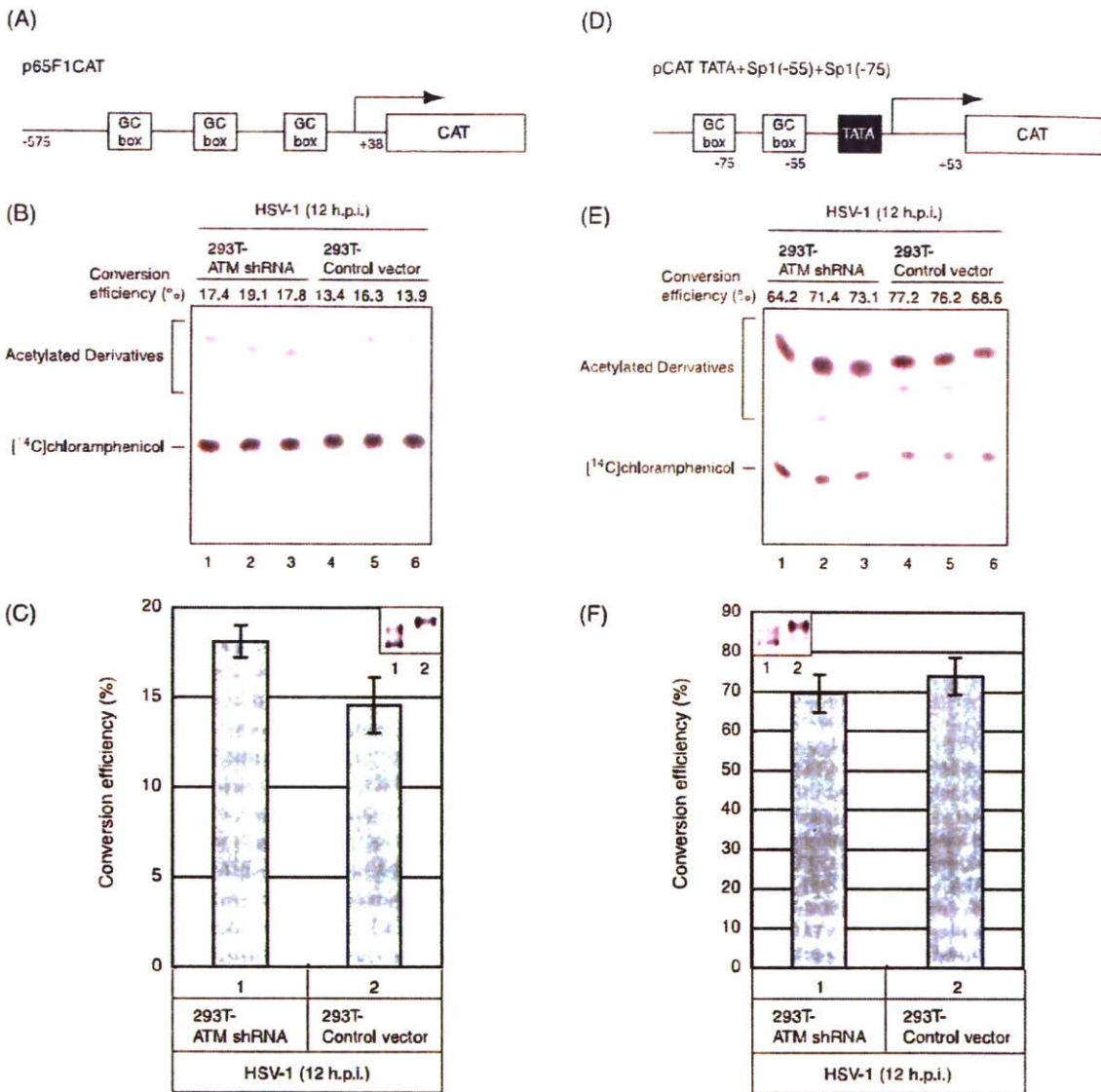pCAT TATA+Sp1(-55)+Sp1(-75)

(B)

(E)

FIG. 9. ATM-dependent Sp1 phosphorylation does not affect Sp1-dependent transcription upon infection. (A and D) Schematic illustration of reporter vectors: p65F1CAT contains three Sp1-binding sites (GC-boxes) of the TATA less p65 promoter sequence (−575 to +38) (A), and pCAT TATA+Sp1(−55)+Sp1(−75) contains two GC-boxes and TATA consensus sequence of HCMV major immediate-early gene (D). (B and E) 293T-ATM shRNA and 293T-Control vector cells were transfected with either p65F1CAT (B) or pCAT TATA+Sp1(−55)+Sp1(−75) (E), infected with HSV-1 at 24 h posttransfection at an MOI of 5, and harvested at 12 hpi. CAT assays were performed as described in Materials and Methods. All transfection experiments were in triplicate. (C and F) Data from three independent experiments in panels B and E were plotted on the graph, respectively. The levels of activity (i.e., the conversion efficiency) were determined by calculating the percentage of the conversion of unacetylated [^{14}C]chloramphenicol to the acetylated form. In order to confirm phosphorylation states and amounts of Sp1 in 293T-ATM shRNA and 293T-Control vector cells infected with HSV-1 at 12 hpi, equal amounts of proteins from each sample were subjected to immunoblot analysis with anti-Sp1 antibody (panels C and F, inset images).

293T-ATM shRNA cells and 293T-Control vector cells (53). Thus, although at least Ser-56 and Ser-101 of Sp1 were phosphorylated dependent on ATM during HSV-1 infection, the Sp1 phosphorylation at both sites does not appear to affect Sp1-dependent transcriptional activity upon HSV-1 infection. Therefore, modification of Sp1 besides phosphorylation at Ser-56 and Ser-101 might induce the reduction of its transcriptional activity during the HSV-1 infection.

IR rapidly activates ATM, which then phosphorylates several transcription factors such as p53, ATF2, and CREB (4, 6, 51). In the present study, high doses of IR (10 and 20 Gy)

induced the hyperphosphorylation of Sp1 in ATM expression-positive cells, but it did not so significantly in ATM expression-silenced cells (Fig. 4), suggesting that Sp1 is phosphorylated in an IR-induced ATM-dependent manner. A previous report demonstrated that IR at low dose (3 to 6 Gy) induces phosphorylation of Sp1 and simultaneously causes an increase in DNA-binding activity of Sp1 (40, 61). Furthermore, coexpression of Sp1 and ATM in *Drosophila* Schneider cells lacking endogenous Sp1 results in an ATM dose-dependent synergistic transactivation of IGF-IR promoter containing Sp1 binding sites, suggesting that Sp1 phosphorylation by ATM might in-

crease its transactivation activity (50). In the present study, however, the transcriptional activity from the Sp1 responsive promoter in ATM-silenced cells upon HSV-1 infection was almost the same as that in ATM-intact cells. Upon HSV-1 infection, Sp1 might undergo phosphorylation besides ATM-dependent phosphorylation or other modification(s). Thus, complex modification(s) of Sp1 caused by HSV-1 infection might mask the functional change by the phosphorylation at Ser-56 and Ser-101.

Kim and DeLuca (31) first documented the hyperphosphorylation of transcription factor Sp1 after HSV-1 infection. From studies conducted with ICP4 deletion mutants, these authors speculated that ICP4 is necessary for the hyperphosphorylation of Sp1 directly or indirectly. Also, they observed partial conversion of Sp1 to the hyperphosphorylated form during infection with wild-type HSV-1 in the presence of phosphonoacetic acid, a specific inhibitor of the viral DNA polymerase (31). We and others have previously demonstrated that HSV infection elicits ATM-dependent DNA damage responses, whereas infection with a UV-inactivated virus or with a replication-defective virus does not, suggesting that viral DNA synthesis is essential for ATM activation (37, 53). In the presence of phosphonoacetic acid, the ATM DNA damage signaling upon infection is blocked at a low multiplicity of infection, although the UL42 gene product, viral early protein, is expressed (37, 53). Therefore, ICP4 may indirectly contribute to Sp1 hyperphosphorylation through expression for viral replication proteins synthesizing viral DNA. Since synthesized viral DNA structure triggers activation of ATM-dependent DNA damage responses upon HSV infection, newly syhthesized viral DNA rather than expressed viral protein(s) appears to be necessary for the hyperphosphorylation of Sp1.

ACKNOWLEDGMENTS

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INSTITUT PASTEUR

Original article

Identification of proteins directly phosphorylated by UL13 protein kinase from herpes simplex virus 1

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Abstract

Herpes simplex virus 1 (HSV-1) UL13 is a viral protein kinase that regulates optimal viral replication in cell cultures. Identification of substrates of protein kinases is a crucial step to elucidate the mechanism by which they function. Using our developed system to analyze the specific protein kinase activity of UL13, we have shown that UL13 protein kinase directly phosphorylates the viral proteins ICP22 and UL49 previously reported to be putative substrates. We also identified UL41 as a previously unreported and novel substrate of UL13. These data will serve as a basis to clarify the mechanism by which UL13 influences viral replication.
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Keywords: Simplex virus; UL13 protein kinase; Phosphorylation

1. Introduction

Herpes simplex viruses (HSVs) are ubiquitous throughout the world and can cause a variety of diseases in humans, including mucocutaneous infections such as herpes genitalis and herpes labialis, potentially sight-impairing herpetic eye disease, and life-threatening herpes encephalitis or disseminated disease [1]. Of the more than 80 viral genes encoded by HSV, this report concerns a viral protein encoded by the HSV-1 *UL13* gene.

The HSV-1 UL13 is a serine/threonine protein kinase that is packaged in the tegument, a virion structural component located between the nucleocapsid and the envelope [2]. UL13 plays a role in viral replication in cell cultures, since UL13 deletion mutants exhibit impaired replication in cell cultures [2]. It is well known that phosphorylation of proteins

by protein kinases is the most common and effective modification that changes the activity of the target proteins. Phosphorylation of target proteins by specific protein kinases regulates many cellular functions such as transcription, translation, cell cycle regulation, protein degradation, and apoptosis [3,4]. Conceivably, HSV utilizes UL13 to regulate its own replicative process and to modify cellular machinery through the phosphorylation of viral and cellular proteins. In fact, UL13 has been reported to play multiple roles in viral gene expression, apoptosis, and nuclear egress [5–7].

UL13 may function by phosphorylating specific viral and cellular protein substrates. Identification of these substrates is a key step for clarification of the mechanisms by which UL13 regulates viral gene expression, apoptosis, and progeny virus egress, and for elucidation of other possible UL13 function(s). Thus far, numerous putative substrates for UL13 including ICP22, Us1.5, ICP0, gI/gE, UL47, UL49, UL13, p60, elongation factor 1δ (EF-1δ), casein kinase IIβ (CKIIβ), RNA polymerase II (RNA pol II), and Us3 have been reported based on the following observations. The phosphorylation and/or posttranslational processing of ICP22, Us1.5, ICP0, gI/gE, UL49, p60, EF-1δ, and RNA pol II have been demonstrated to

be reduced or obviated in cells infected with UL13 mutant viruses [6,8–15], indicating that UL13 mediates the phosphorylation of the putative substrates in infected cells. However, in the experiments with UL13 mutant viruses, UL13 may have directly phosphorylated the putative substrates, or it may have either activated or induced other protein kinase(s) that phosphorylated these putative substrates. Therefore, it is necessary to demonstrate that the substrate is specifically and directly phosphorylated by the enzyme in vitro. Two methods have been reported to show that substrates are phosphorylated by UL13 in vitro. First, ICP0 and gI/gE have been shown to be phosphorylated by UL13 using immunoprecipitation with a polyclonal antibody to UL13 [13,14]. In these in vitro kinase assays, however, it is possible that the protein kinase activity detected using UL13 immunoprecipitation is due to a contaminating kinase(s) either associated with Us3 or coprecipitated by the antibody. Recently, we developed a more reliable system to analyze the specific protein kinase activity of UL13 in vitro [16]. In this system, we express large amounts of recombinant UL13 fused to glutathione S-transferase (GST) in insect cells using a recombinant baculovirus to obtain highly purified UL13 with enzymatic activity (Fig. 1). We also generated a kinase-negative mutant of UL13 in which the invariant lysine 176 was substituted with methionine to eliminate the possibility that kinase activity detected using purified recombinant UL13 is caused by kinase contamination during purification. The use of purified recombinant UL13 and its mutant for in vitro kinase assays enables us to examine a more specific activity of UL13

than that observed during UL13 immunoprecipitation with a polyclonal antibody. In fact, we previously demonstrated that a cellular protein Bid was not phosphorylated directly by Us3, another protein kinase encoded by HSV-1, in an assay system of Us3 kinase activity that used recombinant GST-Us3 and its kinase-negative mutant, even though it has been reported that Bid is efficiently phosphorylated by Us3 immunoprecipitates [17]. Using our developed system, we have shown that EF-1δ and Us3 are phosphorylated directly by UL13 in vitro. Among the numerous putative UL13 substrates reported to date, only EF-1δ and Us3 have been convincingly demonstrated to be phosphorylated directly by UL13 [6,16] and it is unknown at present whether the other reported putative substrates are directly phosphorylated by UL13. In the present study, we examined whether the putative UL13 substrates reported earlier are phosphorylated directly by UL13. We also attempted to identify a previously unreported novel substrate of UL13.

2. Materials and methods

2.1. Cells and viruses

Spodoptera frugiperda Sf9 cells were described previously [16,18]. The recombinant baculoviruses Bac-GST-UL13 and Bac-GST-UL13K176M were described previously [16].

2.2. Plasmids

pMAL-ICP22 was described previously [17]. pMAL-UL41 was constructed by amplifying the entire coding sequence of UL41 from pBC1007 [19] and cloning the DNA fragments into pMAL-c (New England BioLabs) in frame with the maltose binding protein (MBP). pMAL-UL41N and pMAL-UL41C were generated by amplifying the domain containing UL49 codons 1–246 and 215–489, respectively, by PCR from pBC1007 and cloning the DNA fragments into pMAL-c. pMAL-UL49N and pMAL-UL49C were generated by amplifying the domain containing UL49 codons 1–100 and 166–301, respectively, by PCR from pBC1007 and cloning the DNA fragments into pMAL-c. To generate pMAL-ARF6, the entire coding sequence of ARF6 was amplified by PCR from an Epstein–Barr virus-transformed human peripheral blood lymphocyte cDNA library (Clontech) and cloned into pMAL-c. ARF6 is a brefeldin A-insensitive member of the adenosine diphosphate (ADP) – ribosylation factor (Arf) family is a group of structurally related proteins that form a subset of the Ras superfamily of regulatory GTP-binding proteins [20–23].

2.3. Production and purification of MBP fusion proteins expressed in Escherichia coli

MBP fusion proteins (MBP-ICP22, MBP-UL41, MBP-UL41N, MBP-UL41C, MBP-UL49N, MBP-UL49C and MBP-ARF6) were expressed in *Escherichia coli* (*E. coli*) that had been transformed with pMAL-ICP22, pMAL-UL41, pMBP-UL41N,
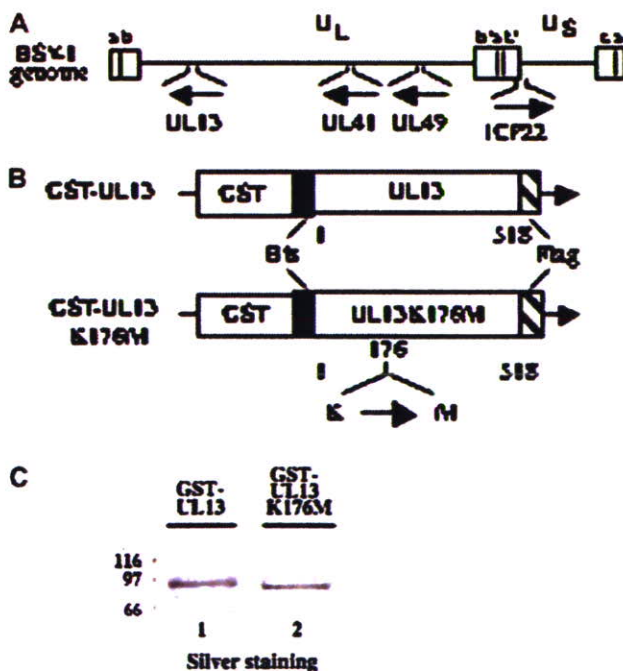

Fig. 1. (A) Schematic diagram of the genome structures of HSV-1 and the location of the *UL13*, *UL41*, *UL49* and *ICP22* genes. (B) Schematic diagram of the predicted amino acid sequence of GST-UL13 and its kinase-negative mutant GST-UL13K176M. The K176M mutation is indicated. (C) A silver-staining gel of purified GST-UL13 (lane 1) and GST-UL13K176M (lane 2) used in the *in vitro* kinase assays.

pMBP-UL41C, pMBP-UL49N, pMBP-UL49C and pMBP-ARF6, respectively, and purified as described previously [16].

2.4. Purification of GST fusion protein from baculovirus-infected cells

GST-UL13 and GST-UL13K176M proteins were purified from Sf9 cells infected with Bac-GST-UL13 and Bac-GST-UL13K176M, respectively, as described previously [16].

2.5. In vitro kinase assays and phosphatase treatment

MBP fusion proteins were captured on amylose beads (New England BioLabs) and used as substrates for *in vitro* kinase assays with GST-UL13 and GST-UL13K176M as described previously [16]. The purified GST-UL13 and GST-UL13K176M used in the *in vitro* kinase assays were electrophoretically separated in a denaturing gel and are shown in Fig. 1C. After the *in vitro* kinase assays, the MBP fusion proteins captured on amylose beads were subjected to phosphatase treatment as described previously [17,19].

3. Results

3.1. UL13 directly phosphorylates ICP22 and UL49 in vitro

We examined whether the putative substrates reported earlier (ICP22 and UL49) were in fact directly phosphorylated by UL13 *in vitro*. ICP22 has been suggested to be a regulator of viral and cellular gene expression, while UL49 is among the most abundant of the tegument proteins. As substrates for these studies, we generated and purified chimeric proteins consisting of MBP fused to the putative substrates (MBP-ICP22, MBP-UL49N and MBP-UL49C). We also used the MBP-ARF6 protein as a control. As kinases for our studies, we used purified UL13 fused to GST (GST-UL13) from Sf9 cells infected with a recombinant baculovirus Bac-GST-UL13. We also used the kinase-negative mutant GST-UL13K176M as a control. The MBP fusion proteins were captured on amylose beads and used as substrates for *in vitro* kinase assays with GST-UL13 and GST-UL13K176M. In these assays, MBP-ICP22, MBP-UL49N, and MBP-UL49C proteins were labeled with [γ-^{32}P]ATP by purified GST-UL13, but MBP-ARF6 was not (Figs. 2B and 3C). When the kinase-negative mutant GST-UL13K176M was used instead of GST-UL13 in the assays, none of the MBP fusion proteins were labeled. Furthermore, labeling of the MBP fusion proteins by GST-UL13 was eliminated by phosphatase treatment (Figs. 2D and 3E). The expression of each MBP fusion protein and identification of each radiolabeled MBP protein band was verified by Coomassie brilliant blue (CBB) staining as shown in Fig. 2A,C and Fig. 3B,D. These results indicate that UL13 specifically and directly phosphorylates ICP22 and UL49 proteins *in vitro*.
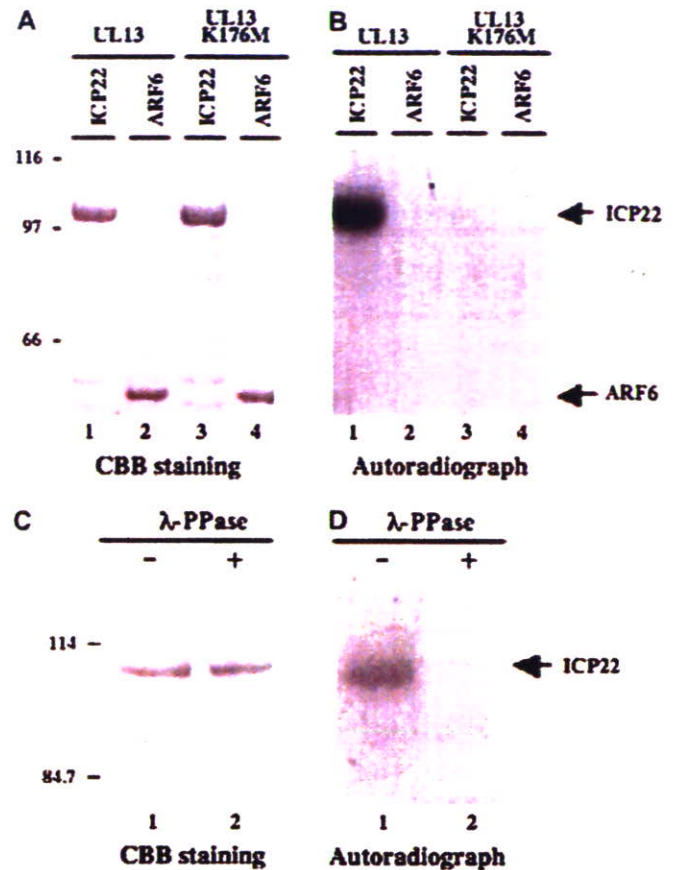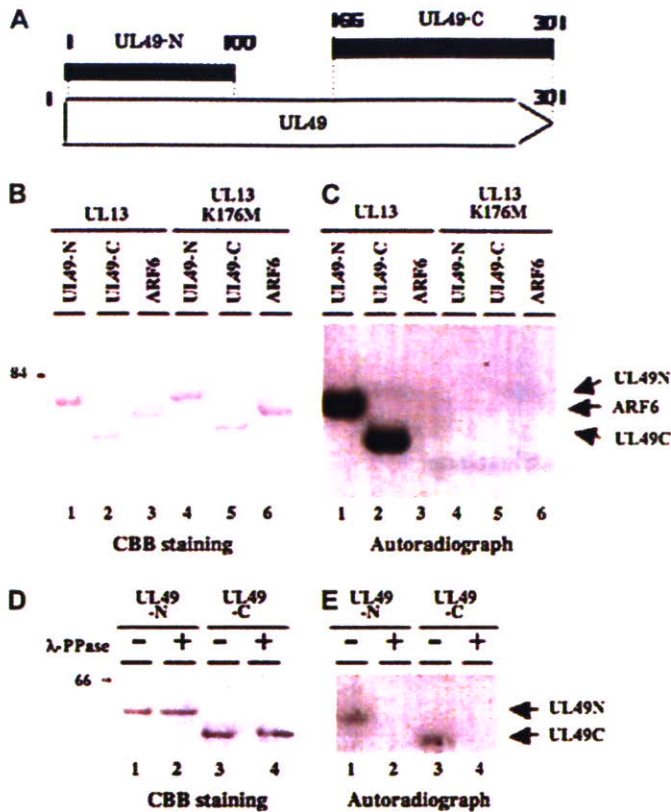

Fig. 2. Autoradiographs of *in vitro* ICP22 phosphorylation. (A) CBB stained images of phosphorylated ICP22. Purified MBP-ICP22 (lanes 1 and 3) and MBP-ARF6 (lanes 2 and 4) were incubated in kinase buffer containing [γ-^{32}P]ATP and purified GST-UL13 (lanes 1 and 2) or GST-UL13K176M (lanes 3 and 4), separated on a denaturing gel, and stained with CBB. Molecular masses (kDa) are shown on the left. (B) Autoradiograph of the gel in panel A. (C) Purified MBP-ICP22 was incubated in kinase buffer containing [γ-^{32}P]ATP and purified GST-UL13 and then either mock-treated (lane 1) or treated with λ-PPase (lane 2), separated on a denaturing gel, and stained with CBB. (D) Autoradiograph of the gel in panel C.

3.2. Identification of UL41 as a novel substrate of UL13

To identify novel viral substrate(s) for UL13, several HSV-1 proteins fused to MBP were expressed in *E. coli* and purified, and the purified MBP fusion proteins were subjected to in vitro kinase assay (data not shown). Among the viral proteins tested, we obtained evidence that UL41 is an in vitro substrate of UL13. This conclusion is supported by the following observations. UL41 is a virion component that mediates indiscriminate degradation of mRNA, subsequently causing shutoff of protein synthesis in infected cells. As shown in Fig. 4C, in the reaction with purified GST-UL13, MBP-UL41, MBP-UL41N and MBP-UL41-C were labeled with [γ-^{32}P]ATP, but MBP-ARF6 was not. When the GST-UL13K176M kinase-negative mutant was used instead of GST-UL13, none of the MBP fusion proteins were labeled. Labeling of MBP-UL41N and MBP-UL41C by GST-UL13 was eliminated by phosphatase treatment (Fig. 4E). The expression of each MBP fusion protein and the identification of each MBP protein-radiolabeled band were
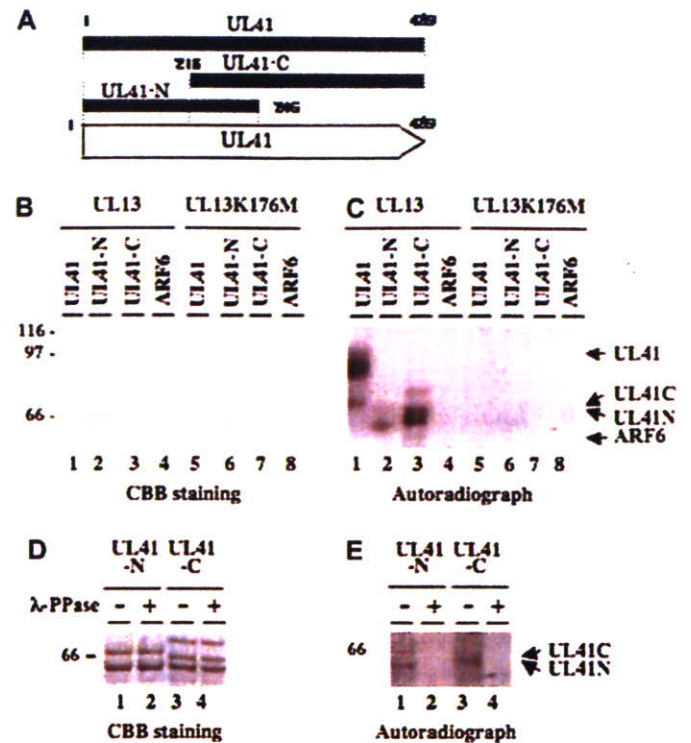
Fig. 3. Autoradiographs of *in vitro* UL49 phosphorylation. (A) Schematic diagram of structures of the UL49 open reading frame. The domains of the UL49 gene used in these studies to generate MBP-UL49 fusion proteins are indicated. (B) CBB stained images of phosphorylated UL49. Purified MBP-UL49N (lanes 1 and 4), MBP-UL49C (lanes 2 and 5), and MBP-ARF6 (lanes 3 and 6) were incubated in kinase buffer containing [γ-^{32}P]ATP and purified GST-UL13 (lanes 1–3) or GST-UL13K176M (lanes 4–6), separated on a denaturing gel, and stained with CBB. Molecular masses (kDa) are shown on the left. (C) Autoradiograph of the gel in panel B. (D) Purified MBP-UL49N (lanes 1 and 2) and MBP-UL49C (lanes 3 and 4) were incubated in kinase buffer containing [γ-^{32}P]ATP and purified GST-UL13 and then either mock-treated (lanes 1 and 3) or treated with λ-PPase (lanes 2 and 4), separated on a denaturing gel, and stained with CBB. (E) Autoradiograph of the gel in panel D.

Fig. 4. Autoradiographs of *in vitro* UL41 phosphorylation. (A) Schematic diagram of structures of the UL41 open reading frame. The domains of the UL41 gene used in these studies to generate MBP-UL41 fusion proteins are indicated. (B) CBB stained images of phosphorylated UL41. Purified MBP-UL41 (lanes 1 and 5), MBP-UL49N (lanes 2 and 6), MBP-UL49C (lanes 3 and 7) and MBP-ARF6 (lanes 4 and 8) were incubated in kinase buffer containing [γ-^{32}P]ATP and purified GST-UL13 (lanes 1–4) or GST-UL13K176M (lanes 5–8), separated on a denaturing gel, and stained with CBB. Molecular masses (kDa) are shown on the left. (C) Autoradiograph of the gel in panel B. (D) Purified MBP-UL41N (lanes 1 and 2) and MBP-UL41C (lanes 3 and 4) were incubated in kinase buffer containing [γ-^{32}P]ATP and purified GST-UL13 and then either mock-treated (lanes 1 and 3) or treated with λ-PPase (lanes 2 and 4), separated on a denaturing gel, and stained with CBB. (E) Autoradiograph of the gel in panel D.

verified by CBB staining as shown in Fig. 4B,D. These results show that UL13 directly phosphorylates UL41 *in vitro*.

4. Discussion

Identification of the substrates of a protein kinase is the first step to clarify the mechanisms by which the protein kinase functions. Once a substrate has been determined, the phosphorylation site(s) of the substrate can be identified by mass spectrometric analysis or mutational analysis, and the biological significance of phosphorylation can be investigated using substrate mutants with amino acid substitution(s) in the phosphorylation site(s). Identification of UL13 substrates has long been hampered by the difficulty of directly demonstrating specific protein kinase activity *in vitro* [24]. For instance, ICP22 was identified as the first putative substrate of UL13 more than a decade ago [15], based on the observation that the phosphorylation and posttranslational processing of ICP22 is obviated in cells infected with UL13 mutant viruses. However, no

studies have so far demonstrated that UL13 directly phosphorylates ICP22 *in vitro*. Our specially developed system to analyze the specific protein kinase activity of UL13 [16] enables us to identify specific UL13 substrates *in vitro*. In the present study, we add to the base knowledge of UL13 substrates and show that previously reported putative substrates of UL13 (ICP22 and UL49) are phosphorylated directly by UL13. Furthermore, our present studies identified UL41, a previously unreported UL13 substrate, as a novel substrate *in vitro*.

Although demonstration that protein substrates are phosphorylated directly by UL13 *in vitro* is important, the protein identified in the present study does not fulfill the requirements to be a natural substrate of UL13 in infected cells. Identification of physiological substrates of a viral protein kinase requires demonstration that the substrate is specifically and directly phosphorylated by the enzyme *in vitro*, and that phosphorylation of the substrate is altered in cells infected with a mutant virus lacking protein kinase activity. We previously identified EF-1δ and Us3 as natural substrates of UL13 that fulfill both of the requirements described above [6,16]. Of the proteins identified to

be phosphorylated directly by UL13 in this study, ICP22 and UL49 have reduced phosphorylation or posttranslational processing in cells infected with UL13 kinase-negative mutant viruses [9,15]. It is therefore likely that ICP22 and UL49 are physiological substrates of UL13 in infected cells. Since phosphorylation of UL41 has not been analyzed thus far in UL13 kinase-negative mutant virus-infected cells, it is not clear at present whether UL41 is a natural substrate of UL13. However, we may speculate that the phosphorylation of UL41 by UL13 regulates the functions of UL41 [25] such as degradation of host mRNAs and the shutoff of host protein synthesis [26] and contributes the optimal viral replication.

In conclusion, we have identified several viral and cellular proteins that are phosphorylated directly by UL13. Taken together with previous reports, some of the identified proteins including ICP22 and UL49 are likely to be physiological substrates of UL13 in infected cells. However, the biological significance of the UL13-mediated phosphorylation of the identified proteins remains unknown. Further studies to resolve this issue are of importance and presently under way in our laboratory. Such studies include the demonstration of altered UL41 phosphorylation in cells infected with UL13 mutant viruses, identification of phosphorylation sites of the UL13 substrates, and investigations of phenotypes of substrate mutants by amino acid substitution(s) in the phosphorylation site(s).

Acknowledgments

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Duration of Neutralizing Antibody Titer after Japanese Encephalitis Vaccination

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Abstract: In paired serum samples collected from 17 children, we measured neutralizing antibody (NTAb) titers after the second series of routine Japanese encephalitis (JE) vaccination in Japan to estimate the duration of NTAb titer when children did not receive the third series of routine vaccination by applying a random coefficient model. We also measured NTAb titers in adult serum samples to confirm the duration of NTAb titer estimated in the analysis of pediatric serum samples. In the absence of the third series of routine vaccination, 18% (3/17), 47% (8/17), 82% (14/17) and 100% (17/17) of children were estimated to become NTAb negative at 5, 10, 15, and 20 years after the second series of routine vaccination, respectively. Of 38 adults, 39.5% (15/38) became NTAb negative; the percentage was somewhat lower than that of antibody-negative children. The results suggested that JE vaccination schedule should be reevaluated in the future.

Key words: Japanese encephalitis vaccine, Japanese encephalitis virus, Routine vaccination in Japan, Duration of neutralizing antibody titer

Japanese encephalitis (JE), or Japanese encephalitis virus (JEV) infection, is an endemic disease in Japan as well as in the other Asian countries. Approximately 50,000 JE patients are reported annually, mainly in Southeast Asia (29). The JE endemic area has been expanding (13–15, 29). JE is an inflammatory disease that affects the central nervous system. Sudden fever, headache, and vomiting occur after 7 to 10 days incubation period. Approximately 30% of JE cases end fatally, and 50% of patients are left with neurological sequelae (9).

The mouse brain-derived JE vaccine was first approved in 1954, and the vaccine strain was switched from the Nakayama strain to the Beijing-1 strain in 1988 in order to enhance the vaccination effectiveness (23), resulting in a dramatic decrease of JE patients. Although only a small number of JE patients have been reported annually in Japan in recent years, the infection rate remains high in swine which is an intermediate host of JEV (6, 9). Human vaccination and JEV anti-

body surveillance are therefore essential tools from the public health point of view (10). If there are neutralizing antibody (NTAb) titers of 1:10 or more, it is thought that natural infection of a JEV can be prevented (2, 8, 16, 19, 29). Moreover, the World Health Organization (WHO) described in its 2006 position paper that NTAb titers of 1:10 or more were accepted evidence of protection against JEV infection (27).

As shown in Fig. 1, JE vaccination had been provided in the three-series routine vaccination program in Japan (9, 23).

However, the Ministry of Health, Labour and Welfare (MHLW) of Japan decided in May 2005 to suspend its recommendation of the routine vaccination with the current mouse brain-derived vaccine until a safer JE vaccine was available because of associated adverse effects including acute disseminated encephalomyelitis (18, 28). In July 2005, MHLW announced that the third series of vaccination would not be resumed even

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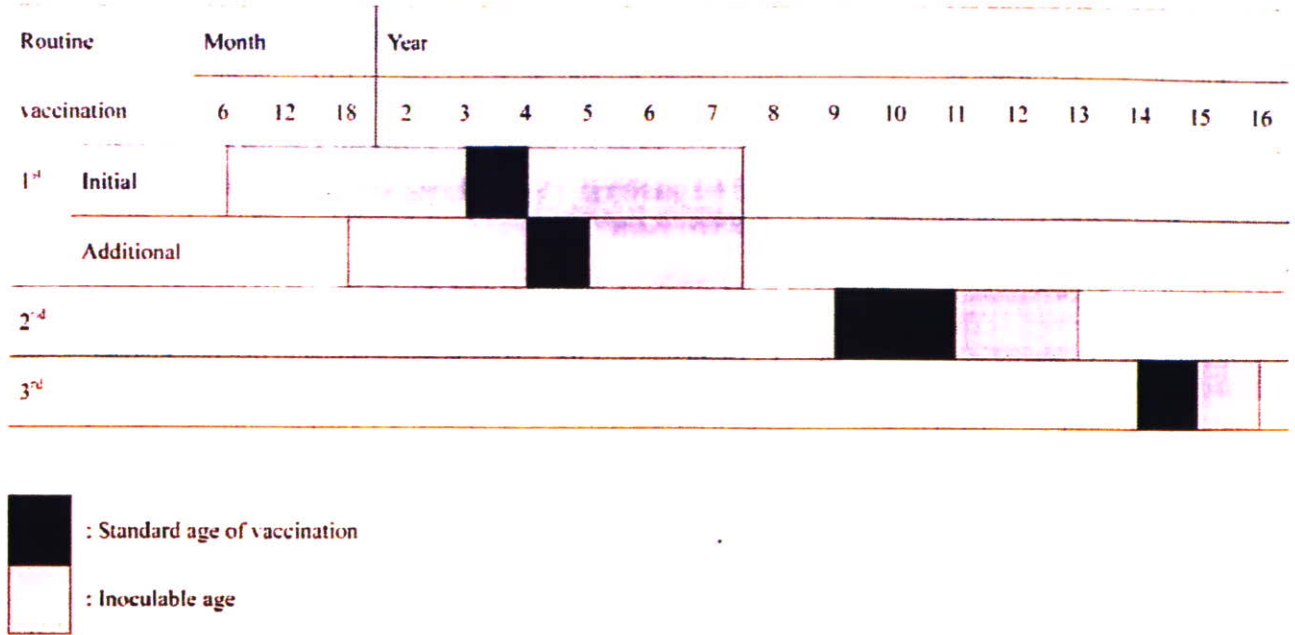
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Fig. 1. In Japan, a JE routine vaccination was generally performed as follows: the first series of routine vaccinations is performed twice at 1 to 4 weeks interval between 6 to 90 months old (standard age is 3 years old) as initial vaccinations and one more time about 1 year after initial vaccinations (standard age is 4 years old) as an additional vaccination; the second series of routine vaccination is performed between 9 and 12 years old (standard age is 9 to 10 years old); the third series of routine vaccination is performed between 14 and 15 years old (standard age is 14 years old).

after a safer vaccine became available.

However, 4 of 57 cerebrospinal fluid samples collected from patients with undiagnosed acute encephalomeningitis were tested JEV genome positive (11), suggesting that the etiology of JEV infection has changed. In 2006, 7 JE patients, including a 3-year-old, were identified in Japan. Many Japanese travel to Southeast Asia where JEV infection is a big concern (3, 21). Considering these facts, maintaining sufficient NTAb to prevent JEV infection is still important for people of all ages in present-day Japan. We are currently developing a Vero cell-derived vaccine to replace the mouse brain-derived vaccine in order to improve vaccine safety and productivity (2, 7, 12, 22). However, little information is available concerning NTAb prevalence based on the JE vaccination schedule shown in Fig. 1 (6). Reviewing the JE vaccination schedule before introducing a Vero cell-derived vaccine will therefore be meaningful.

In the current study, we measured NTAb titers in pediatric serum samples including paired serum samples to estimate the duration of NTAb titer after the current routine JE vaccination program by applying a random coefficient model. We also measured adult serum NTAb titers to support the estimated duration of NTAb titer based on the analysis of pediatric serum samples.

Materials and Methods

Pediatric serum samples after completion of the second series of routine vaccination. Serum samples were collected from 47 children who lived in Hisayama-machi, Fukuoka prefecture, Japan, a potential JE high-risk area based on the prevalence of antibodies against JEV in swine (6, 9), and had completed the second series of routine JE vaccination. The sample collection took place in 1994 and 1997 after obtaining informed consent of the participants to provide their serum samples for measurement of antibodies against infectious diseases. The history of JE vaccination was checked by referring to the mother-child notebook of each participant. The collected samples included paired serum samples (collected in 1994 and 1997) of 17 participants and single serum samples (collected in 1997) of 30 participants. The first samples of paired serum were collected about 2 months after the second series of vaccination in 1994. The second samples of paired serum were collected about 38 months after the second series and before the third series of vaccination in 1997. Each of the single serum samples collected in 1997 consisted of those collected about 2 months after ($n=12$) or about 38 months after ($n=18$) the second series of vaccination. All serum samples were stored at -80 C until

NTAb titers were measured.

Adult serum samples. Thirty-eight adult serum samples were used to determine the accuracy of duration of NTAb estimated in the analysis of pediatric serum samples. All the adult serum samples were collected from visitors at the outpatient vaccination department of the National Hospital Organization Fukuoka National Hospital living in Fukuoka prefecture, Japan, and scheduled to travel to Southeast Asia. Informed consent to provide the serum sample for measurement of NTAbs against JEV was obtained from the visitors. Their ages ranged from 24 to 55, and their history of vaccination was unknown. Most visitors wished to receive an additional JE vaccination prior to their departure. Serum samples were obtained from 18 visitors at least 3 weeks after the additional vaccination. All serum samples were stored at −80 C until NTAb titers were measured.

Neutralizing antibody titer assay. Fifty-percent plaque reduction test with Vero cells was used to measure NTAbs in the serum samples, and 3 points least-squares regression method (3LSRM) described in our previous study was used for analysis (1). The standard deviation in 3LSRM is 0.127 (log) or less and the variation coefficient is 7% or less (1).

Statistical analysis. Geometric mean, standard deviation, and range of NTAb titers after the second series of routine vaccination were separately calculated for the paired serum samples and the single serum samples. For the paired serum samples, the geometric mean titer (GMT) ratio of NTAb titers at about 38 months after the second series of routine vaccination against those at about 2 months after and 95% confidence interval (CI) were calculated. A random coefficient model was applied to the NTAb titers in the paired and single serum samples in order to estimate time when the NTAbs acquired by JE vaccination turned negative (less than 1:10).

$$y_{ij} = \alpha + \beta x_i + a'_i + b'_i x_{ij} + \varepsilon_{ij} \qquad (I)$$

where

$i = 1, ..., t$

$j = 1, ..., n_i$

$a'_i = a_i - \alpha$

$b'_i = b_i - \beta$

$\begin{pmatrix} a_i \\ b_i \end{pmatrix} \sim iidN \left[\begin{pmatrix} 0 \\ 0 \end{pmatrix}, \psi \right], \quad \psi = \begin{pmatrix} \sigma_a^2 & \sigma_{ab} \\ \sigma_{ab} & \sigma_b^2 \end{pmatrix}$

$\varepsilon_{ij} \sim iidN (0, \sigma^2)$

y_{ij} in Equation (I) represents the NTAbs in participant i (1, ..., t) at measuring timepoint j (1, ..., n_i) after logarithmic conversion. α represents the intercept of the entire population, x_i represents the number of years after the last vaccination, β represents the slope of changes in NTAbs in the entire population, a^*_i represents the difference between the intercept of participant i and that of the entire population, b^*_i represents the difference between the slope of participant i and that of the entire population, and ε_{ij} represents the residual error. $\alpha + \beta x_i$ represents the fixed effect of the model (population mean), and $a'_i + b'_i x_{ij} + \varepsilon_{ij}$ represents the deviation from the fixed effect of the model, or the random (intra-participant) effect. Random effects a^*_i and b^*_i were independent and identically distributed (mean (0, 0), variance ψ) and so did residual error ε_{ij} (mean 0, variance σ^2). SAS (SAS Institute, Cary, N.C., U.S.A.) was used for all statistical analyses.

Results

Neutralizing Antibody Titers in Paired and Single Serum Samples

Distribution of NTAb titers in paired and single serum samples collected after the second series of routine JE vaccination is shown in Fig. 2.

Geometric mean of NTAb titers in paired serum samples of 17 fourth graders (aged 9 to 10) at approximately 2 months after the vaccination was $1:10^{2.876}$ (standard deviation, $1:10^{0.737}$; minimum, $1:10^{1.57}$; maximum, $1:10^{3.95}$). When the children became seventh graders (aged 12 to 13) approximately 3 years after the initial analysis, the geometric mean of NTAb titers significantly decreased to $1:10^{2.303}$ (standard deviation, $1:10^{0.826}$; minimum, $1:10^{0.90}$; maximum, $1:10^{3.50}$; GMT ratio, 0.267; two-sided 95% CI, 0.076 to 0.940). All individual paired serum samples showed a flat decrease in NTAb titers; however, there were inter-individual differences in the slope of decrease (range of individual regression coefficient, −0.11 to −1.42) (Fig. 2).

The geometric mean of NTAb titers in single serum samples of 12 children collected only at 2 months after the vaccination was $1:10^{3.693}$ (standard deviation, $1:10^{0.290}$; minimum, $1:10^{3.06}$; maximum, $1:10^{4.10}$) and in serum samples of 18 children collected only at 38 months after the vaccination, it was $1:10^{2.266}$ (standard deviation, $1:10^{0.864}$; minimum, $1:10^{0.97}$; maximum, $1:10^{3.45}$). Neutralizing antibody titers in many of the single serum samples collected at 2 months post-vaccination were higher compared with the paired serum samples collected around the same time. On the other hand, NTAb titers in the single serum samples collected at 38 months post-vaccination were comparable to those in the paired serum samples collected around the same time (Fig. 2).
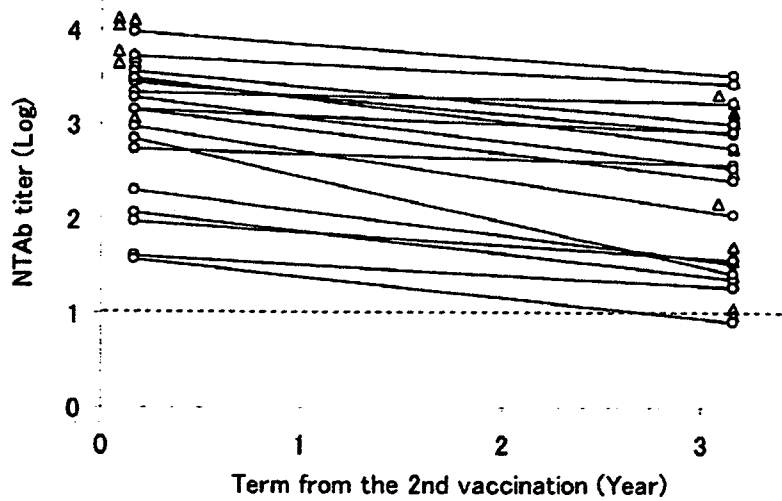
Fig. 2. Neutralizing antibody titers in paired pediatric serum samples: O (n=17) and single pediatric serum samples: Δ (n=30) after second series of routine Japanese encephalitis vaccination.
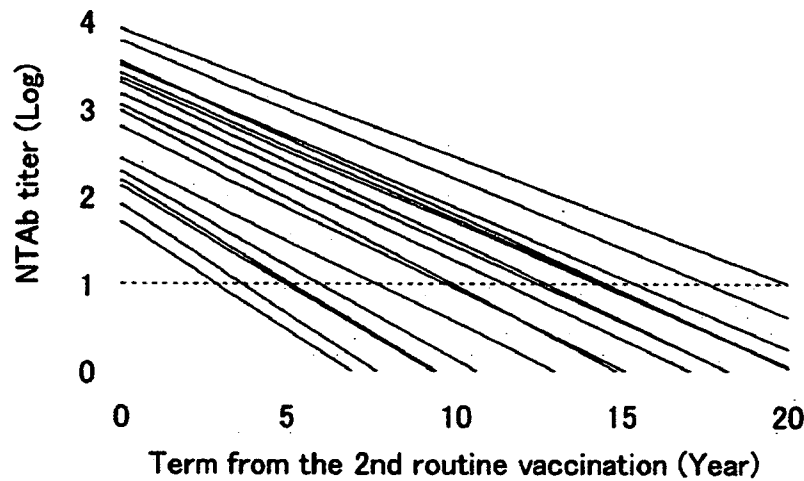

Fig. 3. Estimated individual NTAb titers in pediatric serum samples based on random coefficient model (n=17).

Duration of Neutralizing Antibody Titer after the Second Series of Routine Vaccination

A random coefficient model was applied to the NTAb titers in the paired serum samples collected from 17 children in order to estimate the time until the NTAbs acquired through JE vaccination turned negative (less than 1:10) in individual children (Fig. 3).

At 5, 10, 15, and 20 years after vaccination, 18% (3/17), 47% (8/17), 82% (14/17) and 100% (17/17) of the children, respectively, were estimated to become NTAb negative. Time to negative NTAb titers was also estimated for sensitivity analysis by using single serum samples collected from 30 children (samples of 12 children collected at 2 months post-vaccination and those of 18 children collected at 38 months post-vaccination) in

addition to the said paired serum samples of 17 children (47 samples in total, Fig. 4).

At 5, 10, 15, and 20 years post-vaccination, 15% (7/47), 38% (18/47), 70% (33/47) and 89% (42/47) of children, respectively, were estimated to become NTAb negative. The results were comparable to those estimated by using the paired serum samples of only 17 children. However, the estimates of the 17 paired serum samples calculated by each population were different (Figs. 3 and 4).

Adult Serum Samples

Neutralizing antibody titers in 38 adults aged between 24 and 55 are shown in Fig. 5.

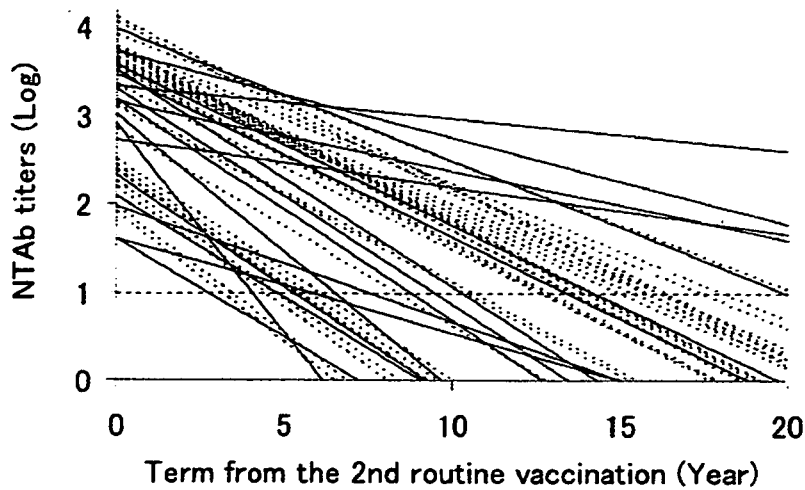Negative NTAb titers were found in 39.5% (15/38)

Fig. 4. Estimated individual NTAb titers in pediatric serum samples based on random coefficient model (*n*=47). The solid lines indicate paired pediatric serum samples (*n*=17) and the broken lines indicate single pediatric serum samples (*n*=30).
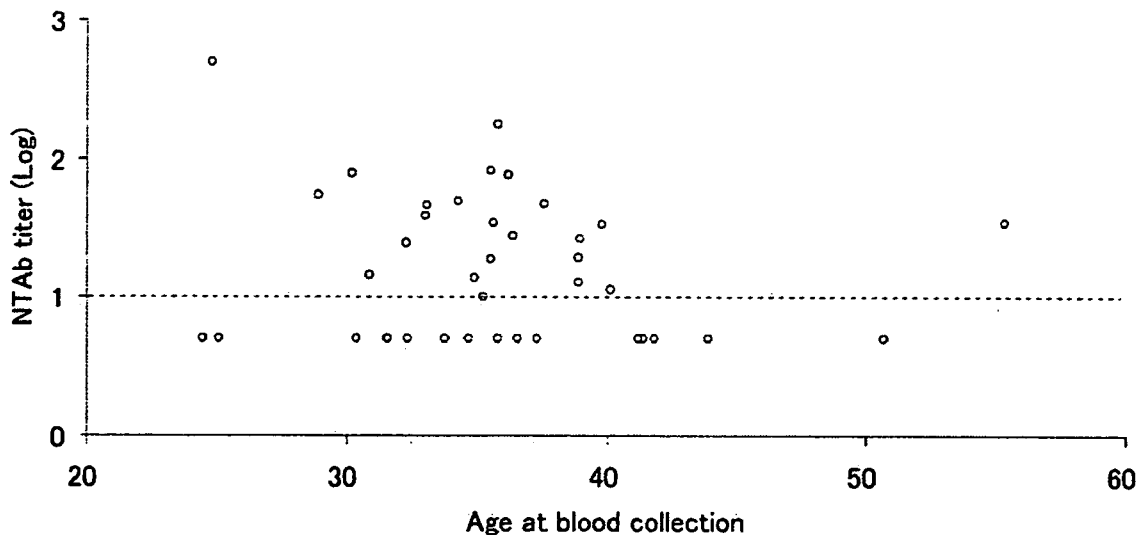

Fig. 5. Distribution of NTAb titers in adult serum samples (*n*=38). Neutralizing antibody titer in a NTAb negative sample is shown as 0.7.

and NTAb titers of $1:10^{1.5}$ or less were found in 65.8% (25/38). Only 2 adults had NTAb titers of $1:10^{2.0}$ or more. In the age-specific analysis, 50.0% (2/4) of those under 30, 29.6% (8/27) of those aged 30 or above and under 40, and 71.4% (5/7) of those aged 40 or above had negative NTAb titers. A higher negative rate was seen in adults aged 40 or above. After additional vaccination, serum samples were collected for further analysis from 18 adults as shown in Fig. 6. All of the 8 volunteers who had had negative NTAb titers had positive NTAb titers.

Discussion

With the revision of the Preventive Vaccination Law in 1994, the JE vaccination program was changed from mass vaccination to individual vaccination in Japan. The JE vaccination rate among children, especially those of the second and third series of vaccination, has been decreased since the revision of the Law. Our current study suggests that the percentage of children with negative NTAb titers would increase to about 18% at 5 years after the second series of vaccination (at the age of 14 to 15) if the third series of vaccination was not pro-
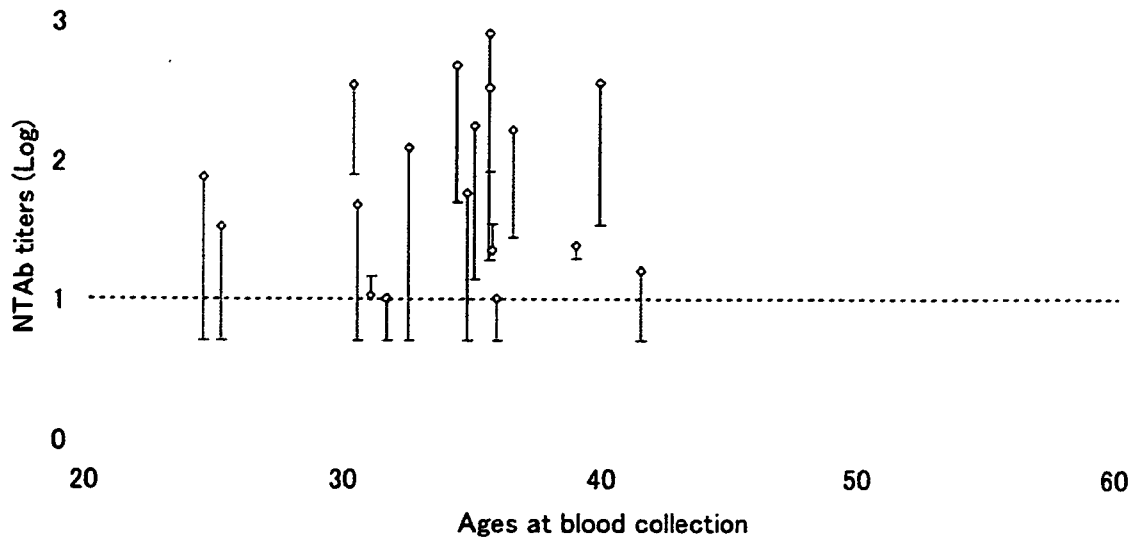
Fig. 6. Distribution of NTAb titers before additional vaccination: — and that of NTAb titers after additional vaccination: ◇ in adults serum samples (n = 18). Neutralizing antibody titer in a NTAb negative sample is shown as 0.7.

vided. One of the paired serum samples (n=17) was found to be NTAb negative at only 3 years after the second series of routine vaccination. Therefore, it is at least highly possible that some children will not be able to maintain the sufficient NTAb titers of 1:10 to prevent JEV infection described by the WHO (27, 29), due to discontinuation of the third series of routine vaccination. Fortunately, no JE patient in the late teens was reported in the first year after MHLW decided to discontinue the third series of vaccination. Considering the pre-vaccination NTAb positive rate of 0.2% (data not shown) among children aged 6 to 90 months (n=468) shown in our previous phase III study of the Vero cell-derived JE vaccine, JE infection risk for children in the present-time Japan is presumably not very high. That children now have fewer chances to get bitten by mosquitoes may be another factor for the low risk of JE infection. However, the infection rate among swine is still high (6, 9). In 2006, 7 JE patients, including a 3-year-old, were identified. According to a study report, the JE virus was detected in the cerebrospinal fluid of 4 of 57 patients with aseptic meningitis (aged 2 to 6) (11). Although the vaccination history of the patients with aseptic meningitis was unknown, the data suggest that the risk of JEV infection is still a matter of concern. The predominant JEV genotype that shifted from Type III to Type I in Japan may have been causing the change of clinical symptoms associated with JE from encephalitis to meningitis (17). Assessment of JE infection risk is quite complicated as described earlier.

We expected to see many antibody-negative adults based on our previous data. In the phase I study of the Vero cell-derived JE vaccine conducted in 2001 in adult

male volunteers living in Osaka City, Japan, aged between 20 and 35, 34.2% (92/269) of the volunteers were antibody negative (12), which is comparable to that shown in the analysis of adult serum samples in the current study. However, the actual antibody negative rate in the serum samples of 38 adult volunteers was slightly lower than the antibody negative rate estimated in the serum analysis of 17 children. Specifically, the antibody negative rate shown in the analysis of adult serum samples was 50.0% in volunteers under 30, 29.6% in those aged 30 or above and under 40, and 71.4% in those aged 40 or above. On the other hand, the estimated antibody negative rate shown in the analysis of serum samples collected from 17 children was 82% at 15 years post-vaccination (at the age of 24 to 25) and 100% at 20 years post-vaccination (at the age of 29 to 30). The slight difference in antibody negative rate was possibly caused by several factors. Approximately half of the adult volunteers had presumably received the third series of vaccination considering the third series vaccination rate of about 50% according to MHLW, although the vaccination history of the volunteers is unknown as described earlier. Some may have previously received JE vaccination prior to their past overseas travel. Some may have acquired natural immunity in Fukuoka, Japan, which is considered a high risk area for infection by JEV judging from the antibody prevalence in swine (6, 9). A linear decrease of NTAbs was assumed in the logarithmic scale since only single or paired serum samples collected from the children were used in the current study; however, decrease in acquired antibodies may be shown by a gentle curve (20). Thus, a complex association of these

factors may have caused the lower antibody negative rate among adults compared to the estimate based on the analysis of pediatric serum samples.

Epidemiological problems associated with the fact that 39.5% of the adult volunteers did not maintain the sufficient NTAb titers to prevent infection described by WHO (27, 29) cannot be ignored at any rate. We measured NTAb titers in serum samples of 18 adult volunteers after the additional vaccination and confirmed that all 8 volunteers who had been NTAb negative eventually turned positive. Based on the estimate that about 18% of children who received the second series of routine vaccination turn NTAb negative at 5 years post-vaccination (at the age of 14 to 15) and the analysis of paired serum samples that identified a child who turned NTAb negative at 3 years post-vaccination, additional vaccinations at intervals of at least 5 years after the second series of vaccination would be ideal in terms of maintaining the sufficient NTAb titers to prevent JEV infection. However, NTAb titers in adults after the additional vaccination became around $1:10^2$ as shown in Fig. 6, suggesting a smaller booster effect compared to that on children. Our previous phase I and III studies also showed comparable results. In the phase I study in adult male volunteers aged between 20 and 35, GMT after three series of vaccination was $1:10^{2.35}$ ($n=30$) with the Vero cell-derived vaccine and $1:10^{2.03}$ ($n=28$) with the current mouse brain-derived vaccine (12). On the other hand, GMT after three series of vaccination was $1:10^{3.96}$ ($n=218$) with the Vero cell-derived vaccine and $1:10^{3.76}$ ($n=221$) with the current vaccine in the phase III study in children aged between 6 to 90 months (data not shown). Children appear to be able to maintain higher NTAb titers that may decrease with age. It may be explained by the inferior booster effect from aging as shown in the case of the influenza vaccination (5). Affected by global warming, the risk of JEV infection in Japan will have little chance of decreasing in the future. The JE vaccine is also considered as a travelers' vaccine, and additional vaccinations are at least strongly recommended for adults who are scheduled to travel to Southeast Asia. Moreover, there is no information regarding the duration of the NTAb titer after additional vaccination in adults. It is suspected that the duration is shorter in adults than in children, because acquired NTAb titers in adults were, after additional vaccination, lower than in children as mentioned above. Therefore, investigation of the duration of NTAb titers in the adult population after additional vaccination would be meaningful in view of the risks related to infections in the elderly and travel to endemic areas.

The duration of NTAb titer in paired serum samples alone ($n=17$) estimated by using a random coefficient

model was different from the duration in paired serum samples and additional single serum samples combined ($n=47$). Estimated value based on a random coefficient model is generally interpreted as weighted mean of population mean profile and data profile. The difference in estimated duration of NTAb titer in the two sets of serum samples is therefore considered as the result of underestimation of the random effect of additional single serum samples and increased weight of observation profile due to sample size increase. However, the estimated duration of NTAb titer in paired serum samples alone ($n=17$) is assumed to be more reliable than the estimated duration in paired serum samples and additional single serum samples combined ($n=47$) since the random coefficient model ignored within-subject variability in the data from the additional single serum samples ($n=30$).

Various methods have been proposed for statistical estimation of duration of antibody titers (24–26). A common problem associated with all the proposed methods is that within-subject variability is ignored. We used the random coefficient model that incorporated a within-subject covariance structure in the current study to solve the problem by taking into account both fixed and random effects. A similar attempt has been made in the Bayesian random-effects model of Coursaget et al. (4) and the Piecewise linear model of Pigeon et al. (20).

The current study has certain limitations in itself since it was designed as a retrospective study in which a limited number of serum samples were used. Duration of NTAb titer will need to be evaluated in a well-designed prospective study in the future when a Vero cell-derived vaccine is introduced. The first series of routine vaccination program, in which 3 sessions of vaccinations are given, cannot be changed in order to ensure basic immunity against JEV. However, the second and third series of routine vaccination need to be reevaluated based on a domestic epidemiological surveillance of JE and a proper assessment of the necessity of the JE vaccine as a travelers' vaccine.

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