

protein. The receptor usage of measles virus has been well characterized; while laboratory strains of measles virus, such as the Edmonston strain, can use either CD46 or SLAM as receptors, wild-type strains, such as the Ichinose strain, can only use SLAM for entry (15, 24, 42, 48, 67). Expression of these receptor molecules in Sf9 cells transfected with the bMON Δ 64/CD46/CALuc or bMON Δ 64/SLAM/CALuc bacmid (Fig. 1B) and subsequent incorporation of the receptors into progeny particles (Ac Δ 64/CD46/CALuc and Ac Δ 64/SLAM/CALuc, respectively) were confirmed by Western blotting (Fig. 5A). CD46 was detected in cells transfected with bMON Δ 64/CD46/CALuc and in the purified particles of Ac Δ 64/CD46/CALuc, whereas SLAM was detected in cells transfected with the bacmid but not in the particles of Ac Δ 64/SLAM/CALuc.

The gp64, CD46, and SLAM proteins are all type I membrane proteins. SLAM has a 77-amino-acid cytoplasmic domain (23), while gp64 and CD46 have only 7- and 33-amino-acid tails, respectively (49, 60). Therefore, we speculated that SLAM may be only inefficiently incorporated into baculovirus particles, due to its large cytoplasmic domain. To examine the effect of the cytoplasmic domain length on incorporation into baculovirus particles, we constructed a mutant SLAM molecule, SLAMcyto7, with a deletion in the C-terminal cytoplasmic domain that preserves only the seven membrane-proximal amino acids. Western blot analysis revealed that SLAMcyto7 was efficiently expressed in Sf9 cells transfected with bMON Δ 64/SLAMcyto7/CALuc and subsequently incorporated into Ac Δ 64/SLAMcyto7/CALuc particles at levels similar to those seen for CD46 inclusion into Ac Δ 64/CD46/CALuc (Fig. 5A).

To determine the efficiency of ligand-directed gene delivery, BHK cells were cotransfected with expression plasmids encoding the measles virus H and F glycoproteins of the Edmonston (EdH and EdF) or Ichinose (IcH and IcF) strain. These cells were inoculated with pseudotype baculoviruses (Fig. 5B). Ac Δ 64/CD46/CALuc exhibited gene delivery specifically to cells expressing EdH and EdF, but not IcH and IcF. Although the efficiency of gene transduction was 10 times lower than that seen with Ac Δ 64/CD46/CALuc, Ac Δ 64/SLAMcyto7/CALuc could also deliver a reporter gene to cells expressing the Edmonston and Ichinose strain glycoproteins but not to control cells. While Ac Δ 64/gp64/CALuc and Ac Δ 64/VSVG/CALuc could effectively deliver a reporter gene to all of the cells examined, Ac Δ 64/SLAM/CALuc was ineffective against all of the cell lines tested, likely due to the lack of SLAM incorporation into the virions.

To confirm ligand-directed gene delivery by Ac Δ 64/CD46/CALuc and Ac Δ 64/SLAMcyto7/CALuc to cells expressing appropriate measles virus glycoproteins, we tested the neutralization of gene transduction by specific monoclonal antibodies against CD46 and SLAM (Fig. 5C). Gene transduction of target cells by either Ac Δ 64/CD46/CALuc or Ac Δ 64/SLAMcyto7/CALuc, but not by Ac Δ 64/gp64/CALuc, could be inhibited in a dose-dependent manner by anti-CD46 and anti-SLAM monoclonal antibodies, respectively. These results indicate that pseudotype baculoviruses can deliver foreign genes to target cells in a ligand-directed manner.

Entry pathway of the pseudotype baculoviruses. Virus entry occurs either by the direct fusion of viral envelope proteins with the host plasma membrane at neutral pH, as seen for measles virus, or following receptor-mediated endocytosis, as seen for AcMNPV and VSV, in which envelope glycoproteins

undergo conformational changes into a fusion-competent state, leading to fusion between viral and host membranes at low pH within endosomes (6, 36, 69). Ammonium chloride and chloroquine, which inhibit endosomal acidification, have been used as entry inhibitors for viruses that penetrate cells through receptor-mediated endocytosis (7). To examine the entry pathways used by the pseudotype baculoviruses, we examined the infectivity of Ac Δ 64/gp64/CALuc, Ac Δ 64/VSVG/CALuc, and Ac Δ 64/CD46/CALuc to BHK cells expressing EdH and EdF in the presence or absence of ammonium chloride or chloroquine (Fig. 6). Although these compounds inhibited gene transduction of BHK cells inoculated with Ac Δ 64/gp64/CALuc or Ac Δ 64/VSVG/CALuc in a dose-dependent manner, gene transduction by Ac Δ 64/CD46/CALuc was not inhibited. In contrast, ammonium chloride treatment enhanced gene expression following Ac Δ 64/CD46/CALuc infection. These results indicate that the pseudotype baculoviruses utilize entry pathways conferred by the nature of the ligand protein replacing gp64.

Morphology of pseudotype baculovirus. To address any alterations in pseudotype baculovirus morphology, we examined the AcMNPV, Ac Δ 64/VSVG/CALuc, and Ac Δ 64/CD46/CALuc virus particles by transmission electron microscopy (Fig. 7A to C). All of the pseudotype baculoviruses exhibited rod shapes and similar sizes, indistinguishable from the wild-type baculovirus. To examine the incorporation of exogenous ligands into the virion, purified Ac Δ 64/VSVG/CALuc and Ac Δ 64/CD46/CALuc particles were examined by immunoelectron microscopy using specific monoclonal antibodies against VSVG and CD46 (Fig. 7E and F). In both pseudotype viruses, gold particles were detected on the virion surface from the stalk to the head domains, indicating that VSVG and CD46 were incorporated into the Ac Δ 64/VSVG/CALuc and Ac Δ 64/CD46/CALuc virus particles, respectively.

DISCUSSION

Baculovirus is a useful tool for gene delivery to mammalian cells due to the large capacity of the virus to incorporate foreign genes, the wide host range, and the lack of replication in mammalian cells, providing minimal toxicity (29, 53, 55, 61, 68). The gp64 envelope glycoprotein, involved in attachment to both insect and mammalian cells, is required for low-pH-triggered membrane fusion following endocytosis during virus entry (6, 8, 12, 14, 20, 21, 31, 40, 41, 66, 69, 70). We have previously demonstrated that the interaction of gp64 with cell surface phospholipids may be important in baculovirus infection of mammalian cells (66). As the recombinant baculoviruses used for gene delivery to mammalian cells in this system retained the gp64 envelope glycoprotein, it was difficult to deliver foreign genes to specific target cells.

To generate a ligand-directed gene-targeting baculovirus vector, we established a bacmid system to produce recombinant baculoviruses in which the gp64 gene was replaced with other ligand genes of interest. The gp64 protein is required for efficient budding from Sf9 cells; the budding of a mutant virus lacking gp64 was reduced to 2% of that seen for wild-type baculovirus (49). Although Ac Δ 64/gp64/CALuc, a pseudotype virus in which gp64 was reintroduced into the gp64 deletion mutant, incorporated two to three times as much gp64 protein as seen in wild-type baculovirus (Fig. 4A), the infectious titers

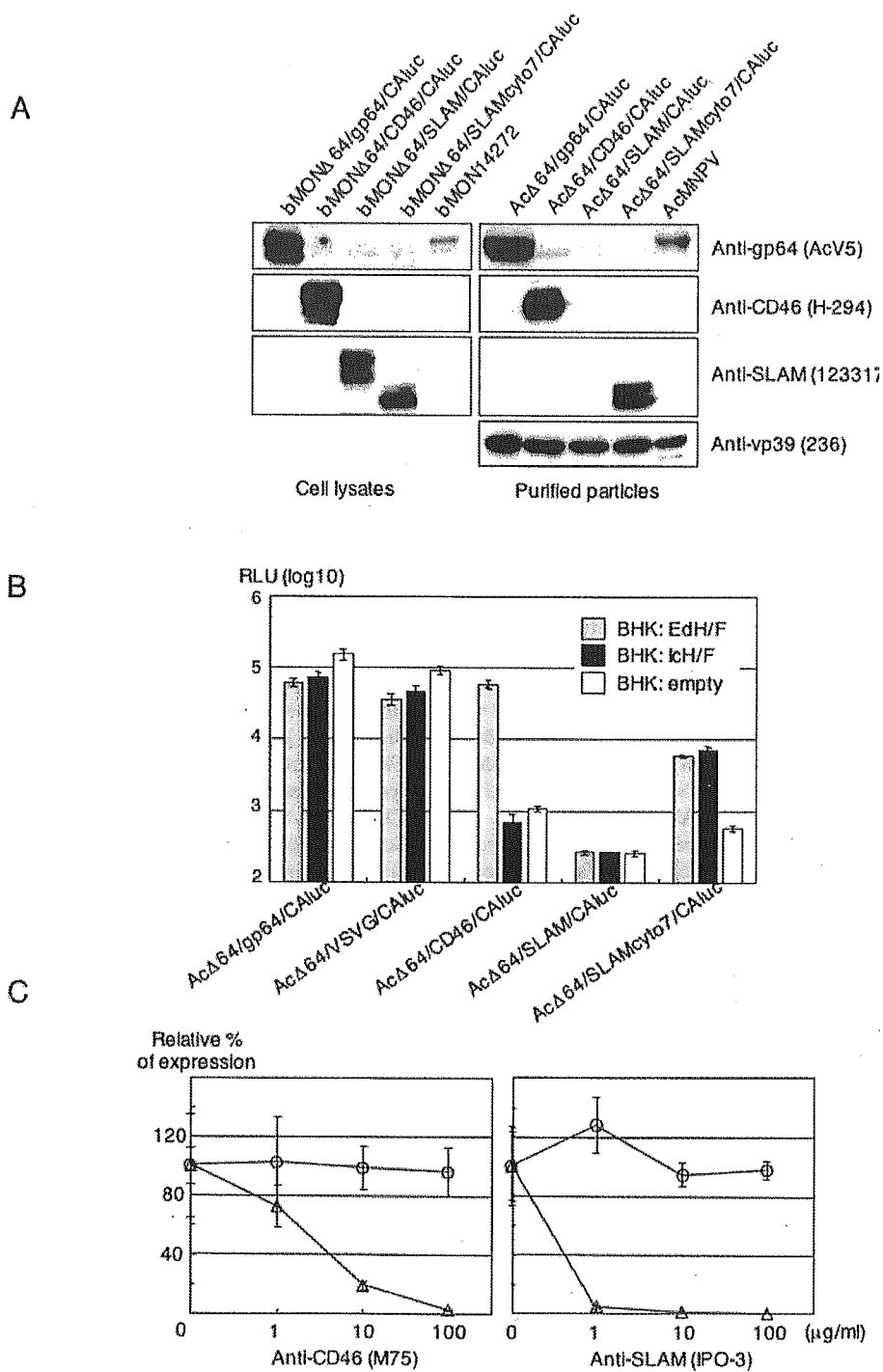


FIG. 5. Ligand-directed gene targeting by pseudotype baculoviruses. (A) The expression of gp64, CD46, SLAM, and SLAMcyto7 in Sf9 cells transfected with the bMON Δ 64/gp64/CALuc, bMON Δ 64/CD46/CALuc, bMON Δ 64/SLAM/CALuc, bMON Δ 64/SLAMcyto7/CALuc, or bMON14272 bacmid was examined by Western blot analysis using monoclonal antibodies specific for gp64 (AcV5), CD46 (H-294), and SLAM (123317), as indicated (left). The incorporation of gp64, CD46, SLAM, and SLAMcyto7 into pseudotype particles, Ac Δ 64/gp64/CALuc, Ac Δ 64/CD46/CALuc, Ac Δ 64/SLAM/CALuc, Ac Δ 64/SLAMcyto7/CALuc, or AcMNPV, was examined by Western blot analysis using monoclonal antibodies specific for gp64, CD46, SLAM, and vp39 (236), as indicated (right). (B) Ligand-directed gene targeting by pseudotype baculoviruses. BHK cells (3×10^4) were co-transfected with expression plasmids encoding measles virus H and F glycoproteins of either the Edmonston (EdH and EdF) or Ichinose (IcH and IcF) strain or with an empty vector and then inoculated with 5×10^6 RIU of Ac Δ 64/gp64/CALuc, Ac Δ 64/VSVG/CALuc, Ac Δ 64/CD46/CALuc, Ac Δ 64/SLAM/CALuc, or Ac Δ 64/SLAMcyto7/CALuc 24 h after transfection. Luciferase expression was determined 24 h after infection. The results shown are the means of three independent assays, and the error bars represent the standard deviations. RLU, relative light units. (C) Neutralization of ligand-directed gene targeting by antibodies specific for viral ligands. Ac Δ 64/gp64/CALuc, Ac Δ 64/CD46/CALuc, or Ac Δ 64/SLAMcyto7/CALuc (10^6 RIU) was preincubated with various concentrations of monoclonal antibodies specific for CD46 (M75) or SLAM (IPO-3) for 60 min at 37°C. Residual activity was determined by measurement of luciferase expression in BHK cells expressing the H and F glycoproteins of the Edmonston or Ichinose strain 24 h postinfection. The values are expressed as the relative percentages of expression. The results shown are the means of three independent assays, with the error bars representing the standard deviations.

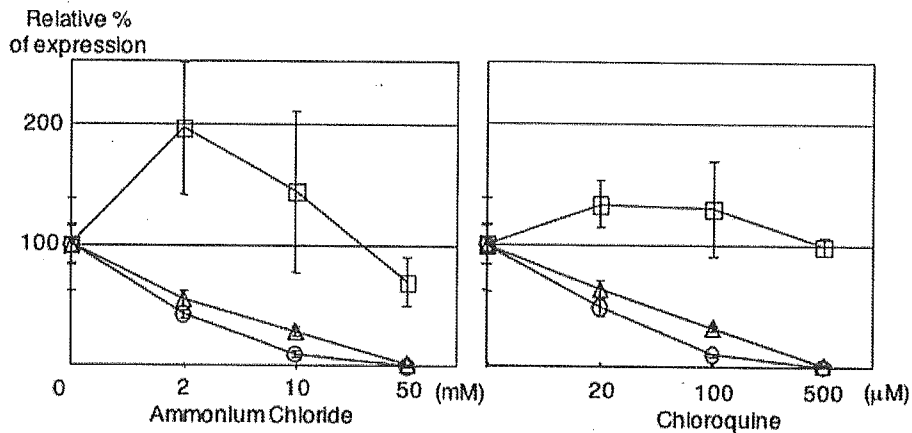


FIG. 6. Effects of lysosomotropic reagents on gene transduction of pseudotype baculoviruses. BHK cells (3×10^4) transfected with expression plasmids encoding the measles virus H and F glycoproteins of the Edmonston strain (EdH and EdF) were pretreated with various concentrations of ammonium chloride or chloroquine for 60 min. The cells were then inoculated with 10^6 RIU of AcΔ64/gp64/CALuc, AcΔ64/VSVG/CALuc, or AcΔ64/CD46/CALuc in the presence of the lysosomotropic reagents. Luciferase expression was determined 24 h postinfection. The results shown are the averages of three independent assays, with the error bars representing the standard deviations.

of the virus, determined by plaque formation in Sf9 cells, were similar. These results suggest that, while the polyhedrin promoter is sufficient to overexpress and incorporate a ligand of interest into the virion, this is not necessarily the best choice to maintain ligand function. The discrepancy between gp64 incorporation and the infectious titer may be attributed to a limited capacity to incorporate functional gp64 into particles and the timing of ligand gene activation. As the polyhedrin promoter is activated in the late stage of infection, baculoviruses budding

in the early stage of infection may be unable to incorporate the ligand expressed by the polyhedrin promoter. Use of the immediate-early promoter for ligand expression may improve the efficiency of incorporation into virus particles. In support of this possibility, the infectious titer of a recombinant AcMNPV in which the gp64 gene was replaced with the F gene from *Lymantria dispar* NPV under the control of the polyhedrin promoter was ~60-fold lower than that of a virus with the F gene under the control of the gp64 promoter (33).

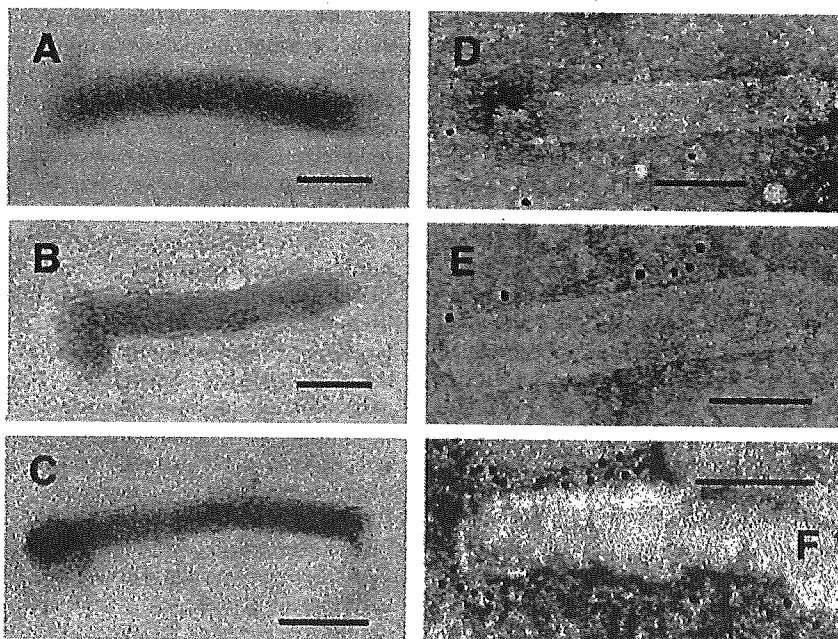


FIG. 7. Electron micrographs of pseudotype baculoviruses. Purified virus particles of wild-type AcMNPV (A), AcΔ64/VSVG/CALuc (B), and AcΔ64/CD46/CALuc (C) were examined by electron microscopy. A typical rod shape was visible in all of the pseudotype baculoviruses. The VSVG or CD46 proteins were observed on the surfaces of AcΔ64/VSVG/CALuc (E) and AcΔ64/CD46/CALuc (F) by immunoelectron microscopy using specific monoclonal antibodies against VSVG and CD46, respectively. AcMNPV treated with the monoclonal antibody against VSVG was used as a control (D). The bars on the panels represent 100 nm.

The replication competency in Sf9 cells of a gp64-null recombinant baculovirus could be rescued by incorporation of the VSVG gene (33, 34). We confirmed that the recombinant baculovirus deleted the gp64 gene and instead incorporated the VSVG gene under the control of the polyhedrin promoter, was replication competent in insect cells, and exhibited a high level of reporter gene transduction into 293T and BHK cells. The compatibility of VSVG in this system may result from similarities of the structural and functional characteristics of VSVG to those of gp64; both proteins are type I membrane glycoproteins, exist in trimeric complexes, and are capable of inducing membrane fusion at low pHs (6, 50, 54). Although the recombinant baculovirus in which gp64 is replaced with VSVG is able to replicate in insect cells and transduce foreign genes into a wide variety of mammalian cells, VSVG was suggested to recognize phosphatidylserine (58) or other ubiquitously expressed molecules other than phosphatidylserine (13) as a receptor(s), making it difficult to confer cell type specificity to gene delivery using VSVG pseudotype baculoviruses.

To establish ligand-directed gene delivery by pseudotype baculoviruses, it is necessary to propagate replication-deficient pseudotype baculoviruses possessing a ligand of interest in Sf9 cells, a replication-competent cell line stably expressing gp64. After three rounds of passage of the pseudotype virus in Sf9 cells, however, replication-competent revertant viruses that had incorporated the gp64 gene were generated. Sf9 cells were established by transfection of Sf9 with a plasmid encoding the gp64 gene lacking any flanking sequences. Southern blot analysis revealed that the gp64 gene was integrated into the genomes of the baculovirus revertants by nonhomologous recombination. Homologous recombination between the AcMNPV genome and either a transfer vector (32, 38), additional AcMNPVs (19), or *B. mori* NPV can occur in insect cells with high frequency (27, 28). In contrast, our data suggest that the revertant viruses were not generated by homologous recombination but by nonhomologous incorporation of the gp64 gene from the Sf9gp64 chromosome into the viral genome. Nonhomologous recombination between plasmid DNA and the baculovirus genome was previously reported upon cotransfection into insect cells (71). A gp64-null virus could propagate in Sf9^{Op1D} cell lines constitutively expressing OpNPV gp64 without incorporating the gp64 gene into the viral genome (33, 34). One possible explanation for our result may be the difference between the gp64 genes of OpNPV and AcMNPV. Although the Sf9gp64 cell line was established by sorting cells expressing a high level of gp64 on the cell surface, the expression level of gp64 might be lower than that of the Sf9^{Op1D} cell line, and the lower level of expression of gp64 might result in the selective amplification of revertants. To circumvent the high-frequency incorporation of foreign DNA into the baculoviral genome, we attempted the lipofection of recombinant bacmids into Sf9 cells instead of amplification of pseudotype baculoviruses in Sf9gp64. Although it is not possible to obtain a high pseudotype virus titer by this method, we can generate pure virus stocks without any contaminating replication-competent baculoviruses incorporating the gp64 gene. To prepare a high-titer stock of a replication-deficient pseudotype baculovirus carrying a foreign ligand, however, it is essential to propagate the pseudotype virus in Sf9 cells expressing gp64 without the transfection of plasmid DNA. Expression of gp64 by RNA trans-

fection, RNA viral vectors, or the RNA replicon system may be able to avoid the incorporation of gp64 DNA into the baculovirus genome.

We constructed a pseudotype baculovirus, Ac Δ 64/CD46/CALuc, bearing human CD46 in place of gp64 on viral particles. CD46 is a multifunctional protein involved in the infection of various microorganisms and the regulation of complement activation (10). CD46, also known as membrane cofactor protein, protects autologous cells from complement attack by serving as a cofactor for factor I-mediated inactivation of C3b and C4b, blocking the complement cascade at C3 activation (2). CD46 also serves as a receptor for human herpes virus 6 (56), group B adenovirus (17, 59, 62), bovine viral diarrhea virus (39), two bacterial strains (*Streptococcus pyogenes* and pathogenic *Neisseria*) (26, 46), and the Edmonston strain of measles virus (15, 42). In this study, we demonstrated that Ac Δ 64/CD46/CALuc exhibited specific reporter gene transfer to and expression in BHK cells expressing the measles virus H and F glycoproteins of the Edmonston strain but not those expressing the Ichinose strain glycoproteins that require SLAM as a receptor (67). Therefore, a CD46 pseudotype baculovirus bearing a suicide gene may be able to eliminate cells expressing pathogen ligands that utilize CD46 as a receptor. Furthermore, CD46 is frequently overexpressed on cancer cells, possibly serving as a mechanism to overcome lysis by complement (16). In support of the potential utility of this vector, the Edmonston strain of measles virus has a potent and selective oncolytic activity (52). CD46 pseudotype baculovirus may also be applicable for the clearance of tumor cells surviving oncolytic measles virus treatment.

Infections with Ac Δ 64/gp64/CALuc and Ac Δ 64/VSVG/CALuc, carrying gp64 and VSVG on the particles, respectively, could be decreased by treatment with ATPase inhibitors, chloroquine, or ammonium chloride, while Ac Δ 64/CD46/CALuc was resistant to treatment. This finding suggests the possibility of constructing a baculovirus vector capable of both targeting and modulating the viral entry pathway, as seen for VSV (7). This is different from influenza virus vectors, where acidic exposure within endosomes is critical for the dissociation of the matrix protein from the ribonucleocapsid (9, 37). In contrast to Ac Δ 64/CD46/CALuc, Ac Δ 64/SLAM/CALuc could not incorporate the full-length SLAM molecule into particles, preventing specific gene transduction. The inability to mediate gene delivery was not due to the absence of an interaction with specific targets, as Sf9 cells expressing SLAM induced membrane fusion with BHK cells expressing the H and F glycoproteins of either the Edmonston or Ichinose strain of measles virus (data not shown). The ligands incorporated efficiently into baculovirus particles, including gp64, VSVG, and CD46, all have relatively short cytoplasmic domains, measuring 7, 29, and 33 amino acids in length, respectively (49, 54, 60). In contrast, SLAM possesses a cytoplasmic tail of 77 amino acids (23). We therefore hypothesized that the length of the ligand cytoplasmic domain may be critical for efficient incorporation into baculovirus particles. Ac Δ 64/SLAMcyto7/CALuc, possessing a mutant SLAM molecule with the C-terminal 70 amino acids of the cytoplasmic domain deleted, efficiently incorporated the mutant SLAM into particles and exhibited specific gene delivery to BHK cells expressing the H and F glycoproteins of both measles virus strains. Although the mechanism by which SLAM

incorporation into baculovirus particles is enhanced by C-terminal truncation of the cytoplasmic domain is not known, one possibility might be a change in SLAM localization. The gp64 protein localizes to the cell surface but is excluded from lipid raft microdomains (72). As VSVG and CD46 also do not associate with lipid rafts (35, 57), proteins localized to lipid raft microdomains may be excluded during virus assembly and budding. Although the cellular localization of SLAM is not known, further studies will be necessary to clarify the relationship between cell surface localization and incorporation into baculovirus particles and to test the involvement of lipid raft microdomains in this process.

Mangor et al. demonstrated that gp64-null baculoviruses pseudotyped with VSVG were not morphologically distinguishable from budded wild-type AcMNPV particles (34). We confirmed that Ac Δ 64/VSVG/CALuc, as well as Ac Δ 64/CD46/CALuc and Ac Δ 64/GFP/CALuc, exhibited a morphology similar to that of AcMNPV. These results indicate that expression of gp64 is not required for the morphogenesis of a rod-shaped structure for budded AcMNPV particles. Immunogold labeling of Ac Δ 64/VSVG/CALuc and Ac Δ 64/CD46/CALuc revealed that the VSVG and CD46 proteins were incorporated into and distributed throughout the whole viral surface. These results are consistent with previous observations that VSVG fusion proteins were distributed throughout the stalk and head domains of baculovirus particles, in contrast to gp64, which was primarily localized in the head domain (11, 44).

In this study, we have demonstrated the capability for ligand-directed gene delivery by pseudotype baculoviruses in vitro. For future in vivo applications of baculovirus vectors for gene targeting to specific organs or virus-infected cells as a method of treatment of inherited or infectious diseases, it is imperative to exhaustively study the transcription of baculoviral genes in mammalian cells for certification of safety. In addition, further studies are needed to establish replication-competent cell lines capable of supporting the propagation of pseudotype viruses without the possibility of replication-competent virus breakthrough by incorporation of gp64 and to optimize the conditions necessary for the efficient incorporation of ligands into recombinant baculovirus particles.

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Involvement of the Toll-Like Receptor 9 Signaling Pathway in the Induction of Innate Immunity by Baculovirus†

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We have previously shown that mice inoculated intranasally with a wild-type baculovirus (*Autographa californica* nuclear polyhedrosis virus [AcNPV]) are protected from a lethal challenge by influenza virus. However, the precise mechanism of induction of this protective immune response by the AcNPV treatment remained unclear. Here we show that AcNPV activates immune cells via the Toll-like receptor 9 (TLR9)/MyD88-dependent signaling pathway. The production of inflammatory cytokines was severely reduced in peritoneal macrophages (PECs) and splenic CD11c⁺ dendritic cells (DCs) derived from mice deficient in MyD88 or TLR9 after cultivation with AcNPV. In contrast, a significant amount of alpha interferon (IFN- α) was still detectable in the PECs and DCs of these mice after stimulation with AcNPV, suggesting that a TLR9/MyD88-independent signaling pathway might also participate in the production of IFN- α by AcNPV. Since previous work showed that TLR9 ligands include bacterial DNA and certain oligonucleotides containing unmethylated CpG dinucleotides, we also examined the effect of baculoviral DNA on the induction of innate immunity. Transfection of the murine macrophage cell line RAW264.7 with baculoviral DNA resulted in the production of the inflammatory cytokine, while the removal of envelope glycoproteins from viral particles, UV irradiation of the virus, and pretreatment with purified baculovirus envelope proteins or endosomal maturation inhibitors diminished the induction of the immune response by AcNPV. Together, these results indicate that the internalization of viral DNA via membrane fusion mediated by the viral envelope glycoprotein, as well as endosomal maturation, which releases the viral genome into TLR9-expressing cellular compartments, is necessary for the induction of the innate immune response by AcNPV.

The baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) has long been used as a biopesticide and as an efficient tool for recombinant protein production in insect cells (39, 42). Subsequently, its efficacy for the delivery of high-level expression of foreign genes under the control of mammalian promoters in infected mammalian cells was also demonstrated (12, 26, 48). Since it causes no visible cytopathic effects, even at high titers, and does not replicate in mammalian cells (49), this baculovirus is now recognized as a useful viral vector, not only for the expression of foreign proteins in insect cells, but also for gene delivery to mammalian cells (4, 9, 12, 16, 26, 28, 37, 45, 48, 49, 53, 54).

AcNPV was also shown to be capable of stimulating interferon (IFN) production in mammalian cell lines and can confer protection from lethal encephalomyocarditis virus infections in mice (18). We demonstrated that intranasal inoculation with AcNPV induces a strong innate immune response and protects mice from a lethal challenge of influenza A and B viruses (1). Furthermore, inoculation with baculovirus induces the secretion of inflammatory cytokines, such as tumor necrosis factor

alpha (TNF- α), interleukin-6 (IL-6), and IL-12, in RAW264.7, a murine macrophage cell line. However, the precise mechanism of induction of the protective immune response by a pretreatment with AcNPV remained unclear.

Members of the IL-1 receptor/Toll-like receptor (TLR) superfamily are key mediators of innate and adaptive immunity (5). Toll, the first member of this superfamily to be identified, was initially discovered as a factor involved in dorsoventral axis formation in fly embryos and was later shown to participate in host defense mechanisms (38). A family of TLRs exists in mammals and has been shown to play an important role not only in the recognition of a wide variety of infectious pathogens and their products, but also in protection of the host from infections with pathogens. So far, 11 TLR family members and their corresponding ligands have been identified, with TLR1 being the only orphan receptor among them. Different TLRs have been shown to mediate immune responses to a variety of different pathogen-derived elements. For example, TLR4, TLR5, and TLR9 are essential for the recognition of lipopolysaccharides (LPS), bacterial flagellin, and bacterial DNA containing unmethylated CpG motifs, respectively (21, 24, 27, 46). TLR2 is implicated in the recognition of peptidoglycan (PGN) and lipopeptides (7, 13, 50, 57), while TLR6 can associate with TLR2 and recognize PGN and lipopeptides derived from mycoplasma (44). On the other hand, TLR3 has been shown to activate immune cells in response to virus-derived double-stranded RNA (6). Although synthetic imidazoquinoline com-

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† This study is dedicated to the memory of Ikuko Yanase.

pounds and guanosine analogs with antiviral activities have been shown to activate TLR7 and TLR8 (25, 36), it was recently demonstrated that single-stranded RNAs from RNA viruses are the natural ligands of these receptors (17, 23). The most recently identified TLR, termed TLR11, senses bacteria that cause infections of the bladder and kidney (60). In summary, TLRs recognize specific components derived from pathogens and activate a signaling cascade that causes proinflammatory cytokine production and subsequent immune responses.

TLRs share a common cytoplasmic Toll-IL-1 receptor (TIR) domain. MyD88, also a TIR domain-containing protein, associates with TLRs and acts as an adapter that recruits IL-1 receptor-associated kinase and TNF receptor-associated factor 6 (TRAF6) to TLRs. Macrophages isolated from MyD88-deficient mice fail to activate NF- κ B and Jun N-terminal protein kinase or to produce inflammatory cytokines in response to microbial components such as lipopeptides, LPS, and CpG-rich bacterial DNA (20, 52), indicating that MyD88 is a critical component in the signaling pathway that leads to the production of inflammatory cytokines.

Viruses are obligate intracellular parasites; accordingly, viral proteins synthesized in host cells bear modifications that reflect the identity and characteristics of the host. Therefore, viral particles do not display exclusively pathogen-associated molecular patterns. Although the mechanisms by which the innate immune response is induced by viral infection are poorly understood, there is increasing evidence suggesting that TLRs function to detect viruses and trigger inflammatory responses. For instance, respiratory syncytial virus and mouse mammary tumor virus activate innate immunity through TLR4 (22, 34, 47), which is a signaling receptor for LPS. Similarly, hemagglutinin from wild-type measles virus was reported to activate TLR2 (10), which also recognizes certain elements of gram-positive bacteria and fungi. Herpes simplex virus type 1 (HSV-1) and human cytomegalovirus have also been shown to recognize TLR2 (15, 35), while vaccinia virus encodes proteins containing amino acid sequences similar to the Toll/IL-1 receptor domain and inhibits IL-1-, IL-18-, and TLR4-mediated signal transduction (11).

It was recently shown that HSV-1 and -2, whose genomes contain abundant CpG motifs, can induce angiogenesis and a variety of diseases, including herpes stromal keratitis, that produce chronic inflammatory responses via a TLR9/MyD88-dependent signaling pathway (33, 40, 61). HSV-1 and -2 are also able to trigger alpha interferon (IFN- α) secretion from plasmacytoid dendritic cells through TLR9/MyD88-dependent signaling (33, 40). The TLR9-mediated recognition of HSV by immunocompetent cells suggests that this recognition pathway may be important for the recognition of other DNA viruses.

For this study, we characterized the innate immune response induced by AcNPV. Peritoneal macrophages and splenic CD11c⁺ dendritic cells obtained from TLR9 or MyD88 knockout mice exhibited severe reductions in proinflammatory cytokine production following stimulation with AcNPV, whereas a significant amount of IFN- α was still detectable in these cells. In addition, the frequency of CpG motifs in the AcNPV genome was similar to that of bacterial DNA and significantly higher than that of mammalian DNA. Furthermore, stimulation by AcNPV was eliminated by a treatment with inhibitors

of endosomal acidification. These results indicate that the internalization of viral AcNPV DNA via membrane fusion by envelope glycoproteins found in the endosome is required for the induction of a TLR9/MyD88-dependent innate immune response.

MATERIALS AND METHODS

Mice and cell culture. C57BL/6 mice were purchased from Clea Japan, Inc., Tokyo, Japan. MyD88-deficient (MyD88^{-/-}) mice were established as previously described (2) and backcrossed more than eight times with C57BL/6 mice. TLR9^{-/-} mice were generated as previously described (24). The mice were injected intraperitoneally with 2 ml of 4% thioglycolate (Sigma-Aldrich Co., St. Louis, Mo.), and cells were harvested 3 days later by peritoneal lavage. The mouse macrophage cell line RAW264.7 was purchased from Riken Cell Bank (Tsukuba, Japan) and maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS), 1.5 mM L-glutamine, 100 U of penicillin/ml, and 100 μ g of streptomycin/ml at 37°C in a 5% CO₂ humidified incubator.

Viruses and reagents. AcNPV was propagated in *Spodoptera frugiperda* (Sf-9) cells in Sf-900II insect medium supplemented with 10% (vol/vol) heat-inactivated FCS. A mutant baculovirus, AcNPV Δ 64, which lacks the gp64 envelope protein and possesses the green fluorescent protein gene under the control of the polyhedrin promoter in the gp64 gene locus, was generated (Y. Kitagawa et al., unpublished data). AcNPV and AcNPV Δ 64 were purified as previously described (1). The inactivation of AcNPV was performed with a Stratilinker 2400 (Stratagene, La Jolla, Calif.) using short-wavelength UV radiation (UVC, 254 nm) at a distance of 5 cm for 30 min on ice (1.6 \times 10⁴ mJ/cm²). The inactivation of infectivity was verified by a plaque assay with Sf-9 cells.

AcNPV DNA was isolated from the purified virions by a treatment with 10 mg of proteinase K (Sigma-Aldrich)/ml and 10% sodium dodecyl sulfate (SDS) in sterile phosphate-buffered saline (PBS) for 2 h at 55°C. The viral DNA was purified by phenol-chloroform-isoamyl alcohol extraction, precipitated at 12,000 \times g, and resuspended in sterile, endotoxin-free Tris-buffered saline. RNAs were removed by incubation with RNase A (10 mg/ml) (Wako Pure Chemical Industries, Osaka, Japan) for 1 h at 37°C, and the viral DNA was extracted as described above. The resultant DNA exhibited a single band by electrophoresis, and neither protein nor chromosomal DNA of insect cells was detected.

Phosphorothioate-stabilized mouse CpG (mCpG) oligodeoxynucleotides (ODN1668) (TCC-ATG-ACG-TTC-CTG-ATG-CT) and human CpG (hCpG) oligodeoxynucleotides (ODN2006) (TCG-TCG-TTT-TGT-CGT-TTT-GTC-GTT) were purchased from Invitrogen (Tokyo, Japan). Guanosine, 2'-deoxy-G, 8-bromo-G, 7-methyl-G, 7-allyl-8-oxo-G (loxoribine) was purchased from Invitrogen (San Diego, Calif.). LPS derived from *Salmonella enterica* serovar Minnesota (Re-595), PGN derived from *Staphylococcus aureus*, monodansylcadaverine (MDC), and chloroquine were purchased from Sigma-Aldrich. Bafilomycin A1 and ammonium chloride were purchased from Wako Pure Chemical Industries. An anti-p39 mouse monoclonal antibody was kindly provided by G. F. Rohrmann. The virus stocks and the other TLR ligands were free of endotoxin (<0.01 endotoxin units/ml), as determined by use of a Pyrodict endotoxin measure kit (Seikagaku Co., Tokyo, Japan).

Production of authentic and truncated forms of gp64 proteins. cDNAs encoding a deletion mutant of gp64 lacking the transmembrane region (gp64 Δ TM) as well as a wild-type version of gp64 were obtained by PCRs with AcNPV DNA as a template. The same 5' primer (5'-CATAAGCITATGGTAAAGCGCTATTGTTTATAT-3') was used to amplify the gp64 and gp64 Δ TM cDNAs, and the 3' primers were 5'-GATTCTAGAATATATTGCTATTACGGTTTCT-3' and 5'-GATTCTAGAATCGAAGTCAATTTAGCGCCAA-3', respectively. cDNAs were subcloned into HindIII and XbaI sites in pIB/V5-His (Invitrogen). The sequences of the recombinant plasmids, pIBgp64/V5-His and pIBgp64 Δ TM/V5-His, were confirmed by DNA sequencing. These plasmids were transfected into Sf-9 cells by the use of Unifector (B-Bridge International, Inc., San Jose, Calif.). After 3 days of incubation, the recombinant gp64 proteins were purified from cell lysates or supernatants by use of a column of nickel-nitrilotriacetic acid beads (QIAGEN, Valencia, Calif.). The protein concentrations were determined by use of a Micro BCA protein assay kit (Pierce, Rockford, Ill.). The recombinant proteins were analyzed by SDS-12.5% polyacrylamide gel electrophoresis (SDS-12.5% PAGE) under reducing conditions, stained with GelCord Blue staining reagent (Pierce), and detected by immunoblotting analysis with an antihexahistidine monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.).

Isolation of peritoneal cells and cytokine production. To evaluate cytokine production from macrophages in vitro, we seeded thioglycolate-elicited perito-

neal cells (PECs) into 96-well plates at a concentration of 2×10^5 cells/well and stimulated them with various doses of AcNPV and loxoribine. After 24 h of incubation, the culture supernatants were collected and analyzed for cytokine production. The concentrations of IL-12 p40 and IFN- α in culture supernatants were determined by enzyme-linked immunosorbent assays (ELISAs). ELISA kits for OptEIA mouse IL-12 p40 Set and mouse IFN- α were purchased from BD Pharmingen (San Diego, Calif.) and PBL Biomedical Laboratories (New Brunswick, N.J.), respectively. Total RNAs were isolated by the use of Sepazol-RNA I (Nacalai Tesque, Kyoto, Japan), electrophoresed, and transferred to nylon membranes. Hybridization was performed with the indicated cDNA probes as previously described (2). cDNA probes specific for IL-12 p40 were established as previously described (31). To determine the effects of infection with AcNPV on cytokine production, we seeded the mouse macrophage cell line RAW264.7 into six-well plates at a concentration of 10^6 cells/well and stimulated them with various TLR ligands, with or without endosomal inhibitors such as chloroquine, baflomycin A1, MDC, and ammonium chloride. For cell stimulation, AcNPV (5 μ g/ml), LPS (10 ng/ml), PGN (2.5 μ g/ml), and mCpG (200 ng/ml) were used.

Preparation of splenic dendritic cells and cytokine secretion. To prepare splenocytes containing dendritic cells (DCs), we cut spleen tissues into small fragments and incubated them with RPMI 1640 containing 400 U of collagenase (Wako)/ml and 15 μ g of DNase (Sigma-Aldrich)/ml at 37°C for 20 min. For the last 5 min, 5 mM EDTA was added, and single-cell suspensions were prepared after red blood cell lysis. CD11c⁺ cells were purified by magnetic cell sorting with anti-CD11c microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer's instructions and were used as splenic DCs. Enriched cells containing >90% CD11c⁺ cells were seeded into 96-well plates at a concentration of 10^5 cells/well and stimulated with various doses of AcNPV or loxoribine. Culture supernatants were collected, and the production of IL-12 p40 and IFN- α was determined by ELISAs.

Indirect immunofluorescence assay and flow cytometric analysis. 293T cells transfected with a plasmid encoding human TLR9 were dislodged with PBS containing 5 mM EDTA 48 h after transfection. The cells were incubated with PBS containing 2% FCS and an anti-Flag (M2) monoclonal antibody (1:1,000) (Santa Cruz Biotechnology) for 1 h at 4°C, washed twice with PBS containing 2% FCS, and further incubated with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (IgG) (Sigma-Aldrich) in PBS containing 2% FCS for 1 h at 4°C. The cells were then fixed with 4% paraformaldehyde for 20 min, and the surface expression of human TLR9 was observed by fluorescence microscopy (UFX-II microscope; Nikon, Tokyo, Japan). Intracellular staining was examined after permeabilization with 0.5% Triton X-100. Stained cells were also analyzed by flow cytometry with a FACSCalibur instrument (Becton Dickinson, San Jose, Calif.), and the data were analyzed with CellQuest software (Becton Dickinson).

NF- κ B-luciferase reporter gene assays with 293T cells. 293T cells were transfected with an NF- κ B-dependent luciferase reporter plasmid (pELAM-Luc) together with human TLR9 expression vectors by the use of Lipofectamine 2000 (Life Technologies, Grand Island, N.Y.). pELAM-Luc (kindly provided by D. T. Golenbock) contains a human E-selectin promoter introduced into the pGL3 reporter plasmid (Promega, Inc., Madison, Wis.). The human TLR9 expression vector (kindly provided by T. H. Chuang) consists of a preprotrypsin signal peptide and a Flag epitope tag followed by an in-frame human TLR9 cDNA sequence (14). At 24 h posttransfection, the cells were stimulated with hCpG DNA (10 μ g/ml) or AcNPV DNA (10 μ g/ml) for 24 h. The luciferase activity was determined as previously described (49) and calculated as the degree of induction compared with an untreated control.

Detection of AcNPV capsid protein in murine macrophage cells by Western blot analysis. RAW264.7 murine macrophage cells (10^6 cells/well) infected with AcNPV at a dose of 40 μ g/ml were washed extensively after 1 h of adsorption and harvested after 4 or 6 h of incubation. The cells were lysed in buffer containing 1% Triton X-100, 135 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1% glycerol, and protease inhibitor cocktail tablets (Roche Molecular Biochemicals, Mannheim, Germany). The lysed sample was separated by SDS-12.5% PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Tokyo, Japan). An anti-p39 mouse monoclonal antibody was used to detect the AcNPV capsid protein, which was visualized with the SuperSignal West Femto chemiluminescent substrate (Pierce).

RESULTS

Immune system activation by AcNPV is not mediated by viral envelope glycoprotein. It was previously reported that an IFN-stimulating preparation purified from Sf-9 cells infected with AcNPV exhibited IFN production both in vitro and in vivo

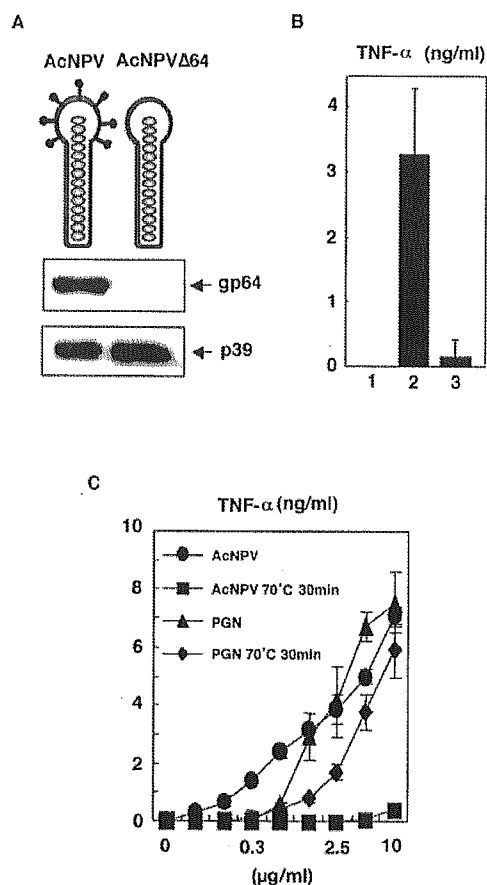


FIG. 1. Immune system activation of macrophages by heat-denatured or gp64-deficient AcNPV. (A) Purified particles of the mutant virus, AcNPV Δ 64, lack gp64, as assayed by immunoblotting. (B) The production of TNF- α in RAW264.7 cells (10^6 cells/well) inoculated with AcNPV (5 μ g/ml) (bar 2) or AcNPV Δ 64 (5 μ g/ml) (bar 3) was determined 24 h after inoculation by a sandwich ELISA. 1 is an uninfected control. Data are shown as means \pm SD. (C) AcNPV and PGN were incubated at 70°C for 30 min. Treated and untreated samples were inoculated into RAW264.7 cells (10^6 cells/well) and incubated for 24 h. The production of TNF- α was determined by a sandwich ELISA. Data are shown as means \pm SD.

and that induction was inhibited by monoclonal antibodies against the AcNPV envelope glycoprotein gp64 (18). To verify these observations, we constructed a mutant baculovirus lacking gp64, which we called AcNPV Δ 64, and examined its ability to stimulate an immune response in RAW264.7 cells, which are highly sensitive to TLR stimulation and respond by producing inflammatory cytokines at a level comparable to that observed in primary macrophages (1). The absence of gp64 in purified particles of AcNPV Δ 64 was confirmed by immunoblotting (Fig. 1A). The mutant virus lost the ability to induce TNF- α production in inoculated RAW264.7 cells (Fig. 1B), a result that is consistent with the previous observation that gp64 appears to play an important role in the induction of the immune response by AcNPV (18). Because some microbial products are known to induce cytokine production in macrophages, it was important to eliminate the possibility that contamination with microbial products contributed to the immune system

activation by AcNPV. Although the stimulation of macrophages by AcNPV was completely eliminated by incubation at 70°C for 30 min (Fig. 1C), stimulation by the bacterial components PGN and LPS was resistant to heat treatment (Fig. 1C and data not shown). These data indicate that the activation of macrophages by AcNPV is mediated by heat-labile viral components rather than by LPS and PGN.

To further verify the involvement of gp64 in immune system stimulation by baculovirus, we prepared expression plasmids encoding both wild-type gp64 and a C-terminally truncated gp64 protein (gp64ΔTM) with a C-terminal His₆ tag to allow for purification. Upon transfection of Sf9 cells, both recombinant proteins were detected, while gp64ΔTM was efficiently secreted into the culture supernatant (Fig. 2A). The protein from cells expressing gp64ΔTM was purified by column chromatography, producing a single band corresponding to gp64ΔTM and comparable to viral gp64 (Fig. 2B). We also tried to obtain the wild-type gp64 protein from the cell lysates but could not purify it to a homogeneous band (data not shown).

The activities of AcNPV, gp64ΔTM, and PGN on RAW264.7 cells were then examined. A dose-dependent induction of TNF-α and IL-6 was observed for RAW264.7 cells treated with AcNPV and PGN, whereas cytokine production was not observed for cells treated with gp64ΔTM (Fig. 2C). In addition, gp64ΔTM was not able to induce IFN-α production in RAW264.7 cells (Fig. 2D). Furthermore, the pretreatment of macrophage cells with gp64ΔTM inhibited immune system activation by AcNPV but had no effect on the activation by PGN (Fig. 2E), suggesting that the gp64ΔTM protein still retained some of the biological functions of the wild-type gp64 protein, at least in terms of its interaction with host cells. These results indicated that gp64 is an essential element of AcNPV-induced immune system activation in RAW264.7 cells but that it does not directly participate in the reaction. Viral components other than gp64 may be more directly involved in this process.

AcNPV induces inflammatory cytokine production through a MyD88/TLR9-dependent pathway. Immune cells from MyD88- or TLR-deficient mice are unresponsive to TLR ligands, as assayed by their levels of cytokine production (5). Therefore, we used PECs and splenic CD11c⁺ DCs obtained from MyD88- and TLR-deficient mice to determine whether or not the TLR signaling pathway is responsible for the activation by AcNPV. Thioglycolate-elicited PECs were isolated from wild-type, MyD88^{-/-}, TLR2^{-/-}, TLR4^{-/-}, and TLR9^{-/-} mice and examined by ELISA and Northern blot analysis for the induction of IL-12 following exposure to AcNPV. Wild-type macrophages inoculated with AcNPV produced large amounts of IL-12 in a dose-dependent manner, whereas MyD88- or TLR9-deficient macrophages had severely reduced IL-12 production (Fig. 3A). PECs from TLR2^{-/-} and TLR4^{-/-} mice produced IL-12 at wild-type levels in response to AcNPV (Fig. 3A).

Loxoribine is a potent inducer of cytokine production in macrophages and functions through a TLR7-dependent pathway (36). PECs from wild-type, TLR2^{-/-}, TLR4^{-/-}, and TLR9^{-/-} mice all produced IL-12 in response to loxoribine, whereas no IL-12 production was observed in PECs from MyD88^{-/-} mice (Fig. 3A). The transcription of IL-12 p40

mRNA was also impaired in MyD88- and TLR9-deficient macrophages stimulated with AcNPV (Fig. 3B). We further examined the response of splenic CD11c⁺ DCs to AcNPV and loxoribine. Wild-type and TLR4^{-/-} splenic CD11c⁺ DCs produced IL-12 in response to AcNPV in a dose-dependent manner, whereas the production of IL-12 was severely impaired in MyD88^{-/-} and TLR9^{-/-} mice (Fig. 3C). In response to loxoribine, splenic CD11c⁺ DCs from TLR4^{-/-} and TLR9^{-/-} mice exhibited higher IL-12 production levels than wild-type cells, whereas the production of IL-12 was completely inhibited in MyD88^{-/-} mice (Fig. 3C). These results indicate that AcNPV induces the production of inflammatory cytokines in immunocompetent cells through a MyD88/TLR9-dependent pathway.

AcNPV produces IFN-α through a MyD88/TLR9-independent pathway. IFNs are important mediators of the early host defense against various viral infections. Since AcNPV has also been shown to be a potent inducer of IFN-α (Fig. 2D) (18), we investigated whether IFN-α production induced by AcNPV is dependent on the MyD88 and TLR9 signaling pathways. Although IFN-α induction by the TLR9 ligand, CpG oligonucleotides, was completely abolished in PECs and splenic CD11c⁺ DCs derived from MyD88^{-/-} or TLR9^{-/-} mice (data not shown), IFN-α production in response to AcNPV was less impaired (Fig. 4A). This contrasted sharply with the complete loss of IL-12 production observed for these cells (Fig. 3). Macrophages from MyD88^{-/-} and TLR9^{-/-} mice exhibited a slight reduction in IFN-α and IFN-β mRNA transcription in response to AcNPV (Fig. 4B). These results indicate that AcNPV induces the production of inflammatory cytokines in immunocompetent cells through a MyD88/TLR9-dependent pathway, while other MyD88/TLR9-independent pathways are also involved in the production of IFNs.

AcNPV DNA stimulates immune system activation in macrophage cell lines. CpG motifs present in the genomes of many bacteria are unmethylated, whereas eukaryotic genomes are much more likely to undergo methylation. Previous work demonstrated that bacterial DNAs and certain oligonucleotides containing unmethylated CpG dinucleotides can stimulate PECs and DCs (19, 32). In addition, TLR9 is essential for the immune response to CpG-rich DNA, since TLR9-deficient mice are refractory to such stimulation (24). The frequency of bioactive CpG motifs in the AcNPV genome was similar to that observed for *Escherichia coli* and HSV DNAs (61) and significantly higher than that in murine and entomopoxvirus DNAs (Table 1).

To determine the methylation status of the AcNPV genome, we digested DNAs isolated from AcNPV, Sf-9 cells, *E. coli*, and 293T cells with the restriction enzyme HpaII, which cannot cleave when the cytosine adjacent to the cleavage site (CC↓GG) is methylated. While DNA isolated from 293T cells was refractory to HpaII digestion, DNAs from AcNPV, Sf-9 cells, and *E. coli* were sensitive to HpaII digestion, indicating that most of the CpG dinucleotides in AcNPV were unmethylated (Fig. 5A).

To determine the ability of AcNPV DNA to stimulate an immune response in vitro, we purified the viral DNA from virions. RAW264.7 cells were then treated with purified viral DNA or PGN with or without liposomes (Fig. 5B). The transfection of viral DNA with liposomes resulted in the production

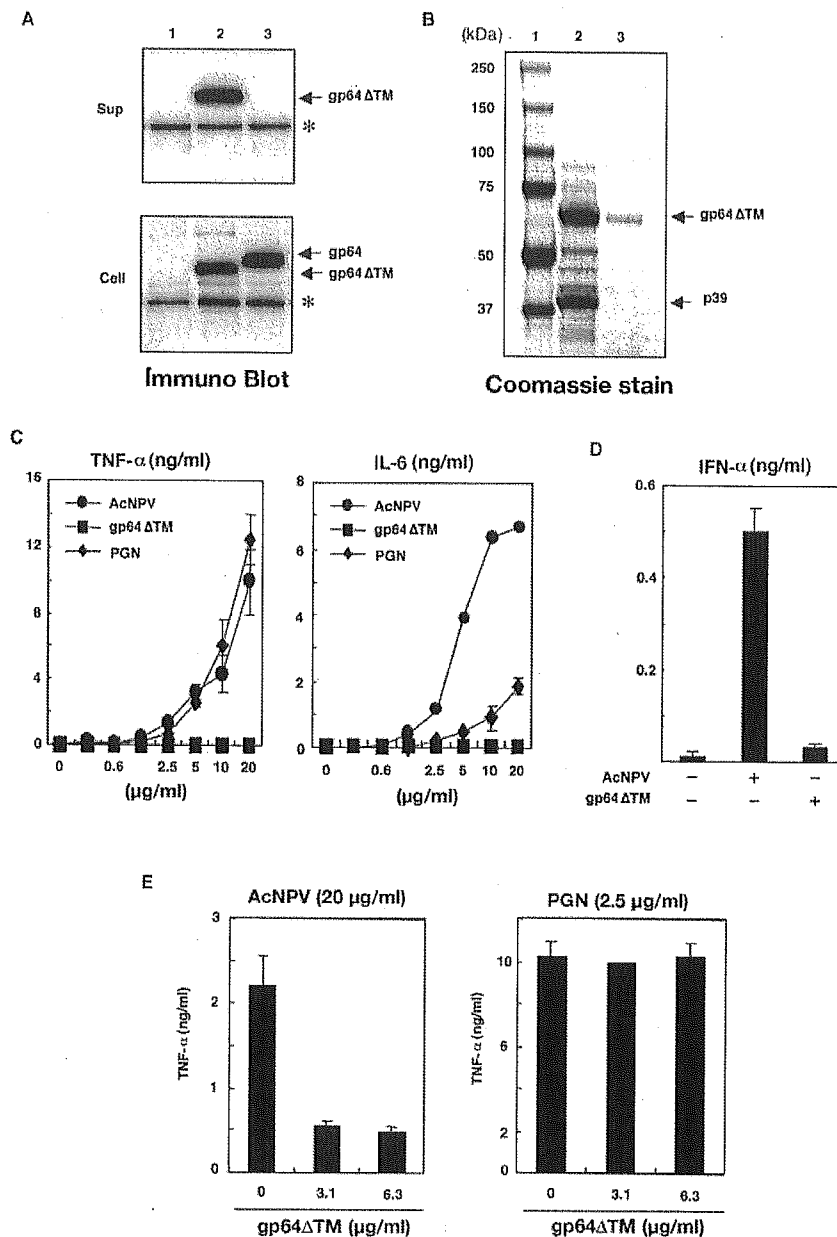


FIG. 2. Immune system activation by AcNPV in macrophages is not mediated by gp64. (A) Wild-type gp64 and a deletion mutant lacking the transmembrane region of the gp64 envelope protein (gp64ΔTM) were expressed in Sf-9 cells. Whole-cell lysates and culture supernatants were subjected to SDS-PAGE under reducing conditions and visualized by immunoblotting with an anti-hexahistidine monoclonal antibody. Lane 1, cells transfected with pIB/V5-His; lanes 2 and 3, cells transfected with pIBgp64ΔTM/V5-His and pIBgp64/V5-His, respectively. The heavy chains of the antibody are indicated by asterisks. (B) Purified AcNPV virions (lane 2) and gp64ΔTM (lane 3) were analyzed by SDS-PAGE and Coomassie blue staining. Lane 1, molecular mass markers. (C) Activation of mouse macrophage RAW264.7 cells (10^6 cells/well) treated with the indicated amounts of AcNPV or gp64ΔTM. The production of TNF- α and IL-6 in culture supernatants after 24 h of incubation was determined by sandwich ELISAs. PGN was used as a positive control. Data are shown as means \pm SD. (D) Production of IFN- α in RAW264.7 cells (10^6 cells/well) inoculated with AcNPV (5 μ g/ml) or gp64ΔTM (5 μ g/ml), as determined by a sandwich ELISA after 24 h of incubation. Data are shown as means \pm SD. (E) Production of TNF- α in RAW264.7 cells (10^6 cells/well) inoculated with AcNPV (20 μ g/ml) or PGN (2.5 μ g/ml), with or without a pretreatment with the indicated amounts of gp64ΔTM for 2 h at 37°C. After 24 h of incubation, the production of TNF- α in culture supernatants was determined by a sandwich ELISA. Data are shown as means \pm SD.

of TNF- α , but this effect was not observed in the absence of liposomes. The enhancement of TNF- α production by liposomes was not observed in cells treated with PGN, and the addition of liposomes alone did not elicit TNF- α production

(Fig. 5C). These results indicate that the internalization of viral DNA is necessary for the activation of the AcNPV-mediated TLR9 signaling pathway. Thus, the impaired immune system activation by AcNPVΔ64 in macrophages may result from a

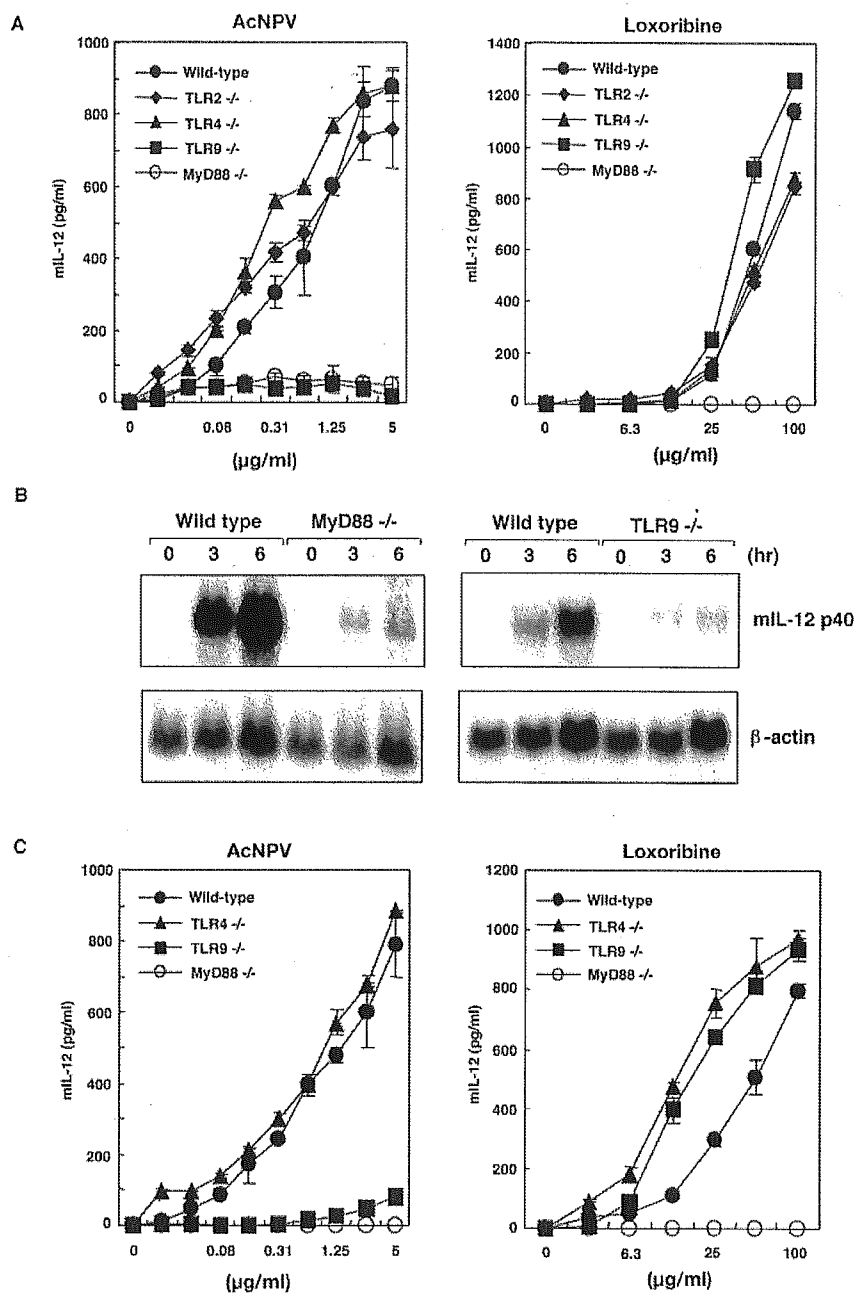


FIG. 3. AcNPV activates PECs and DCs in a MyD88/TLR9-dependent manner. (A) PECs (2×10^5 cells/well) from wild-type (C57BL/6) or MyD88-, TLR2-, TLR4-, or TLR9-deficient mice were stimulated with the indicated amounts of AcNPV or loxoribine. The production of IL-12 p40 in culture supernatants was measured by a sandwich ELISA. Data are shown as means \pm SD. (B) Northern blot analysis of murine macrophage cells stimulated with AcNPV. PECs (6×10^6 cells/well) from wild-type or MyD88- or TLR9-deficient mice were stimulated with AcNPV (10 μ g/ml) for the indicated times. Total RNAs were extracted and subjected to Northern blot analysis. (C) Splenic CD11c⁺ DCs were prepared from wild-type or MyD88-, TLR4-, or TLR9-deficient mice and enriched by magnetic cell sorting. Splenic DCs (10^5 cells/well) were stimulated with the indicated amounts of AcNPV or loxoribine for 24 h. The production of IL-12 p40 in supernatants was measured by a sandwich ELISA. Data are shown as means \pm SD.

failure to internalize viral DNA via gp64-mediated membrane fusion.

To further confirm that viral DNA activates the signaling pathway following internalization via gp64, we inactivated

AcNPV by UV irradiation and examined the production of TNF- α in RAW264.7 cells. UV irradiation diminished the AcNPV-mediated induction of TNF- α , but the addition of liposomes restored the activation (Fig. 5C). These results

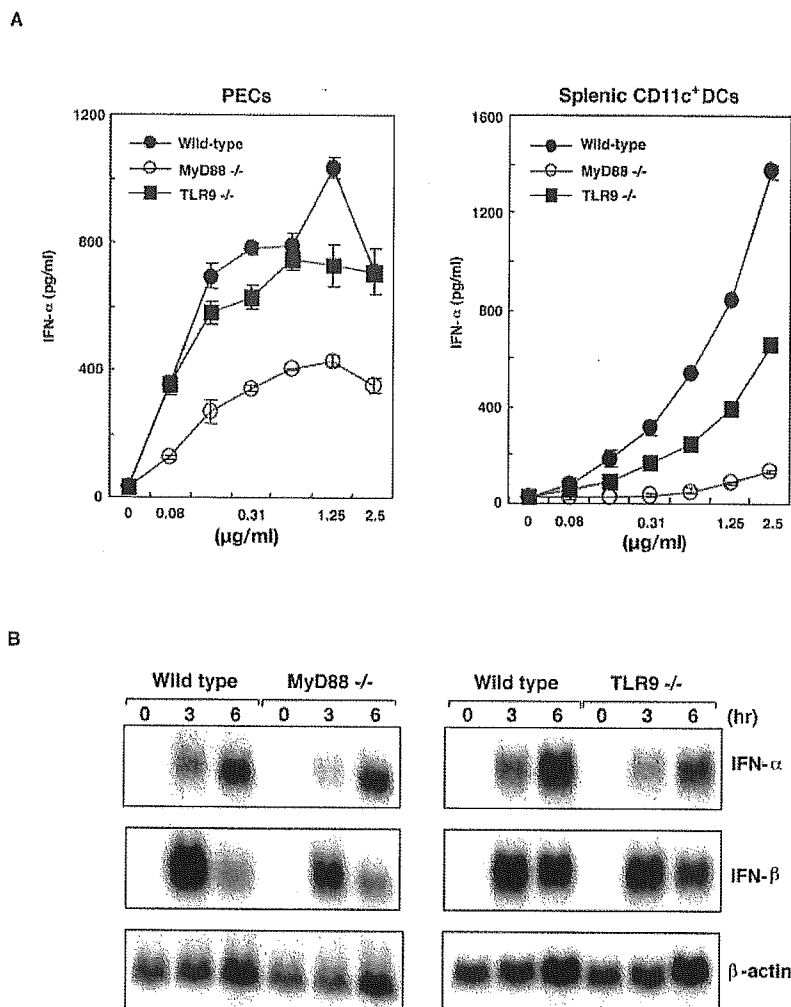


FIG. 4. IFN production by AcNPV is mediated by a MyD88/TLR9-independent process. (A) PECs (2×10^5 cells/well) and splenic CD11c⁺ DCs (1×10^5 cells/well) were prepared from wild-type or MyD88- or TLR9-deficient mice and stimulated with the indicated amounts of AcNPV or loxoribine for 24 h. The production of IFN- α in culture supernatants was measured by a sandwich ELISA. Data are shown as means \pm SD. (B) Northern blot analysis of murine macrophage cells stimulated with AcNPV. PECs (6×10^6 cells/well) from wild-type or MyD88- or TLR9-deficient mice were stimulated with AcNPV (10 μ g/ml) for the indicated times. Total RNAs were then extracted and subjected to Northern blot analysis.

suggest that the denaturation of gp64 by UV irradiation impaired the fusion capability of the envelope protein, thus inhibiting the internalization of viral DNA into the cell via membrane fusion.

AcNPV DNA induces NF- κ B activation through human TLR9. Signaling via TLRs occurs through the sequential recruitment of the adapter molecule MyD88 and the serine-threonine kinase IL-1 receptor-associated kinase, which leads to the activation of mitogen-activated protein kinases and the nuclear factor NF- κ B (51). To assess whether or not the expression of human TLR9 confers cellular responsiveness to AcNPV DNA, we transfected 293T cells with a human TLR9 expression plasmid and a pELAM luciferase reporter plasmid together with AcNPV or hCpG, which was used as a positive control (Fig. 6A). Although NF- κ B activation was not observed for cells transfected with undigested AcNPV DNA,

TABLE 1. CpG motif frequencies in AcNPV and other genomes^a

Motif	Frequency of appearance				
	<i>E. coli</i>	Mouse	HSV-1	AcNPV	AmEPV
CACGTT	1.30	0.11	0.76	0.90	0.17
AGCGTT	1.70	0.17	0.42	1.12	0.15
AACGTC	0.60	0.11	0.73	0.98	0.17
AGCGTC	1.30	0.15	0.85	0.85	0.15
GGCGTC	1.40	0.15	4.0	1.10	0.02
GGCGTT	2.50	0.15	1.51	1.37	0.10
Average	1.53	0.14	1.38	1.05	0.13

^a The frequency at which each CpG hexamer appeared in the *E. coli*, mouse, HSV-1, AcNPV, and *Amsacta moorei* entomopoxvirus genomes was determined by using published sequence data. The GenBank accession numbers for the complete genomes of AcNPV and AmEPV are NC 001623 and NC 002520, respectively. The complete genomes of *E. coli* K-12 and HSV-1 and mouse chromosome sequences were described previously (61).

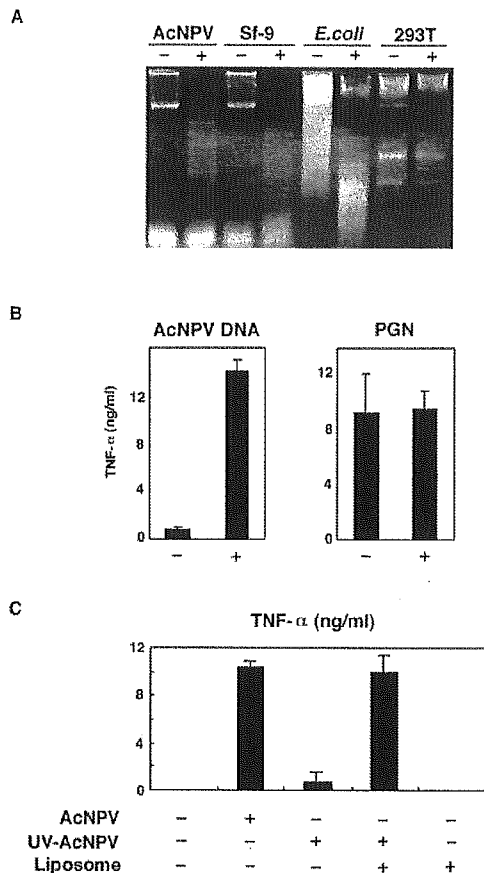


FIG. 5. Activation of mouse macrophage cell line by AcNPV DNA. (A) Methylation status of genomic DNA. Genomic DNAs obtained from AcNPV, Sf-9 cells, *E. coli*, and 293T cells were digested with the methylation-sensitive restriction enzyme HpaII. Undigested (-) and digested (+) samples were analyzed by agarose gel electrophoresis. (B) RAW264.7 cells (10^6 cells/well) were treated with AcNPV DNA (5 μ g/ml) or PGN (2.5 μ g/ml) in the absence (-) or presence (+) of liposomes for 24 h, and the production of TNF- α in culture supernatants was determined by a sandwich ELISA. Data are shown as means \pm SD. (C) Activation of RAW264.7 cells (10^6 cells/well) inoculated with untreated or UV-inactivated AcNPV (5 μ g/ml) in the presence or absence of liposomes was assessed by the production of TNF- α in culture supernatants. Data are shown as means \pm SD.

HindIII-digested viral DNA and hCpG exhibited significant NF- κ B activation, suggesting that undigested viral DNA is incapable of penetrating cells by transfection. No activation of NF- κ B was observed in 293T cells cotransfected with a human TLR2 or TLR4 expression plasmid when stimulated with digested AcNPV DNA (data not shown).

Recent work demonstrated that the endogenous expression of TLR3, TLR7, TLR8, and TLR9 was mainly detected in the cytoplasmic vesicles of macrophages (58). To examine the localization of transiently expressed TLR9, we transfected 293T cells with a TLR9 expression plasmid and examined TLR9 expression by immunofluorescence microscopy and cell sorting. The expression of TLR9 in the cytoplasm was three times higher than that at the cell surface (Fig. 6B and C). These results indicate that the introduction of AcNPV DNA into the

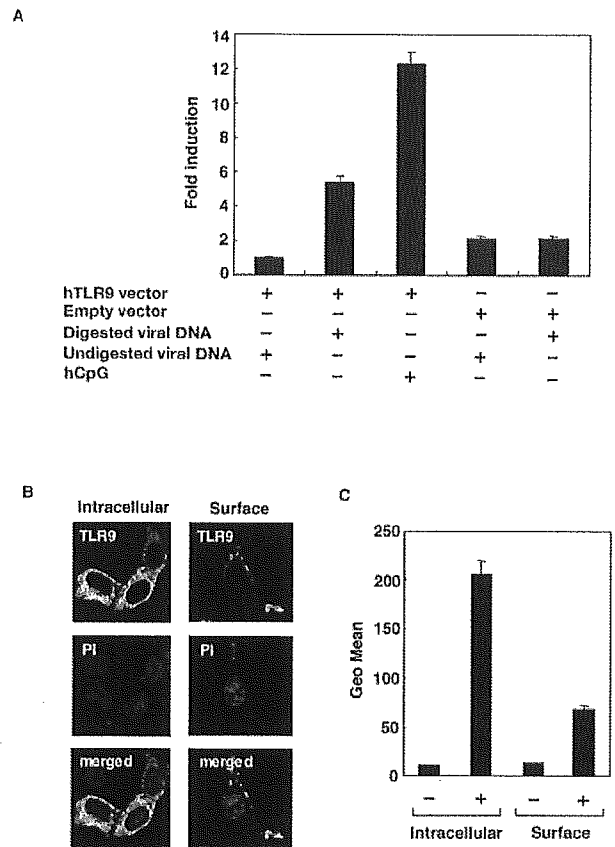


FIG. 6. AcNPV DNA induces NF- κ B activation through human TLR9. (A) 293T cells were transfected with an empty or human TLR9 expression vector together with a pELAM luciferase reporter plasmid. Twenty-four hours after transfection, the cells were stimulated with digested or undigested AcNPV DNA (10 μ g/ml). hCpG (10 μ g/ml) was used as a positive control. The luciferase activity was determined at 24 h posttransfection and expressed as the level of induction compared with that detected in cells transfected with the human TLR9 expression vector alone. Data are shown as means \pm SD. (B) Immunofluorescence micrographs of 293T cells transfected with an N-terminal Flag-tagged human TLR9 expression vector and stained with an anti-Flag (M2) monoclonal antibody. The intracellular (left) and cell surface (right) expression of TLR9 is shown. Nuclei were stained with propidium iodide (PI). Samples were observed by confocal microscopy. (C) The surface and intracellular expression of human TLR9 in 293T cells transfected with an N-terminal Flag-tagged human TLR9 expression vector (+) or an empty vector (-) and stained with an anti-Flag monoclonal antibody was examined by fluorescence-activated cell sorting.

cytoplasm is specifically detected by human TLR9 and results in the activation of NF- κ B.

AcNPV requires endosomal maturation to induce immune system activation in macrophages. To further explore the role of endocytosis in the signal transduction pathway triggered by AcNPV DNA, we examined the effect of endosomal maturation or acidification inhibitors. As shown in Fig. 7A, chloroquine was able to inhibit immune system activation of RAW264.7 cells treated with AcNPV and mCpG oligonucleotides in a dose-dependent manner, but no inhibition of LPS or PGN activation was observed. Other inhibitors of endoso-

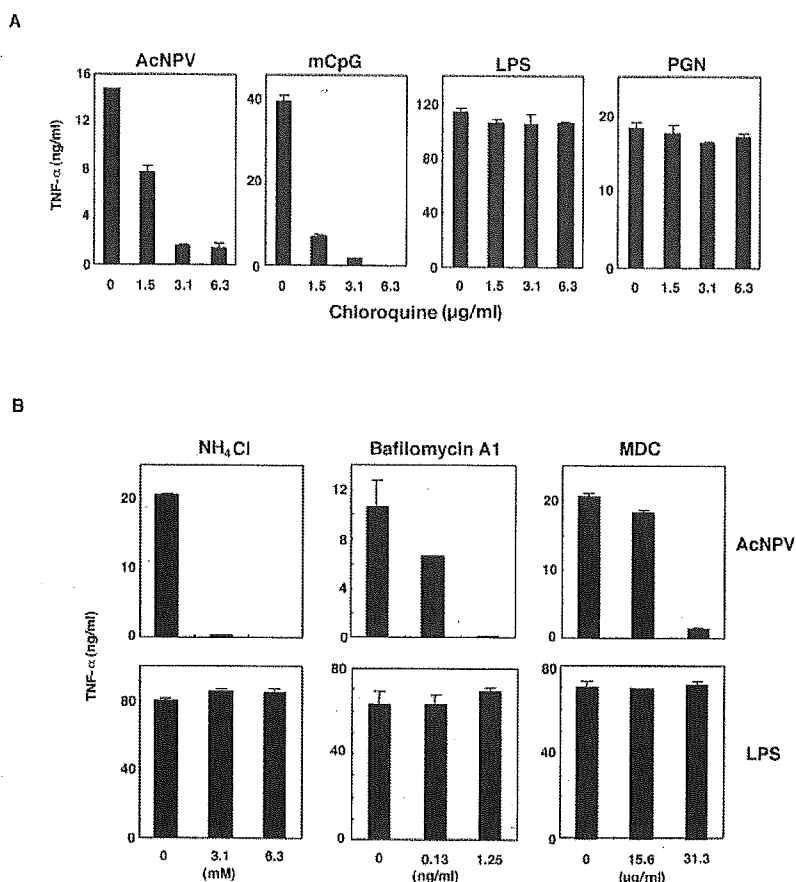


FIG. 7. AcNPV requires endosomal maturation to induce immune system activation in macrophages. (A) RAW264.7 cells (10^6 cells/well) were stimulated with AcNPV ($5 \mu\text{g/ml}$), mCpG (200 ng/ml), LPS (10 ng/ml), or PGN ($2.5 \mu\text{g/ml}$) at the indicated concentrations of chloroquine. After 24 h of incubation, the production of TNF- α in culture supernatants was determined by a sandwich ELISA. Chloroquine was added to the cells 2 h before stimulation. Data are shown as means \pm SD. (B) RAW264.7 cells (10^6 cells/well) were treated with AcNPV ($5 \mu\text{g/ml}$) or LPS (10 ng/ml) and with the indicated concentrations of endosomal maturation inhibitors. After 24 h of incubation, the production of TNF- α in culture supernatants was determined by a sandwich ELISA. The inhibitors were added to the cells 2 h before stimulation. Data are shown as means \pm SD.

mal maturation, such as ammonium chloride, bafilomycin A1, and MDC, inhibited AcNPV-induced, but not LPS-induced, immune system activation (Fig. 7B). Together with our other data, these results indicate that endosomal acidification and/or maturation is a key step in AcNPV-induced immune system activation via TLR9, a process that requires the release of the viral genome into TLR9-expressing cytoplasmic vesicles following the internalization of viral DNA by endocytosis through gp64-mediated membrane fusion.

AcNPV penetrates macrophages via the phagocytic pathway.

To further confirm that baculovirus was internalized into macrophages, we inoculated RAW264.7 cells with a recombinant baculovirus carrying a luciferase gene under the control of a mammalian promoter, AcCAGluc (49). As shown in Fig. 8A, the expression of luciferase was observed in 293T cells, but not RAW264.7 cells, that were infected with AcCAGluc. The viral capsid protein was clearly detected by immunoblotting for both 293T and RAW264.7 cells infected with AcNPV, but the protein level was greatly diminished in RAW264.7 cells by 6 h postinoculation, probably as a result of degradation (Fig. 8B).

These results suggest that baculovirus can penetrate into different cells via gp64-mediated endocytosis but that it translocates into different subcellular compartments in different cells. In 293T cells, the nucleocapsid was apparently able to reach the nucleus, where the reporter gene was efficiently transcribed following uncoating. However, in the immunocompetent RAW264.7 cells, the nucleocapsid appeared to have been trapped by the phagocytic pathway, and degraded viral DNA was then translocated into TLR9-expressing intracellular compartments (58).

DISCUSSION

We have previously demonstrated that intranasal inoculation with AcNPV induces a strong innate immune response that protects mice from a lethal challenge with influenza virus (1). The lungs of mice inoculated with AcNPV exhibited a marked infiltration of macrophages, which presumably inhibit the growth of influenza virus in the lung tissues. The baculovirus envelope glycoprotein gp64 contains mannose, fucose,

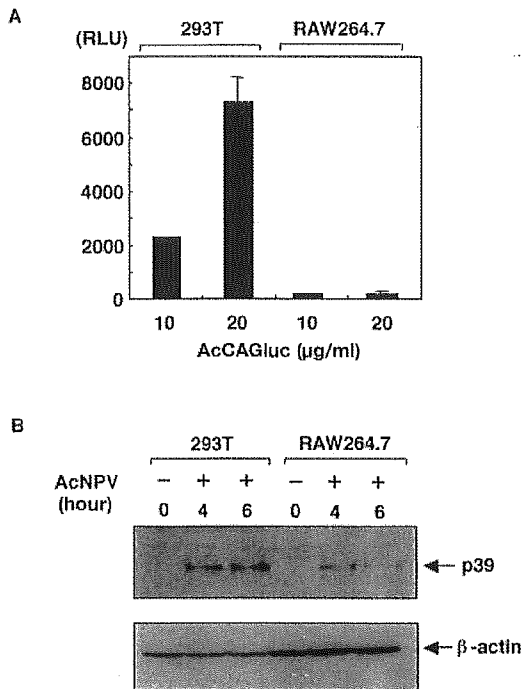


FIG. 8. AcNPV penetrates macrophages through the phagocytic pathway. (A) 293T and RAW264.7 cells (10^6 cells/well) were inoculated with a recombinant baculovirus possessing the luciferase gene under the control of the CAG promoter, AcCAGluc (49) (10 and 20 μ g/ml). Cells were harvested 24 h after infection, and relative luciferase activities were determined. (B) 293T and RAW264.7 cells (10^6 cells/well) were inoculated with AcCAGluc (40 μ g/ml), washed extensively after 1 h of adsorption, and harvested after 4 or 6 h of incubation. The presence of the p39 capsid protein in cells inoculated with AcNPV was determined by immunoblotting with an anti-p39 monoclonal antibody.

and *N*-acetyl-glucosamine modifications but no detectable galactose or terminal sialic acid residues (29). The mannose receptor (MR) recognizes a range of carbohydrates present on the surfaces and cell walls of microorganisms. MR is primarily expressed on macrophages and DCs and is involved in MR-mediated endocytosis and phagocytosis. In addition, MR plays a key role in host defense and the induction of innate immunity (8). Therefore, it is tempting to speculate that gp64 interacts with MR through its mannose modifications in macrophages and DCs of mice inoculated with AcNPV. However, our data contradict such a model; instead, we show that it is AcNPV DNA, not the gp64 glycoprotein, that induces immune system activation in a MyD88/TLR9-dependent manner.

Recently, it was shown that plasmacytoid DCs (pDCs) naturally produce IFN- α in response to viruses (30). HSV-1 and -2, whose genomes contain abundant CpG motifs, are able to induce the production of IFN- α in pDCs. The HSV-induced production of IFN- α in pDCs derived from MyD88- and TLR9-deficient mice was completely eliminated (33, 40). The recognition of the HSV genome by TLR9 was shown to be mediated by an endocytic pathway that can be inhibited by chloroquine or bafilomycin A1. In this study, we demonstrated that AcNPV induces proinflammatory cytokines through a

MyD88/TLR9-dependent signaling pathway, whereas signaling molecules other than MyD88 may participate in IFN- α production in response to AcNPV. Recently, MyD88-independent TLR signaling events involving TIR domain-containing adaptor inducing IFN- β (TRIF) were described (59). Therefore, it is possible that the TRIF pathway is one means by which AcNPV induces MyD88-independent IFN production. However, future studies are needed to clarify the precise mechanisms of this induction.

While UV irradiation of AcNPV abolishes its ability to stimulate an immune response, the addition of liposomes is able to restore this activity. UV-inactivated HSV is capable of inducing the production of IFN- α in pDCs (40), indicating that viral replication is not required for the HSV-induced immune response. In contrast, UV irradiation of AcNPV abolishes immune stimulation in macrophages, while internalization of the inactivated virus by liposomes restores the activity. These results, in conjunction with our data for AcNPV Δ 64, indicate that the AcNPV-induced production of cytokines in immunocompetent cells requires a fusion process mediated by gp64 that leads to internalization of the viral genome into the cells.

Recently, several viral envelope glycoproteins were shown to induce immune system activation through TLRs (10, 22, 34, 47). However, gp64 does not directly participate in a TLR-mediated immune response. TLR family members are expressed differentially at very low levels on the surfaces of different immune cells and appear to respond to different stimuli (43). A recent study indicated that LPS and CpG-rich DNA activate TLRs in distinct cellular compartments (3). Internalization and endosomal maturation are required for CpG-rich DNA to activate TLR9, but not for LPS to activate TLR4 on the plasma membrane. We showed here that the inhibition of endosomal maturation by a treatment with chloroquine abolishes the immune system activation of AcNPV in a dose-dependent manner. These results imply that immune system activation by AcNPV through TLR9 requires membrane fusion via gp64 as well as the liberation of the viral genome into cytoplasmic vesicles expressing TLR9.

Interestingly, Lund et al. demonstrated that the TLR7-mediated immune recognition of single-stranded RNAs from vesicular stomatitis virus and influenza virus requires endosomal acidification (41). The recognition of HSV-1 and HSV-2 viral DNAs through a TLR9/MyD88-dependent pathway in pDCs also requires endosomal acidification (40). These data indicate that TLR7 and TLR9 expressed in the endosomal or lysosomal compartments of immunocompetent cells recognize the viral genome entering the cell through receptor-mediated endocytosis or phagocytosis, leading to the secretion of inflammatory cytokines and IFNs. However, the precise mechanisms by which viral genomes translocate to TLR-expressing compartments are still unknown.

Since the first report on the immunostimulatory potential of bacterial DNA, which found that the main immunogenic fraction of mycobacterial lysates consists of genomic DNA (55, 56), substantial progress has been made towards understanding the immunostimulatory potency of CpG-rich DNA motifs, which are more common in bacteria than in vertebrates. For instance, TLR9 was shown to be responsible in vivo for immune system stimulation by oligodeoxynucleotides containing unmethylated CpG motifs (24). Like bacteria, AcNPV contains a significant

number of potentially bioactive CpG motifs. Interestingly, the frequency of CpG motifs in HSV DNA, which has been shown to be involved in the induction of angiogenesis in stromal keratitis (61), was similar to that in *E. coli* DNA. In contrast, the frequency of CpG motifs in the genome of an insect poxvirus was much lower than that for AcNPV (Table 1).

In conclusion, we have demonstrated that AcNPV has the ability to induce innate immune system activation through a MyD88/TLR9-dependent pathway. The molecular mechanisms of viral uptake, intracellular processing, and the induction of potent antiviral activity in immune cells require further investigation. However, the strong immune response induced by AcNPV makes it a promising candidate for a novel, adjuvant-containing vaccine vehicle against infectious diseases. In particular, our findings raise the possibility that AcNPV may be harnessed therapeutically to induce a host immune response against various infectious diseases caused by pathogens invading the respiratory tract.

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Hepatitis E Virus Transmission from Wild Boar Meat

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We investigated a case of hepatitis E acquired after persons ate wild boar meat. Genotype 3 hepatitis E virus (HEV) RNA was detected in both patient serum and wild boar meat. These findings provided direct evidence of zoonotic foodborne transmission of HEV from a wild boar to a human.

Hepatitis E virus (HEV), a causative agent of human hepatitis E, is a single-stranded positive-sense RNA virus recently classified as the sole member of the genus *Hepevirus* in the family *Hepeviridae* (1,2). HEV is transmitted primarily by the fecal-oral route through contaminated drinking water. However, recent studies have demonstrated that various animal species have serum antibodies to HEV, suggesting that hepatitis E is a zoonotic disease (3). In Japan, 4 hepatitis E cases have been linked directly to eating raw deer meat (4), and several cases of acute hepatitis E have been epidemiologically linked to eating undercooked pork liver or wild boar meat (5,6). These cases provide convincing evidence of zoonotic food-borne HEV transmission. We report direct evidence of HEV transmission from a wild boar to a human.

The Study

A 57-year-old woman came to Iizuka Hospital on March 12, 2005, with malaise and anorexia. Although she was a healthy hepatitis B virus carrier and negative for serologic markers of hepatitis A and C, testing upon admission showed elevated levels of liver enzymes (alanine aminotransferase 752 IU/L, aspartate aminotransferase 507 IU/L, and γ -glutamyl transpeptidase 225U/L). A serum sample collected on March 16 was positive for both immunoglobulin M (IgM) and IgG antibodies to HEV when tested by an antibody enzyme-linked immunosorbent assay using recombinant viruslike particles (7). This

led to the diagnosis of hepatitis E. The hepatitis was typical, acute, and self-limiting, and the patient recovered by the end of March.

The patient's husband traditionally hunted boar for food 3 or 4 times a year, and she had eaten boar meat on 2 occasions. With her husband, she ate the meat as part of a hot pot on December 28, 2004, 11 weeks before her illness, and again, grilled, on January 19, 2005, along with 10 other people (including her husband) 8 weeks before her illness. Disease did not develop in the other 10 people. Except for this wild boar meat, the patient had not eaten meat or liver from other wild animals. Since she had not traveled abroad in the past 30 years, transmission must have occurred in Japan. Two portions of meat from the wild boar (meats 1 and 2) eaten on December 28, 2004, and 1 portion from the other wild boar (meat 3) eaten on January 19, 2005, remained and were frozen.

Juice was obtained from the sliced meat by centrifugation at $10,000 \times g$ for 15 min. The supernatant was used for RNA extraction. A nested reverse transcription-polymerase chain reaction (RT-PCR) was conducted to amplify part of open reading frame 2 (ORF2), which corresponds to nucleotides (nt) 5939–6297 of the genotype 1 HEV genome (GenBank D10330), with external sense primer HEV-F1 (5'-TAYCGHAAAYCAAGGHTGGCG-3') and antisense primer HEV-R2 (5'-TGYTGTTTRTCR-TARTCCTG-3'). A nested PCR was conducted with internal sense primer HEV-F2 (5'-GGBGTBGCNGAGGAGG-AGGC-3') and internal antisense primer HEV-R1 (5'-CGACGAAATYAATTCTGTGCG-3'). This procedure allows amplification of HEV 1, 3, and 4 genotypes. A PCR product of 359 bp including the primer sequences was obtained from meat 3 by nested PCR. However, meats 1 and 2 were negative. HEV RNA was not detected in the patient's serum by the same amplification method. This may have resulted from an extremely small amount of RNA.

New primers for the nested RT-PCR were designed for a region within the 359 base region based on the meat 3 sequences, which corresponded to nt 5983–6243. The first PCR was performed with external sense primer HEV-WB-F1 (5'-ACCTCTGGCCTGGTAATGCT-3') and antisense primer HEV-WB-R2 (5'-GAGAAGCGTATCAGCAAGGT-3'). The nested PCR was performed with internal sense primer HEV-WB-F2 (5'-TATTCATGGCTCTCCTGTCA-3') and internal antisense primer HEV-WB-R1 (5'-ACAGTGTCAGAGTAATGCCT-3'). These primers allowed amplification of 281 nt, including the primer sequences from the patient serum collected on March 16, 2005. In contrast, meats 1 and 2 were negative with these new primers.

To further analyze the RNA in the patient serum and meat 3, RNA genomes encoding an entire ORF2 were

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amplified as overlapping segments, nucleotide sequences were determined, and phylogenetic analysis was carried out with avian HEV as an outgroup. Avian HEV is a causative agent of chicken hepatitis-splenomegaly syndrome (8). Two sequences, 1 from the patient (DQ079629) and the other from meat 3 (DQ079630), were classified into genotype 3 (Figure). Only 1 nt difference was observed in the 1,980 nt of the entire ORF2; the nucleotide sequence identity was 99.95%. The difference was not accompanied by any amino acid changes. These data demonstrated that HEV infection was transmitted from the wild boar meat to the patient on January 19, 2005.

Conclusions

Currently, deer, pig, and wild boar are suspected sources of foodborne zoonotic transmission of HEV in Japan, and genotypes 3 and 4 of HEV are believed to be indigenous (4–6,9,10). Direct evidence for transmission of genotype 3 HEV from animals to humans was observed in acute hepatitis in 4 persons who had eaten uncooked deer meat that contained $\approx 10^7$ copies of HEV RNA (4). However, the rare finding of HEV antibody-positive deer in Japan suggest that deer are not the major zoonotic reser-

voir of HEV in this country (11). In contrast, high antibody-positive rates in domestic pig and wild boar, including HEV genotypes 3 and 4, have been frequently detected, suggesting that persons who eat uncooked meat are at risk for infection with HEV (12,13). This report is the first to provide direct evidence of zoonotic foodborne genotype 3 HEV transmission from wild boar to a human.

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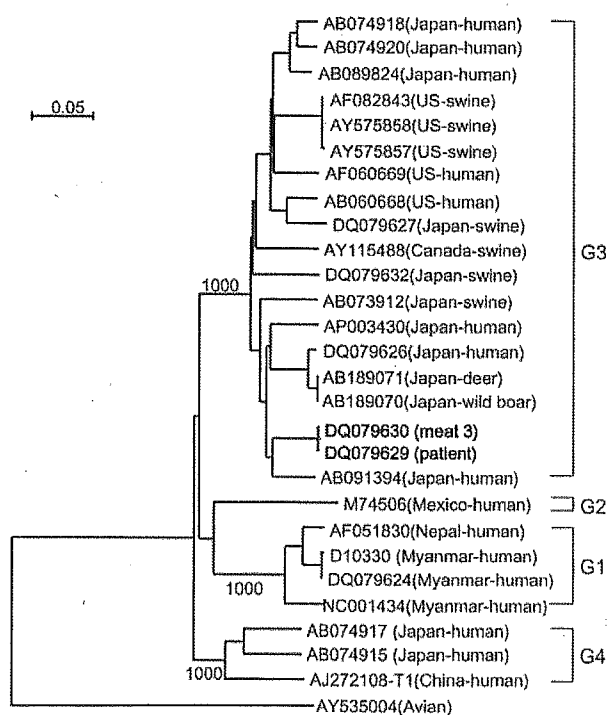


Figure. Phylogenetic tree of hepatitis E virus (HEV) reconstructed with avian HEV as an outgroup. Nucleotide sequences of the entire open reading frame 2 were analyzed by the neighbor-joining method. The bootstrap values correspond to 1,000 replications. The 2 nucleotide sequences characterized in this study are shown in **bold**. The horizontal scale bar at the top left indicates nucleotide substitutions per site.