INTRODUCTION

Virus infections often extensively induce characteristic changes in the morphology and motility of infected cells, such as rounding, filopodium, syncytium and migration, which trigger physical interactions between cells to facilitate virion spread and to promote virus virulence (Jouvenet et al., 2006; Sanderson et al., 1998; Sherer et al., 2007; Valderrama et al., 2006; Weir & Fisman, 2004).

Baculoviridae is a diverse family of viral pathogens that are mainly infectious to insects of the orders Lepidoptera, Diptera and Hymenoptera (Rohrmann, 2013). The baculovirus genome consists of a large, circular, dsDNA molecule that ranges in size from 80 to 180 kbp. Baculovirus nucleocapsids are rod shaped and are enclosed within a lipid envelope. In a typical baculovirus life cycle, two nucleocapsids are rod shaped and are enclosed within a lipid envelope. In a typical baculovirus life cycle, two

The GenBank/EMBL/DDBJ accession numbers for the rho1, rac and cdc42 sequences of S. litura are KJ547698, KJ547699 and KJ547700, respectively.

Baculovirus induction usually causes aggregation of host cells, as observed in Ld652Y cells that were infected with the archetype species of baculovirus, Autographa californica multiple nucleopolyhedrovirus (AcMNIV) (Griffiths et al., 1999), a group I alphabaculovirus. In addition, cell aggregation has been detected in several group II alphabaculovirus–host cell combinations including Spodoptera exigua multiple nucleopolyhedrovirus (SeMNIV)-infected Se301 cells (Pijlman et al., 2002), Helicoverpa armigera nucleopolyhedrovirus (HearNPV)-infected HzAM1 cells (Jakubowska et al., 2009) and Spodoptera litura nucleopolyhedrovirus (SplNPV)-infected SpLi-221 cells (Yu et al., 2008). To date, the underlying molecular mechanism and biological significance of baculovirus-induced cell aggregation remains to be determined.

Cell aggregation is the result of cell migration and direct cell–cell contact, which are associated with the reorganization of cellular actin (Briere & Yap, 2013; Friedl, 2004). The actin cytoskeleton is mainly manipulated by the Rho family of GTPases, which control signal transduction pathways that link membrane receptors to the cytoskeleton (Sit & Manser, 2011; Taylor et al., 2011). In mammalian cells, the Rho family of GTPases consists of 23 highly evolutionarily conserved intracellular signalling molecules (Colicelli, 2004), of which Rho, Rac and Cdc42 are the most studied members. Rho GTPases usually switch between active GTP-bound forms and inactive GDP-bound forms. The active forms can target actin polymerization to discrete sites on the plasma membrane, which induces membrane protrusions. Additionally, GTP-bound forms can interact with and activate downstream target proteins, such as ROCK (orthologue of Rok in mammalian cells), mDia and Pak, to regulate many cell processes, such as morphogenesis,
wound healing, cell–cell adhesion, metastasis and endosome trafficking (Fukata & Kaibuchi, 2001; Heasman & Ridley, 2008).

The movement pattern of a single cell can be classified into mesenchymal or amoeboid movement (Friedl, 2004). Mesenchymal movement is lamellipodium-dependent (Bergert et al., 2012; Friedl, 2004), whereas amoeboid movement is bleb-dependent (Bergert et al., 2012; Friedl, 2004; Otto et al., 2011). Three Rho GTPases are key regulators of cell–cell contact and E-cadherin-mediated cell–cell adhesion in many mammalian cells and fruit fly cells both in vitro and in vivo (Braga et al., 1997; Harden et al., 1999; Takaishi et al., 1997; Warner & Longmore, 2009; Yamada & Nelson, 2007). A previous study indicated that Rac can stimulate lamellipodium formation (Heasman & Ridley, 2008), whereas RhoA activation is essential for bleb formation (Bergert et al., 2012). Cdc42 is essential for filopodium formation (Heasman & Ridley, 2008), which helps cells explore the extracellular matrix and navigate to appropriate targets (Wood & Martin, 2002).

We report that SpltNPV infection induced amoeboid movement of SpLi-221 cells, and Rho1 (the orthologue of RhoA in Insecta) was activated in the infected cells. The Rho1/Rok (the orthologue of ROCK in Insecta), but not Cdc42 or Rac1, signalling pathway was essential for the cell aggregation that is induced by infection of baculoviruses, in at least one of their lineage. Additionally, inhibition of the Rho1 signalling pathway attenuated SpltNPV propagation in vitro, suggesting that Rho1 or the Rho1-dependent cell aggregation may facilitate SpltNPV spread.

RESULTS

SpltNPV infection induces SpLi-221 cell aggregation and is mediated by an amoeboid mechanism

Upon SpltNPV infection, SpLi-221 cells exhibited obvious cytopathic effects (CPEs), such as cell rounding and cell aggregation (Fig. 1a). The extent of cell aggregation observed in this study was first evaluated using an aggregation assay. As shown in Fig. 1(b), the infected cells aggregated at 24 and 48 h post-infection (p.i.). Significantly higher cell aggregation indices were observed in the infected cells (0.6 and 0.65 at 24 and 48 h p.i., respectively) when compared with the mock-infected cells (approx. −0.1 at both 24 and 48 h p.i.) (P<0.01). The negative aggregation index of the mock-infected cells may have been due to cell propagation, whereas the infected cells did not propagate because cell cycles were likely arrested. Baculoviruses can block the cell cycle in G2/M or S phases (Braunagel et al., 1998; Ikeda & Kobayashi, 1999; Saito et al., 2002; Zhou et al., 2004).

No significant differences in aggregation indices were observed between the infected cells at 24 and 48 h p.i., which suggests that the cell aggregation process was completed by 24 h p.i.

Scanning electron microscopy (SEM) analysis was performed to further investigate the morphology of the cell aggregation. As shown in Fig. 1(c), membrane blebs were observed on surfaces of SpltNPV-infected SpLi-221 cells at 24 h p.i. (black arrowheads). In contrast, the surfaces of mock-infected cells were smooth, and filopodia and lamellipodia were observed (white arrowhead and white arrow, respectively). This observation suggested that SpltNPV may induce amoeboid movement of SpLi-221 cells.

The life cycle of baculoviruses is temporally regulated, and each successive stage of viral replication is dependent upon the proper expression of genes during the preceding stage (Friesen, 1997). Baculovirus late gene expression is dependent upon the onset of viral genome replication (Guarino et al., 2002). To further investigate the roles of early genes, DNA replication and late genes in the baculovirus-induced cell aggregation, the effect of aphidicolin, an inhibitor of viral DNA replication, on cell aggregation was examined. Approximately 2.5 × 10^5 SpLi-221 cells were treated with 10 μg aphidicolin ml⁻¹ followed by SpltNPV infection. No obvious cell aggregation was observed in the aphidicolin-treated cells (Fig. 1d). The cell aggregation was significantly attenuated compared with the mock (DMSO)-treated cells (0.17 vs 0.62 at 24 h p.i.; Fig. 1e) (P<0.01), which suggests that viral DNA replication or late viral gene expression may contribute to the cell aggregation process.

Characterization of rho1, cdc42 and rac cDNAs from SpLi-221 cells

Cell aggregation that is induced by viral infection requires cell migration and direct cell–cell contact. The Rho family of GTPases, including Rho1, Rac1 and Cdc42, are key regulators of cell migration and cell–cell adhesion in mammalian and fruit fly cells (Braga et al., 1997; Harden et al., 1999; Takaishi et al., 1997; Yamada & Nelson, 2007). In this study, nucleotide sequences of cDNAs of Spodoptera litura rho1, rac and cdc42 were first identified by gene fishing and RACE. The conserved regions among orthologues of rho1, cdc42 and rac from Spodoptera frugiperda, Bombyx mori and Drosophila melanogaster were used to design degenerate primers. PCR amplification was performed to acquire the conserved regions of target genes in SpLi-221 cells, and 3’ RACE and 5’ RACE were performed to acquire the complete gene cassettes. The ORFs of S. litura rho1, rac and cdc42 genes are 579 nt, 579 nt and 576 nt, which encode 192 aa, 192 aa and 191 aa, respectively.

The predicted amino acid sequences of the S. litura Rho1, Rac and Cdc42 genes were aligned with orthologues from Homo sapiens and several selected insects that belong to orders Diptera, Hymenoptera, Coleoptera and Homoptera. The amino acid sequence percentage identities were calculated using AlignX (Invitrogen). The percentages of sequence identity for these selected proteins ranged from

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80 to 100 % (data not shown), which reflected the high conservation of Rh1, Rac and Cdc42.

SplitNPV-induced cell aggregation is Rh1-dependent

To investigate the roles of Rh1, Cdc42 and Rac in SplitNPV-induced cell aggregation, RNA interference (RNAi) was applied to knock down target gene expression. The SpLi-221 cells were first transfected with the dsRNAs that corresponded to S. litura rho1, cdc42 and rac genes. EGFP dsRNA was used as a non-specific control. After 24 h, the cells were infected with SplitNPV. As shown in Fig. 2(a), at 24 h p.i., the silencing of Rh1 expression strongly reduced the extent of cell aggregation. In contrast, marked cell aggregation was observed in the cells that were treated with Rac, Cdc42 or EGFP dsRNAs. A significant difference in the aggregation index (one-way ANOVA, \( \text{LSD}_{0.01} \)) was detected between the Rh1 dsRNA-treated cells (−0.02) and the EGFP dsRNA-treated cells (0.58) (Fig. 2b). No significant differences were observed in the cell aggregation indices among the Rac (0.54), Cdc42 (0.58) and EGFP dsRNA-treated cells (one-way ANOVA, \( \text{LSD}_{0.05} \)) (Fig. 2b). The efficiency of RNAi was confirmed by reverse transcriptase PCR (RT-PCR) and Western blotting. Both mRNA transcription and protein expression levels of rho1, rac and cdc42 were markedly knocked down in the corresponding dsRNA-treated cells compared with the EGFP dsRNA-treated cells (Fig. 2c, d). These results indicate that the SplitNPV-induced cell aggregation is Rh1-dependent, whereas the closely related Rac and Cdc42 are not responsible for this process.

These results indicate that Rh1 may be activated upon viral infection. To test this possibility, a pull-down assay was performed on lysates from SplitNPV-infected SpLi-221
cells using Rho-binding domain-bound agarose beads. As expected, the amount of Rho1-GTP increased approximately 5.8-fold in the infected cells compared with the mock-infected cells (Fig. 2e).

Two highly similar rac genes have been identified in the Drosophila genome: rac1 and rac2 (Hakeda-Suzuki et al., 2002). A third gene, mtl, encodes a closely related protein that is structurally similar to both Cdc42 and Rac1; however, this gene functions like Rac1 or Rac2 (Hakeda-Suzuki et al., 2002). In the Rac-knockdown cells, other Racs may compensate for the function of S. litura Rac. To confirm whether Racs were essential for SpltNPV-induced cell aggregation, recombinant AcMNPV viruses expressing dominant isoforms of Rac and Rho1 under the D.

Fig. 2. SpltNPV-induced cell aggregation is Rho1-dependent. (a) A morphology study of the dsRNA-treated SpLi-221 cells at 24 h p.i. (m.o.i. = 5 TCID50 per cell). Bars, 20 μm. (b) Aggregation index analyses were performed at 24 h p.i. to calculate the extent of cell aggregation in the dsRNA-treated SpLi-221 cells. Results are mean ± SD values. Statistical analyses were performed using one-way ANOVA and LSD tests to compare the Rho1, Rac and Cdc42 dsRNA-transfected cells with the EGFP (control) dsRNA-transfected cells. (c, d) The respective transcription (c) and protein expression (d) levels of rho1, rac and cdc42 were detected by RT-PCR and Western blotting analyses at 24 h p.i. (m.o.i. = 5 TCID50 per cell) to confirm that these genes were successfully silenced by the corresponding RNAi treatment. (e) Western blotting analyses were performed to detect the amounts of Rho1-GTP in the mock- and SpltNPV-infected SpLi-221 cells at 24 h p.i. (m.o.i. = 5 TCID50 per cell). Densitometry was performed using Quantity One image software (Bio-Rad). dsEGFP (control), dsRho1, dsRac and dsCdc42 indicate the EGFP, Rho1, Rac and Cdc42 dsRNA-treated cells, respectively. The figure shows one representative set of Western blot analyses out of three independent experiments.
melanogaster heat-shock protein 70 promoter, vRacN17 and vRho1N19, were constructed (Fig. 3a). A previous study demonstrated that the overexpression of dominant-negative isoforms, RacN17 and Rho1N19, could overwhelm the background activation of Rac1 and Rho1, respectively (Hakeda-Suzuki et al., 2002). In addition, as Rho1 was found to be essential for SpLi-221 cell aggregation in an RNAi assay (Fig. 2a, b), vRho1N19 was used as a marker or a positive control to confirm the superinfection assay was reliable. SpLi-221 cells were first infected with SpltNPV at an m.o.i. of 5 TCID50 per cell for 2 h and were then superinfected with vRacN17, vRho1N19 or vAcWT (a wild-type AcMNPV) at an m.o.i. of 5 TCID50 per cell for 1 h. vRho1N19 was used as a positive control and vAcWT was used as a negative control. As shown in Fig. 3(b), vRho1N19 inhibited the SpltNPV-induced cell aggregation. Neither vRacN17 nor vAcWT had this capability. The SpltNPV-infected cells aggregated normally; however, the vAcWT-infected cells underwent apoptosis because SpLi-221 cells were non-permissive to AcMNPV, which is consistent with a previous study (Lin et al., 2010). The aggregation indices of the vRho1N19-superinfected cells (0.1) and the vAcWT-superinfected cells (0.49) were significantly different (one-way ANOVA, LSD0.01) (Fig. 3c). No significant differences were observed between the SpltNPV/vAcWT- and SpltNPV-infected cells (one-way ANOVA, LSD0.05) (Fig. 3c), which suggested that AcMNPV infection did not inhibit the SpltNPV-induced cell aggregation. These results indicate that the SpltNPV-induced cell aggregation is Racs-independent.

**Fig. 3.** SpltNPV-induced cell aggregation is not Racs-dependent. (a) The schematic shows the genes that were cloned into the lacZ locus of bMON14272 to generate vRho1N19 and vRacN17. The inserted genes are shown in each bacmid. (b) The morphologies of SpltNPV- and vAcWT-infected SpLi-221 cells and SpltNPV/vAcWT-, SpltNPV/vRho1N19- and SpltNPV/vRacN17-superinfected SpLi-221 cells at 24 h p.i. (m.o.i.=5 TCID50 per cell). Bars, 20 μm. (c) Aggregation index analyses were performed at 24 h p.i. to quantify the extent of cell aggregation in the SpltNPV-infected and SpltNPV/vAcWT- (control), SpltNPV/vRho1N19 (positive control)- and SpltNPV/vRacN17-superinfected SpLi-221 cells. Results are mean ± SD values. Statistical analyses were performed using one-way ANOVA and LSD tests to compare the SpltNPV-infected, SpltNPV/vRho1N19- and SpltNPV/vRacN17-superinfected SpLi-221 cells with SpltNPV/vAcWT-superinfected SpLi-221 cells.
**SpltNPV-induced cell aggregation is Rho1/Rok-dependent**

Multiple effectors are downstream from the Rho1 signalling pathway in mammalian cells (Bishop & Hall, 2000). The serine/threonine protein kinase Rok is the most extensively studied effector in the Rho family. The inhibitor of Rok, Y-27632, was used to investigate whether the SpltNPV-induced cell aggregation was dependent on the Rho1/Rok signalling pathway. Approximately 2.5 × 10^5 SpLi-221 cells were treated with 250 μM Y-27632 24 h prior to SpltNPV infection (m.o.i. = 5 TCID<sub>50</sub> per cell), and this treatment was continued throughout the infection. As shown in Fig. 4(a), the extent of cell aggregation in the Y-27632-treated cells was attenuated. The aggregation index of the Y-27632-treated cells was 0.17 and was significantly lower than that of the water-treated cells (0.68) (Fig. 4b). Therefore, the SpltNPV-induced cell aggregation is Rho1/Rok-signalling-dependent.

**SpltNPV production and bleb formation in SpLi-221 cells is markedly impaired by Rho1 dsRNA treatment in vitro**

Previous studies have demonstrated that RhoA activation facilitates viral infection; however, RhoA can restrict viral replication in certain types of infection. In respiratory syncytial virus (RSV) infection, syncytium formation and maturation of RSV filamentous virions require RhoA activation (Gower et al., 2005). RhoA activation inhibits human immunodeficiency virus (HIV) replication in vitro (Wang et al., 2000). Proteomic studies have indicated that Rho1 is associated with BVs of AcMNPV and HearNPV (Hou et al., 2013; Wang et al., 2010). To investigate whether the Rho1-dependent cell aggregation had any effect on baculovirus infection, BV production was determined. Rho1 dsRNA-treated SpLi-221 cells were infected with SpltNPV at either an m.o.i. of 5 TCID<sub>50</sub> per cell (to initially infect most of the cells) or an m.o.i. of 0.5 TCID<sub>50</sub> per cell (to initially infect less than 30% of the cells). At the higher m.o.i., no significant differences in BV production were observed at 120 h p.i. between the Rho1 dsRNA-treated cells and the EGFP dsRNA-treated cells (P>0.05) (Fig. 5a), and occlusion bodies (OBs) appeared in the Rho1 dsRNA-treated cells (Fig. 5b). However, at the lower m.o.i., Rho1 dsRNA treatment decreased BV production by approximately 40% compared with the EGFP dsRNA treatment at 120 h p.i. (P<0.05) (Fig. 5a). These results suggest that the SpltNPV-induced cell aggregation may benefit SpltNPV spread. In the early phase of baculovirus infection, nucleocapsids bud out of the plasma membrane to form BVs; therefore, it is possible that the bleb formation was due to budding. To rule out this possibility, SpltNPV-infected SpLi-221 cells were treated with Rho1 or EGFP dsRNA. These cells were observed using SEM. In the EGFP dsRNA-treated cells, blebs were observed at 24 h p.i. (Fig. 5c, white arrowheads), whereas no bleb formation was observed in the Rho1 dsRNA-treated cells. Because BVs were produced in both cases, we argue that the SpltNPV-induced blebs are Rho1-dependent and not the result of BV budding.

**DISCUSSION**

Cell aggregation has been observed in many virus-infected vertebrate systems, such as adenovirus-infected A549 cells (Leland & Ginocchio, 2007), vaccinia virus-infected BS-C-1 cells (Doceul et al., 2010) and tiger frog virus-infected ZF-4 cells (Luo et al., 2009). However, the biological significance and molecular basis of vertebrate and invertebrate virus-induced cell aggregation are poorly understood. To the best of our knowledge, this is the first report that the Rho1 signalling pathway is activated and necessary for cell aggregation in an invertebrate virus-cell system, and that bleb-driven cell migration occurs in SpltNPV-infected insect cells. In addition, inhibiting cell aggregation by silencing the Rho1 signalling pathway slightly decreased SpltNPV spread in vitro.

Blebs and lamellipodia are features of amoeboid movement and mesenchymal movement, respectively (Bergert et al., 2012; Friedl, 2004; Otto et al., 2011). In this study, SEM revealed that blebs appeared on the surfaces of SpltNPV-infected SpLi-221 cells, which suggests that the SpltNPV-infected cells migrate with amoeboid movement. Recent studies have suggested that bleb-based cell migration is a common feature of cell migration; bleb-driven migration produces faster migration speeds than the lamellipodium-driven process (Croft & Olson, 2008). Therefore, our data...
suggest that increased migration after virus infection is a possibility, but further experiments are needed to firmly establish whether this is the case or not.

A previous study indicated that the early and late viral protein SpltNPV-IAP4 is required for SpltNPV-induced cell aggregation (Yu et al., 2008). However, many factors may contribute to baculovirus-induced cell aggregation, including viral DNA replication and certain viral proteins. After the SpltNPV-infected SpLi-221 cells were treated with the viral DNA replication inhibitor aphidicolin, cell aggregation, which had appeared in the DMSO-treated cells, was not observed. Baculovirus late gene expression requires viral DNA replication (Guarino et al., 2002); therefore, our results suggest that viral DNA replication or late viral proteins are essential for the aggregation of infected SpLi-221 cells and early viral proteins such as SpltNPV-IAP4 are not sufficient to induce cell aggregation. Certain reports have pointed out that insect cells such as Se301 cells tend to aggregate at pH 7.0 (Jakubowska et al., 2009). However, in our research, the pH of the medium was pH 6.5. More importantly, the SpltNPV-induced cell aggregation could be inhibited by Rho1 dsRNA-treatment, while the infected cells aggregated obviously after EGFP dsRNA-treatment. As the pH condition was the same in both RNAi treatments, the aggregation phenomenon studied here did not result from change of pH.

Using RNAi and dominant-negative experiments, we found that Rho1, but not Cdc42 or Rac1, was responsible for the cell aggregation. Rok is a downstream effector of Rho1. Treatment with Y-27632 markedly reduced the cell aggregation. During amoeboid movement in mammalian cells, RhoA signalling promotes actin-myosin contractility through ROCK (Croft & Olson, 2008). In addition, we observed that Y-27632 inhibited SeMNPV-induced aggregation of host Se301 cells in vitro (data not shown). Therefore, we argue that the Rho1/Rok signalling pathway plays a pivotal role in cell aggregation at least in the group II baculovirus-insect cell system.

We found that Rho1 was activated in SpLi-221 cells during SpltNPV infection. Certain viruses, such as hepatitis B virus, can activate RhoA via the viral protein HBx (Feng et al., 2011). It is unclear whether the SpltNPV-encoded protein can directly induce Rho1 activation. A recent study indicated that XIAP and cIAP1 can mediate RhoA activation in mammalian cells (Hornburger et al., 2014). Since silencing of SpltNPV-IAP4 or Rho1 inhibits SpltNPV-induced cell aggregation and orthologues of the SpltNPV-IAP4, XIAP and cIAP1, can mediate RhoA activation, it would be interesting to investigate whether the SpltNPV-IAP4 mediates cell aggregation via Rho1 signalling. Alternatively, SpltNPV may indirectly induce Rho1 activation. The PI3K/AKT signalling pathway, which is activated

Fig. 5. SpltNPV spread and bleb formation of SpLi-221 cells is impaired by Rho1 dsRNA treatment. (a) BV production assay was performed for the dsRNA-treated SpLi-221 cells that were infected with SpltNPV at an m.o.i. of 5 or 0.5 TCID50 per cell at 120 h p.i. Results are mean ± sd values (*P<0.05, Student’s t-test). (b) Optical microscopy observations of OB formation in dsRNA-treated SpLi-221 cells at 48 and 72 h p.i. (m.o.i.=5 TCID50 per cell). Bars, 20 μm. dsEGFP (control) and dsRho1 indicate the EGFP and Rho1 dsRNA-treated cells, respectively. (c) SEM results for the dsRNA-treated SpLi-221 cells at 24 h p.i. dsEGFP (control) and dsRho1 indicate the EGFP dsRNA- and Rho1 dsRNA-treated SpLi-221 cells, respectively. Blebs are indicated by white arrowheads. Bars, 10 μm.

(a) dsEGFP/ dsRho1

(b) dsEGFP/ dsRho1

(c) dsEGFP/ dsRho1
by baculovirus infection, can induce RhoA activation in certain mammalian cell types (Liu et al., 2013; Xiao et al., 2009).

In the process of cadherin-mediated cell–cell adhesion, Rho1 is required to facilitate the establishment and maturation of cell–cell contact (Braga et al., 1997; Takaishi et al., 1997; Yamada & Nelson, 2007). Additionally, RhoA activation is essential for bleb formation in amoeboid movement (Bergert et al., 2012). Therefore, it is reasonable to infer that SpltNPV-induced cell aggregation shares similar mechanisms with Rho1/Rok pathway-dependent cell–cell contact and amoeboid movement.

Rac1 is required for cell–cell contact establishment via the Arp2/3 complex (Yamada & Nelson, 2007), and Cdc42 is required to regulate amoeboid movement via the Arp2/3 complex and myosin light chain 2 (MLC2) in certain mammalian cell types (Gadea et al., 2008). The Arp2/3 complex mediates the branching of filamentosous actin and is regulated by the Wiskott-Aldrich syndrome protein (Oda & Eto, 2013). MLC2 provides actomyosin contractility, which is required for amoeboid movement and cell–cell contact formation (Bergert et al., 2012; Yamada & Nelson, 2007). Racs and Cdc42 may be required for baculovirus-induced cell aggregation. However, according to our research, Racs and Cdc42 are not essential for this process. A Wiskott-Aldrich syndrome protein-like baculoviral protein, Pp78/83, has been demonstrated to be an activator of the Arp2/3 complex (Goley et al., 2006). In addition, ROCK can activate MLC2 (Amano et al., 1996). Therefore, in baculovirus-infected cells, the function of Rac and Cdc42 may not be required and may be replaced by the function of Rok and Pp78/83.

Virus-induced CPEs trigger physical interactions between cells and usually facilitate virion spread and virus virulence (Jouvenet et al., 2006; Sanderson et al., 1998; Sherer et al., 2007; Valderrama et al., 2006; Weir & Fisman, 2004). Upon SpLiNPV infection at high m.o.i., the silencing of Rho1 did not affect SpLiNPV replication in SpLi-221 cells. However, when SpLi-221 cells were infected by SpLiNPV at low m.o.i., the titre of progeny virions from Rho1 dsRNA-treated cells decreased by approximately 40 % at 120 h p.i. Although this decrease was only statistically significant when the titres were compared using fold change instead of absolute value, our result still implies that Rho1 and the Rho1-dependent cell aggregation may play a role in the spread efficiency of baculovirus in vitro. The absence of an obvious effect of Rho1 on SpLiNPV propagation in vitro may be due to the inability of our SpLiNPV-SpLi-221 combination to magnify such a subtle difference. Therefore, to better uncover the biological significance of baculovirus-induced cell aggregation, further studies should pay attention to other systems, such as SeMNPV-Se301 cells and HearNPV-HzAM1 cells, where the aggregation effect is also observed. On the other hand, cell aggregation can be considered as an enhancement of cell–cell contact. Therefore, the cell aggregate, which was observed in vitro, may not bind to form cell clusters in vivo. Thus, it may be possible that the cell aggregation in vitro reflects an enhancement of some kind of cell–cell contact in vivo. A previous study indicated that there are some phenoloxidases that can impair baculovirus BVs in insect haemolymph (Shelby & Popham, 2006). And in mammalian cells, a study pointed out that when cells were treated with antiretroviral drugs the cell-to-cell spread of HIV was much more efficient than the cell-free spread (Sigal et al., 2011). Therefore, if the contact among larva such as haemocytes is increased, cell-to-cell spread may benefit the spread of baculovirus in vivo.

**METHODS**

**Cells, insect larvae and viruses.** The *S. litura* cell line TUAT-SpLi221 (SpLi-221) (Yanase et al., 1998) was donated by Dr. Zihui Su (JT Biohistory Research Hall, Osaka, Japan). The *S. exigua* cell line Se301 (Hara et al., 1995) was donated by Professor Just M. Vlak (Wageningen University, the Netherlands). Both cell lines were grown in TNN-FH medium (pH 6.5) (Hink, 1970), which was supplemented with 10 % FBS (Gibco), penicillin (100 μg ml⁻¹) and streptomycin (30 μg ml⁻¹) at 27 °C. The *S. litura* and *S. exigua* larvae were reared on an artificial diet at 27 °C under a 14/10 h light/dark cycle and constant humidity (60 %) (Li et al., 2002). The SpLiNPV G2 strain was isolated from the ZSU strain (Fang et al., 2001). SpLiNPV OBs were purified from infected *S. litura* larvae as described previously (Fang et al., 2001). BV stocks were harvested from SpLiNPV-infected SpLi-221 cells. In these cells, BV titres were determined using a TCID50 end point dilution assay (O’Reilly et al., 1992). The appearance of OBs in cells indicated positive infection, and these cells were counted at 7 days p.i. The SeMNPV US1 strain was propagated in *S. exigua* larvae and in Se301 cells as described by Gelernter & Federici (1986). Time zero was defined as the time when the viral inoculum was added. Aphidicolin, an inhibitor of viral DNA replication, and Y-27632, an inhibitor of Rok, were purchased from Sigma-Aldrich.

**Aggregation assay.** The SpLi-221 cells (2.5 × 10⁵) were seeded into 12-well plates. After 2 h of cell attachment onto the bottoms of the plates, the extent of cell convergence was approximately 5 %. SpLi-221 cells were infected with SpLiNPV at an m.o.i. of 5 TCID₅₀ per cell. For RNAi or drug inhibition assays, the cells were first treated with dsRNA (Rho1, Rac, Cdc42 or EGFP dsRNA) or drugs (DMSO, aphidicolin, Y-27632, water). At least two photographs were taken using an ECLIPSE TE2000 U microscope (Nikon) and the numbers of single cells and cell clusters on the photographs were counted at different time points. Each treatment was performed with at least three biological repeats, then the extent of cell aggregation of each biological repeat was calculated and data were analysed with a t-test or one-way ANOVA and LSD test. In this study, three or more cells contacting each other were considered a cluster, whereas two contacted cells were considered two single cells because daughter cells are grouped in the final stage of mitosis. The extent of cell aggregation was calculated according to the following formula, which is similar to previous studies (Gebbink et al., 1993; Inohara & Raz, 1995): aggregation index = 1 − Nt/Nc. Nc (control) and Nt (test) represent the number of single cells plus clusters pre- and post-infection, respectively.

**SEM.** Approximately 2.5 × 10⁵ SpLi-221 cells were seeded into 12-well culture plates. The cells attached evenly onto a piece of coverslip within the well, and the cell convergence was approximately 5 %. Before SpLiNPV infection at an m.o.i. of 5 TCID₅₀ per cell, SpLi-221 cells were treated with or without dsRNA (Rho1 or EGFP dsRNA). The cells on the coverslip were fixed, dehydrated, dried and coated with platinum at different time point as described by Lehmann et al.
RT-PCR analysis. SpI-221 cells were harvested at different time points p.i. Total RNAs were isolated using an RNeasy Mini kit (Qiagen) and were treated with the RNase-free DNase Set (Qiagen) to digest contaminating DNAs. RT-PCR was performed using an iScript cDNA Synthesis kit (Bio-Rad). Briefly, first-strand cDNAs were synthesized using 1 μg of total RNA as a template. After PCR amplification of the cDNAs using gene-specific primers, the PCR products were analysed by electrophoresis using 1% agarose gel. The sequences of the primer pairs that were used for the RT-PCR analyses were as follows: SpI-221-rho-1 (5′-AAATCTGGCTGCATTGTCG-3′), SpI-221-rho-2 (5′-TTACACGAGGACACCTAGCTTCT-3′), SpI-221-rac-1 (5′-GGTATTGGTGGAGGAGGTGTCG-3′), SpI-221-rac-2 (5′-CCGTTTACACGACCCTTGCTTGCT-3′), SpI-221-cdc42-1 (5′-TTGTATGATGGAGGATGTCG-3′), SpI-221-cdc42-2 (5′-CGGATTCGCGAACTTGCTTGCTG-3′), actin-2 (5′-GGTACCCCTGCGTTGGGATGAC-3′).

Western blotting. Cell samples were lysed using RIPA buffer [25 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1% SDS] (Thermo Scientific). The total protein concentration was determined using a BCA Protein Assay kit (Thermo Scientific). The protein samples were incubated at 70°C for 10 min, subjected to electrophoresis in 10% NuPAGE Novex Bistris Gels with MES SDS running buffer, and transferred onto PVDF membranes (Millipore) according to the manufacturers’ protocols. Western blotting was performed according to standard procedures, as described by Sambrook & Russell (2001). The primary polyclonal antibodies anti-RhoA (human, 1:1000), anti-Rac1 (human, 1:1000) and anti-Cdc42 (human, 1:400) were purchased from Proteintech. The mouse monoclonal anti-actin antibody was purchased from Abcam. HRP-conjugated anti-rabbit IgG (1:5000) and anti-mouse IgG (1:5000) were purchased from CWBio. Signal visualisation was accomplished using an enhanced chemiluminescence system (ECL; Amersham Biosciences).

Rho1 activation assay. Approximately 5 × 10^6 SpI-221 cells were infected with SpltNPV at an m.o.i. of 5 TCID₅₀ per cell. At different time points p.i., the cells were washed with ice-cold TBS [20 mM Tris/HCl (pH 7.5) and 150 mM NaCl] and were analysed using a Rho Activation Assay kit (Millipore). Briefly, GTP-bound Rho1 was precipitated using Rho-binding-domain-bound agarose beads, and the amount of Rho1-GTP was determined by Western blotting using anti-RhoA (Proteintech; 1:1000). A densitometry analysis was performed using Quantity One® image software (Bio-Rad).

Construction of recombinant viruses. The hsp70 promoter has been proven to be able to drive exogenous protein expression efficiently in SpI-221 cells via the Bac-to-Bac® Baculovirus Expression System (Invitrogen) technology (Lin et al., 2010). The hsp70 promoter, also known as heat shock promoter, is an autoregulatory promoter that remains unexpressed until the temperature is increased above normal range. It is then induced and expressed at higher levels to help cells cope with the heat stress. Thus, it is widely used for the expression of proteins of interest in insect cells, such as SpI-221 cells, which are commonly used for the production of baculovirus proteins. The expression of hsp70 promoter allows for tight control of protein expression, ensuring that the protein is synthesized only when needed, which is beneficial for the production of recombinant proteins.
to generate pUC18-Rho1N19 and pUC18-RacN19. The mutagenic primer pairs rho1-N19-U (5'-GGGGTACCATGGTGGATCCGCGGTCGG-AAAATAATATTGATGCGCTCGGACGCGGTTGTTATAAAAAAAAAAT- TGCCCTGCTAC-3')/rho1-N19-D (5'-GGGGTACCATGGTGGATCCGCGGTCGG-AAAATAATATTGATGCGCTCGGACGCGGTTGTTATAAAAAAAAAAT- TGCCCTGCTAC-3') and rac-N17-U (5'-GGGGTACCATGGTGGATCCGCGGTCGG-AAAATAATATTGATGCGCTCGGACGCGGTTGTTATAAAAAAAAAAT- TGCCCTGCTAC-3') and rac-N17-D (5'-GGGGTACCATGGTGGATCCGCGGTCGG-AAAATAATATTGATGCGCTCGGACGCGGTTGTTATAAAAAAAAAAT- TGCCCTGCTAC-3') were used. pUC18-Rho1N19 and pUC18-RacN19 were subsequently digested using BamHI and KpnI, and the resulting fragments of HSP70-Rho1N19-SV40 and HSP70-RacN17-SV40 were cloned into pFB1-PH-PG (Wu et al., 2006) to generate the donor plasmids pFB1-Rho1N19-PG and pFB1-RacN17-PG, respectively. Then, the two donor plasmids were transformed into DH10Bac (Invitrogen) to generate vRho1N19 and vRacN17 as described previously (Wu et al., 2006). All of the constructs were verified by PCR analysis and DNA sequencing.

SpLi-221 cells (2.5 × 10⁴) were infected with SpLTNPV (m.o.i. = 5 TCID₅₀ per cell) for 2 h and superinfected with WT AcMMPV vAcWT (Wu et al., 2006) to induce Rho1N19 and RacN17 expression. Three hours later, the infected cells were heat-shocked at 42 °C for 30 min to induce Rho1N19 and RacN17 expression.

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