Role of G protein and protein kinase signalling in influenza virus budding in MDCK cells

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Recently, we have shown that influenza virus budding in MDCK cells is regulated by metabolic inhibitors of ATP and ATP analogues (Hui & Nayak, Virology 290, 329–341, 2001). In this report, we demonstrate that G protein signalling stimulators such as sodium fluoride, aluminium fluoride, compound 48/80 and mastoparan stimulated the budding and release of influenza virus. In contrast, G protein signalling blockers such as suramin and NF023 inhibited virus budding. Furthermore, in filter-grown lysophosphatidylcholine-permeabilized virus-infected MDCK cells, membrane-impermeable GTP analogues, such as guanosine 5′-O-(3-thiotriphosphate) or 5′-guanylylimidodiphosphate caused an increase in virus budding, which could be competitively inhibited by adding an excess of GTP. These results suggest that the G protein is involved in the regulation of influenza virus budding. We also determined the role of different protein kinases in influenza virus budding. We observed that specific inhibitors or activators of protein kinase A (H-89 and 8-bromoadenosine 3′,5′-cyclic monophosphate) or of protein kinase C (bisindolylmaleimide I and Ro-32-0432) or of phosphatidylinositol 3-kinase (LY294002 and wortmannin) did not affect influenza virus budding. However, the casein kinase 2 (CK2) inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole decreased virus budding. We further observed an increase in the CK2 activity during the replication cycle of influenza virus, although Western blot analysis did not reveal any increase in the amount of CK2 protein in virus-infected cells. Also, in digitonin-permeabilized MDCK cells, the introduction of CK2 substrate peptides caused a down-regulation of virus budding. These results suggest that CK2 activity also regulates influenza virus budding.

Introduction

Influenza virus morphogenesis involves a number of steps that require the transport of viral components – the haemagglutinin (HA), neuraminidase (NA), matrix proteins (M1 and M2) and viral ribonucleoprotein (vRNP) – to the assembly site, interaction among the viral components, budding from the membrane and release of virus particles (reviewed in Nayak, 1996, 2000; Nayak & Barman, 2002; Nayak & Hui, 2002). It is becoming evident that steps involved in these processes are regulated by host components. However, the role of host components, particularly in the budding process, is poorly understood. Recently, the concept of a virus budding process that is the topological reverse of endocytosis has been proposed for human immunodeficiency virus (HIV) and Ebola virus (Patnaik et al., 2000). Furthermore, budding and particle release of HIV and other retroviruses appear to involve participation of the components of the vacuolar protein sorting pathway (for a review, see Freed, 2002). Also, it has recently been shown that lipid rafts are involved in the budding process of many enveloped viruses including influenza virus (reviewed in Nayak & Barman, 2002). We recently found that influenza virus budding is an active process and requires ATP hydrolysis (Hui & Nayak, 2001). Since ATP is involved in regulating many cellular processes, including membrane signalling and protein kinase (PK) activities, and since PKs have been shown to regulate different phases of the virus infectious cycle, we have investigated the role of G protein membrane signalling and PKs in influenza virus budding.

A family of heterotrimeric (αβγ) guanine nucleotide-binding regulatory proteins (G proteins) are involved in the regulation of transmembrane signal transduction (for a review see Helmreich, 2001). The G proteins operate by utilizing a...
guanine nucleotide-binding and -hydrolysing cycle. Agonist–ligand receptor interaction causes the exchange of guanosine 5′-diphosphate (GDP) for guanosine 5′-triphosphate (GTP) at the ζ subunit of the G protein (Gζ) and leads to subsequent dissociation of Gζ from the βγ subunit (Gβ and Gγ). This GTP-bound Gζ is the active state of the G protein, which is terminated by hydrolysis of the bound GTP by an intrinsic GTPase activity. There is increasing evidence that heterotrimeric G proteins and PKs participate in the regulation of apical membrane dynamics such as membrane ruffling, endocytosis and exocytosis (reviewed in Ceresa & Schmid, 2000; Cavalli et al., 2001). Moreover, Gζ, Gα, and GTPase activating protein GAP-43 are enriched in lipid rafts (Arni et al., 1998; Moffett et al., 2000), regions on the apical plasma membrane that function as the budding site of influenza virus (Scheiffele et al., 1999; Barman & Nayak, 2000; Zhang et al., 2000; Barman et al., 2001).

In the present study, we have investigated the role of G proteins and PKs in regulating influenza virus budding from the apical cell surface. Our results show that in WSN virus-infected Madin–Darby canine kidney (MDCK) cells, G protein signalling stimulators (such as fluorides, compound 48/80, mastoparan or GTP analogues) increased virus budding, and that G protein signalling blockers (suramin or NÔF023) decreased virus budding. In addition, the introduction of the casein kinase 2 (CK2) inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), or CK2-immunodepleted cytosol into permeabilized cells inhibited virus budding, whereas inhibitors of protein kinase A (PKA), protein kinase C (PKC), or phosphatidylinositot 3-kinase (PI3K) did not affect virus budding.

Methods

Viruses and cells. All experiments were carried out using MDCK cells obtained from the ATCC and maintained in tissue culture medium containing Dulbecco’s modified Eagle’s medium (DMEM; Gibco BRL) supplemented with 10% heat-inactivated foetal bovine serum (FBS; Atlanta Biologicals), 10 U/ml penicillin G and 10 µg/ml streptomycin and propagated at 37 °C in a 5% CO2-saturated atmosphere with a resulting pH of 7.4 in the tissue culture medium. Influenza virus strain A/WSN/33 (H1N1) was used in these experiments and prepared by infecting MDCK cells at an m.o.i. of 0.005 and incubating in virus growth medium containing minimum essential medium (MEM) supplemented with 0.2% BSA, 0.075% NaHCO3, and 0.0015% DEAEx-dextran at 37 °C. At 36 h post-infection (p.i.), culture fluids were harvested, clarified by centrifugation and then stored at −80 °C. Stock virus titre was 1×108 p.f.u./ml.

Chemicals, drugs and peptides. All chemicals, drugs and peptides used in these experiments were purchased from Sigma or Calbiochem. Sodium fluoride (NaF), compound 48/80, suramin, NF023, and 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP) were dissolved in water. H-89, LY294002 and DRB were dissolved in absolute ethanol. Ro-32-0432 and bisindolylmaleimide I (BIM) were dissolved in DMSO. The residual solvents (less than 1%) had no toxic effect on the cell monolayer. Aluminium fluoride (AlF3) treatment on MDCK cells was performed as follows: (i) 40 µM aluminium chloride (AlCl3) plus 30 mM NaF (Brewer & Roth, 1995) or (ii) 50 µM aluminium ammonium sulfate [Al(NH4)2(SO4)2] plus 10 mM potassium fluoride (KF) (Ikonen et al., 1996). The GTP and GTP analogues guanosine 5′-O-(3-thiotriphosphate) (GTP/3S) and 5′-guanylylimidodiphosphate (GMP-PNP) were dissolved in serum-free DMEM just before use. G protein signalling activator mastoparan (INLKALAAALKIL), PKA inhibitor 6–22 amide (TYADFIASGRTGRRNAI), PKC inhibitor peptide 19–36 (RFARKGR/QKGNV-HEVKNN and CK2 substrate peptide (RRADDSDDDDDD) were dissolved in water.

Virus infection and sample collection. For virus infection, MDCK cells were seeded at a cell density of 1×105 cells/35 mm dish and maintained in 2 ml of culture medium for 30 h. Prior to infection, cells were washed with PBS* (PBS plus 0.5 mM MgCl2 and 1 mM CaCl2). In all experiments, cells were infected with WSN virus at an input m.o.i. of 1 in virus dilution buffer (PBS* supplemented with 0.2% BSA, 0.005% DEAEx-dextran, 10 U/ml penicillin G and 10 µg/ml streptomycin). After adsorption for 1 h at 37 °C, the residual unabsorbed virus was removed and cells were washed five times with DMEM containing 0.2% BSA. The infected cells were incubated in 2 ml DMEM containing 0.2% BSA for 13 h at 37 °C. Sialidase (NA; final concentration 0.1 ml/ml) was added 2.5 h prior to removal of culture medium. Infected cells were then washed five times with 2 ml of DMEM containing 0.2% BSA to remove residual virus. The fifth wash was collected as the experimental background. The new medium, with or without the drug, was added and harvested after a 15 min or 1 h incubation for the virus assay.

LPC permeabilization of filter-grown MDCK cells. The lysophosphatidylcholine (LPC) permeabilization method was carried out as described previously (Hui & Nayak, 2001). Briefly, MDCK cells (1×106) were seeded on Transwells (24 mm diameter, 0.4 µm pore size; Costar), infected with WSN virus at an m.o.i. of 1 and incubated for 13 h. The Transwell filters containing the cell monolayers were permeabilized from the basal side by LPC (Avanti Polar Lipids). The Transwells were then washed and treated with DMEM with or without GTP analogues from the basal chamber for 20 min at 37 °C. Subsequently, the Transwells were incubated with 1.5 ml DMEM with 0.2% BSA and 2.5 ml DMEM in the upper and lower compartments, respectively, and virus released in the upper chamber after 15 min was assayed.

Plaque assay. Determination of the amount of influenza virus released from infected cells into the medium was carried out by plaque assay on monolayers of MDCK cells in 35 mm tissue culture dishes using 2 ml agar overlay MEM supplemented with 0.6 % low melting point agarose, 0.075% NaHCO3, and 0.0015% DEAEx-dextran. Visible plaques were counted after 3 days at 33 °C and p.f.u./ml were calculated.

Western blotting. Proteins were resolved by SDS–PAGE (10% gel) and transferred electrophoretically to Trans-Blot nitrocellulose membrane (Bio-Rad). Membranes were blocked in 1% blocking solution and probed with anti-C2K2 monoclonal antibodies (Santa Cruz Biotechnology; diluted 1:500) overnight at 4 °C. The membrane was then incubated with anti-mouse monoclonal antibodies (diluted 1:1200) for 1 h at room temperature and antigens finally visualized on film by enhanced chemiluminescence (ECL; Amersham Biosciences).

Digitonin permeabilization of filter-grown MDCK cells. Digitonin permeabilization of the basolateral membrane of MDCK cells was carried out as described by Esparris-Ogando et al. (1994), and the cytosol support of permeabilized MDCK cells was carried out according to the procedures of Pimplikar et al. (1994) and Ikonen et al. (1996). Briefly, MDCK cells were seeded, grown and infected on the Transwell as
described previously (Hui & Nayak, 2001). The Transwell filters containing the cell monolayers were permeabilized from the basal side with digitonin (250 µg/ml) for 15 min at room temperature. The Transwells were washed twice with PBS® on the apical side and with transport medium [115 mM potassium acetate (KOAc), 25 mM HEPES, pH 7.4; 2.5 mM MgCl₂, 1 mM DTT, 5 mM EGTA and 2.5 mM CaCO₃] on the basal side. Cells were then incubated with 250 µl (2 mg) of HeLa or MDCK cytosol with or without an ATP-regenerating system (1 mM ATP, 8 mM phosphocreatine and 50 µg/ml creatine phosphokinase) in the basal chamber and with PBS® containing 0.1 U/ml NA in the upper compartment. After 45 min at 37 °C incubation, the upper compartment was washed five times and the fifth wash was collected for the experimental background. The Transwells were then incubated with 0.5 ml DMEM plus 0.2% BSA in the upper compartment and with cytosol in the lower compartment and virus released into the upper chamber after 15 min was assayed.

### Permeabilization assessment
The effectiveness of permeabilization was assessed by the cell viability assay (trypan blue uptake) and the release of cytosolic lactate dehydrogenase (LDH) from the basal side as previously described (Hui & Nayak, 2001).

### Preparation of HeLa cytosol
The preparation of HeLa cytosol was carried out as described previously (Pimplikar et al., 1994). Briefly, HeLa cells were grown in suspension in Eagle's Minimal Essential Medium (MEM; Sigma) supplemented with 1 g/l glucose and 10% newborn calf serum (Gemini Bio-Products) to a density of 4 × 10⁶/ml. Cells were pelleted by centrifugation, washed with ice-cold PBS and resuspended in cold KOAc swelling buffer (1 mM MgCl₂, 1 mM DTT, 1 mM EGTA and 1 mM cytochalasin D). After swelling for 5 min on ice, cells were centrifuged, the supernatant removed, protease inhibitor cocktail (Sigma) added and the cells broken by a glass Dounce homogenizer with 10 strokes. Finally, 1/10 volume of 10 × KOAc buffer (1 x KOAc buffer: 115 mM KOAc, 25 mM HEPES, pH 7.4; 2.5 mM MgCl₂ and 10 mM DTT) was added to the homogenate and further homogenized with 20 strokes. Using this procedure, > 80% of the cells were broken, as judged by light microscopy. The homogenate was centrifuged at 14 000 g for 10 min followed by another centrifugation at 171 000 g for 90 min. The supernatant was frozen in small aliquots at −80 °C. This procedure routinely yielded ~ 14 ml containing ~ 12.5 mg/ml cytosolic protein from 10 l of cell suspension.

### Preparation of MDCK cytosol
The preparation of MDCK cytosol was carried out as described previously (Ikonen et al., 1996). Confluent MDCK monolayers from 40 150 cm² dishes (total 7 × 10⁶ cells) were trypsinized. Trypsin was then inactivated by first resuspending the cells in culture medium containing 10% FBS, pelleting and washing again with 30 ml of ice-cold PBS supplemented with 1 mg/ml soybean trypsin inhibitor. The cell pellet was resuspended in cold MDCK swelling buffer (1 mM MgCl₂; 1 mM EGTA) and incubated on ice for 10 min. After pelleting the cells, a protease inhibitor cocktail containing cytochalasin D (final concentration 1 µM) and DTT (final concentration 1 mM) was added and the cells broken by sonication. The solution containing cell fragments was then adjusted to an isotonic condition by adding 1/10 vol. of 10 × KOAc stock. Following a 20 min centrifugation at 600 g at 4 °C, the supernatant was further centrifuged for 1 h at 200000 g at 4 °C. The cytosol (~ 2.5 ml) containing ~ 12 mg/ml protein was aliquoted and stored at −80 °C.

### Immunodepletion of CK2
To deplete CK2, 150 µg anti-CK2α antibody was coupled to 0.1 g protein A–Sepharose beads (Amersham Biosciences) in PBS by mixing for 2 h at 4 °C. The beads were then washed with PBS and incubated with cytosol (10 mg) overnight on ice with intermittent mixing. After removing the beads by centrifugation, the cytosol was used in a digitonin permeabilization assay. The depletion was monitored by determining the loss of CK2 activity.

### Preparation of membranes and cytosol from MDCK cells for assaying enzymatic activity
The membranous and cytosolic fraction of MDCK cells was prepared by a modification of a previously described method (Hansen & Casanova, 1994). Briefly, MDCK cells were rinsed and scraped off in PBS, pelleted in a microfuge and resuspended in 1 ml of ice-cold 3 mM imidazole (pH 7.4) and 300 mM sucrose. The resuspended cells were passed at least 40 times through a 25-gauge syringe. Greater than 90% of the cells were lysed under these conditions, as assessed by trypan blue staining. The nuclei and cell debris were then pelleted by centrifugation at 14000 g for 5 min and the supernatant further centrifuged at 105 000 g for 1 h. The pellet and supernatant were then taken to represent membrane and cytosolic fractions, respectively.

### PKC activity assay
PKC activity assays were performed as described previously (Hui & Yung, 1992; Yung et al., 1994). The reaction mixture (25 µl) containing 30 mM Tris–HCl buffer, pH 7.5, 6 mM magnesium acetate, 0.25 mM EGTA, 0.4 mM CaCl₂, 40 µg/ml phosphatidylerosine, 8 µg/ml dioleoylglycerol, 1 mg/ml histone IIIS, 0.12 mM [γ-³²P]ATP (Perkin-Elmer Life Sciences) and membrane or cytosol fractions containing 10 µg of protein were incubated at 30 °C for 8 min. Phosphorylated substrates were quantified as described (Huang & Huang, 1991). Twenty µl of the reaction mixture was spotted on to a line of origin (1.5 cm from the bottom) on an instant thin-layer chromatography strip (1 × 9.5 cm, type SG; Gelman Sciences), which had previously been spotted with ATP (50 mM) in 15% trichloroacetic acid (TCA). After chromatography for 6 min at room temperature in a beaker containing 5% TCA and 0.2 M KCI, the strips were air dried and the origin (1.5 cm above the line of origin) excised for counting in a scintillation counter.

### CK2 activity assay
Assays for CK2 activity were performed as described previously (Perich et al., 1992). Briefly, 50 µl of reaction mixtures containing 50 mM Tris–HCl, pH 7.5, 12 mM MgCl₂, 100 mM NaCl, 1 mM CK2 peptide substrate, 25 µM [γ-³²P]ATP and 10 µg isolated protein (membrane or cytosol) were incubated at 30 °C for 8 min. Reactions were stopped by adding 90 µl 10% TCA and 10 µl BSA and incubated on ice for 10 min. The precipitated protein was removed by centrifugation and 12 µl of supernatant was spotted on to pieces of P81 phosphocellulose (Whatman Int.). The phosphocellulose pieces were washed with an excess of 75 mM phosphoric acid, air dried and counted in a scintillation counter.

### Statistical analysis
The results were obtained from at least three separate experiments, each using triplicate culture plates in each experiment. Unless otherwise specified, all data were expressed as mean ± standard deviation (SD) of n values (number of experiments) and where SD bars are not apparent, the SD value was less than the symbol used. The significance of the difference (P) between values was compared using the Student’s t-test, and P < 0.05 or less was considered significant.

### Results

#### G protein-acting drugs alter virus budding
To investigate the role of G proteins in the regulation of influenza virus budding, MDCK cells were infected at an m.o.i. of 1 and incubated without drugs for 13 h, when optimal virus budding occurred (Hui & Nayak, 2001). At 13 h p.i., infected
compared with mock treatment, the treatment of compound 48 (probably interacting with Gαi, Gαo and GαS) increases virus budding. However, fluoride ion (F−) and phosphorylation play an important role in the process of influenza virus budding. The results of the above experiments strongly suggested that GTP plays an important role in influenza virus budding. To examine the role of GTP analogues, we used basolateral-surface LPC-permeabilized MDCK cells (Hui & Nayak, 2001), since GTP and the GTP analogues GTPγS (hydrolysis-resistant GTP analogue) and GMP-PNP (non-hydrolysable GTP analogue) cannot gain entry into intact cells (Cockcroft & GomPERTS, 1985). MDCK cells were grown and infected on a supporting microporous membrane and permeabilized by LPC from the basolateral surface (Hui & Nayak, 2001). Permeabilized cells were then incubated with 1 mM GTPγS or GMP-PNP, which keep the G protein in an active state because they can bind but are not hydrolysed (Terry et al., 1995). The results showed that the virus budding rate after treatment with GTPγS and GMP-PNP in LPC-permeabilized cells increased to 147 ± 16% and 178 ± 27%, respectively, compared with 100% in LPC-permeabilized cells with mock treatment (Fig. 2). The enhancing effect of GTPγS and GMP-PNP could be competitively inhibited by the presence of excess GTP. A high concentration of GTP (10 mM) decreased the virus budding rate for both GTPγS and GMP-PNP to 102 ± 3% and 99 ± 7%, respectively (Fig. 2). Taken together, these results suggest that G protein signalling is involved in regulating the influenza virus budding process.

PKA inhibitor and activator do not alter virus budding

In Fig. 1(A), NaF and AlF−4, which stimulate G protein signalling, increased virus budding. However, fluoride ion (F−) has previously also been shown to increase the level of adenosine 3′,5′-cyclic monophosphate (cAMP) in MDCK cells (Eker et al., 1994). Since our previous findings indicated that ATP was a key molecule involved in regulating virus budding (Hui & Nayak, 2001), we wanted to determine whether PKs and phosphorylation play an important role in the process of influenza virus budding. Moreover, PKA is a cAMP-dependent activator.

GTP analogues in LPC-permeabilized infected cells increase virus budding
Table 1. Rate of virus budding after treatment with different drugs

The virus budding rate in the presence of drug after either 15 min or 1 h was compared with mock-treated cells (100%). The numbers in parentheses represent the number of independent experiments. Experiments were carried out as shown in Figs 1 and 3. *P < 0.05 and **P < 0.001 vs mock treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dosage</th>
<th>15 min</th>
<th>1 h</th>
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<tbody>
<tr>
<td>Mock</td>
<td>—</td>
<td>100</td>
<td>100</td>
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<tr>
<td>G protein signalling stimulator</td>
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<tr>
<td>NaF</td>
<td>10 mM</td>
<td>268 ± 35 (4)**</td>
<td>312 ± 39 (7)**</td>
</tr>
<tr>
<td></td>
<td>50 mM</td>
<td>329 ± 21 (3)**</td>
<td>ND</td>
</tr>
<tr>
<td>AlF₃ [AlCl₃ + NaF]</td>
<td>40 µM</td>
<td>193 ± 23 (3)**</td>
<td>193 ± 37 (3)**</td>
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<tr>
<td>AlF₃ [AlNH₄(SO₄)₃ + KF]</td>
<td>50 µM</td>
<td>222 ± 40 (3)**</td>
<td>ND</td>
</tr>
<tr>
<td>Compound 48/80</td>
<td>1 µg/ml</td>
<td>191 ± 19 (3)**</td>
<td>191 ± 12 (3)**</td>
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<td></td>
<td>10 µg/ml</td>
<td>300 ± 21 (3)**</td>
<td>ND</td>
</tr>
<tr>
<td>Mastoparan</td>
<td>50 µM</td>
<td>218 ± 8 (4)**</td>
<td>226 ± 26 (3)**</td>
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<td></td>
<td>250 µM</td>
<td>555 ± 38 (3)**</td>
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<td>G protein signal blocker</td>
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<td>Suramin</td>
<td>200 µg/ml</td>
<td>86 ± 2 (4)*</td>
<td>68 ± 1 (3)**</td>
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<td>750 µg/ml</td>
<td>64 ± 6 (3)**</td>
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<td>NF023</td>
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<td>84 ± 6 (4)*</td>
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<td>30 µM</td>
<td>80 ± 3 (3)*</td>
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<td>H-89</td>
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<td>Br-cAMP</td>
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<td>100 ± 6 (3)</td>
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<td></td>
<td>1 mM</td>
<td>117 ± 9 (3)</td>
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<td>97 ± 13 (4)</td>
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<td>PI3K inhibitor</td>
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<td>20 µM</td>
<td>87 ± 11 (3)</td>
<td>87 ± 8 (3)*</td>
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<td>Wortmannin</td>
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<td>50 µM</td>
<td>90 ± 8 (3)</td>
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<tr>
<td>CK2 inhibitor</td>
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<tr>
<td>DRB</td>
<td>5 µM</td>
<td>95 ± 2 (4)*</td>
<td>93 ± 3 (3)</td>
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<tr>
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<td>10 µM</td>
<td>89 ± 4 (4)*</td>
<td>74 ± 5 (3)*</td>
</tr>
<tr>
<td></td>
<td>50 µM</td>
<td>77 ± 6 (4)*</td>
<td>67 ± 7 (3)**</td>
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Protein kinase, which is a downstream effector of heterotrimeric G protein (for a review, see Helmreich, 2001). Therefore, to test whether the budding process was affected by PKA, we treated the infected MDCK cells with the highly specific and membrane-permeable PKA inhibitor H89, which decreases the PKA activity, or with the phosphodiesterase-resistant cAMP analogue 8-Br-cAMP, which increases the cAMP level and in turn enhances PKA activity. In addition, 8-Br-cAMP has been shown to stimulate apical endocytosis in MDCK cells (Eker et al., 1994). However, neither H89 (100 and 500 nM) nor 8-Br-cAMP (0.1 and 1 mM) had any significant effect on virus budding (Table 1). These data indicate that PKA does not play a significant role in influenza virus budding.

PKC inhibitors do not alter virus budding

PKC is a large superfamily of related PKs, which carry out diverse regulatory roles in many cellular processes (for a review, see Pears, 1996). PKC has been shown to be involved in plasma membrane dynamics such as membrane ruffling and endocytosis (reviewed in Ceresa & Schmid, 2000). Therefore, we treated the infected MDCK cells with the highly specific membrane-permeable PKC inhibitors such as BIM or Ro-32-0432. However, neither BIM (50 and 200 nM) nor Ro-32-0432 (50 and 200 nM) exhibited any significant effect on virus budding (Table 1). The cytosolic and membranous PKC activity decreased in cells treated with the PKC inhibitors (data not shown in the table).
shown). Although PKC has been shown to play a role in influenza virus mRNA translation (Kurokawa et al., 1990) and virus entry (Root et al., 2000), our data indicate that, like PKA, PKC does not play a significant role in influenza virus budding.

**PI3K inhibitors do not alter virus budding**

Besides PKC, PI3K has been found to regulate various steps in endocytic trafficking (for a review, see Ridley, 2001). Therefore, we treated the infected MDCK cells with the highly specific membrane-permeable PI3K inhibitors, LY294002 and wortmannin. However, neither LY294002 (5 and 20 µM) nor wortmannin (25 and 50 nM) had any significant effect on virus budding (Table 1). These data indicate that PI3K does not play a significant role in influenza virus budding.

**CK2 inhibitor decreases virus budding**

Previous studies have indicated that CK2 activity is associated with influenza virus particles (Tucker et al., 1990; for a review, see Hui, 2002) and that the viral PA protein in the influenza polymerase complex is phosphorylated by CK2 (Sanz-Ezquerro et al., 1998; Perales et al., 2000). Therefore, we wanted to know whether the CK2 plays any role in virus budding. When virus-infected cells were treated with the

![Graph showing inhibitory effect of GTP analogues on virus budding.](image1)

**Fig. 2.** Inhibitory effect of GTP analogues on virus budding. Filter-grown MDCK cells were infected at an m.o.i. of 1 and incubated for 13 h. Cells were subsequently permeabilized with LPC as described in Methods. Permeabilized cells were treated with different GTP analogues for 20 min from the basal side. Cells were then washed and incubated for 15 min. Samples from the apical chamber were collected and virus titres were determined by p.f.u. assay. Results are presented as the percentage of mock-treated non-permeabilized cells (100%). Data represent mean ± SD (n = 3). *P < 0.001 vs mock treatment on LPC-permeabilized cells.

![Graph showing effect of the CK2 inhibitor DRB on virus budding.](image2)

**Fig. 3.** Effect of the CK2 inhibitor DRB on virus budding. WSN-infected MDCK cells were incubated without drugs for 13 h. Infected cells were then washed and treated with or without DRB for 15 min (grey bars) or 1 h (black bars) at different concentrations, as indicated in the figure. Release of infectious virus into the culture supernatant was determined by p.f.u. assay. The results are presented as the percentage of mock-treated cells (100%). Data represent mean ± SD (n = 3 or 4, see Table 1). *P < 0.05, **P < 0.001 vs mock treatment.

![Graph showing CK2 activity required for virus budding.](image3)

**Fig. 4.** CK2 activity is required for virus budding. WSN-infected (closed circles or squares) and non-infected (open circles or squares) MDCK cells were incubated without drugs. Both cytosolic (A) and membranous (B) CK2 activities were determined at indicated time-points. Note that membranous CK2 activity represents a small fraction of the total CK2 activity. (C) Western blot analysis of CK2α protein in virus-infected cells at 0 and 14 h p.i. MDCK cells, infected with WSN, were lysed with RIPA buffer, resolved by SDS–PAGE (10% gel) and immunoblotted with monoclonal anti-CK2α antibodies. Bands were analysed by ECL development.
Table 2. Rate of virus budding after treatment of digitonin-permeabilized MDCK cells with peptides

The numbers in parentheses represent the number of independent experiments. Experiments were done out as shown in Fig. 5(A).

<table>
<thead>
<tr>
<th>Digiton permeabilized</th>
<th>Cytosol ATP-regeneration system*</th>
<th>Peptide</th>
<th>CK2†</th>
<th>PKA‡</th>
<th>PKC§</th>
<th>Budding rate after 15 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100 (3)†</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>35 ± 11 (3)#</td>
</tr>
<tr>
<td>+</td>
<td>HeLa</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>60 ± 3 (3)‡</td>
</tr>
<tr>
<td>+</td>
<td>HeLa</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>62 ± 6 (3)‡</td>
</tr>
<tr>
<td>+</td>
<td>MDCK</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>80 ± 5 (3)‡</td>
</tr>
<tr>
<td>+</td>
<td>HeLa</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>82 ± 9 (3)‡</td>
</tr>
<tr>
<td>+</td>
<td>HeLa</td>
<td>+</td>
<td>20 µM</td>
<td>-</td>
<td>-</td>
<td>65 ± 5 (3)‡</td>
</tr>
<tr>
<td>+</td>
<td>HeLa</td>
<td>+</td>
<td>100 µM</td>
<td>-</td>
<td>-</td>
<td>50 ± 4 (3)#</td>
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<tr>
<td>+</td>
<td>HeLa</td>
<td>+</td>
<td>-</td>
<td>20 nM</td>
<td>-</td>
<td>79 ± 7 (3)‡</td>
</tr>
<tr>
<td>+</td>
<td>HeLa</td>
<td>+</td>
<td>-</td>
<td>200 nM</td>
<td>-</td>
<td>78 ± 11 (3)‡</td>
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<tr>
<td>+</td>
<td>HeLa</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>2 µM</td>
<td>85 ± 8 (3)‡</td>
</tr>
<tr>
<td>+</td>
<td>HeLa</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>20 µM</td>
<td>81 ± 6 (3)‡</td>
</tr>
</tbody>
</table>

* ATP-regeneration system in cytosol: 1 mM ATP, 8 mM phosphocreatine and 50 µg/ml creatine phosphokinase.
† CK2 substrate peptide (RRADDSDDDDD), Kᵦ = 19 µM (Marin et al., 1994).
‡ PKA inhibitor 6–22 amide (TYADFIASGRTGRRNAL), Kᵦ = 1.7 nM (Cheng et al., 1986).
§ PKC inhibitor peptide 19–36 (RFARKGALRQKNVHEVKN), Kᵦ = 147 nM (House & Kemp, 1987).
# P < 0.05 and *P < 0.001 vs mock treatment on digitonin-permeabilized cells (with HeLa cytosol and ATP-regeneration system).

membrane-permeable CK2 inhibitor DRB at different concentrations (5, 10 and 50 µM) for 15 min or 1 h, the virus budding rate from MDCK cells decreased with increasing concentrations of DRB (Fig. 3, Table 1). CK2 activities in infected and uninfected cells after treatment with 50 µM DRB were determined in parallel and the results indicated that both the cytosolic and membranous CK2 activities decreased in virus-infected cells after DRB treatment (data not shown) and corresponded to the decrease in virus budding.

We next examined whether CK2 activity changed during the virus infectious cycle. The CK2 activity assay indicated that both the cytosolic and membranous CK2 activities increased during influenza virus infection (Fig. 4A, B). It should be noted that the maximum increase in CK2 activity was observed around 15 h p.i., which is consistent with the time of maximum virus budding (Hui & Nayak, 2001). CK2 is a tetramer composed of two types of subunit with the general structure α₂β₂ or αxβ₂ (reviewed in Allende & Allende, 1995). The α (42–44 kDa) and x' (38 kDa) subunits are catalytic subunits, whereas the β (26–41 kDa depending on cell type) subunit is a regulatory subunit. By Western blot analysis of the CK2α subunit, we did not find any significant change in the amount of CK2α subunit in infected MDCK cells (Fig. 4C). However, we failed to detect CK2α x' subunit in MDCK cells by Western blotting (data not shown), suggesting that MDCK cells do not express the CK2α x' subunit. These data indicate that the enzymatic activity, but not expression of the CK2 protein, was stimulated during influenza virus infection.

**CK2 substrate peptides in digitonin-permeabilized infected cells decrease virus budding**

Since the above results suggested that CK2, but not PKA, PKC or PI3K, was involved in the influenza virus budding process, we wanted to determine whether the CK2 substrate peptide could compete with and decrease virus budding. Therefore, we developed an in vivo digitonin-permeabilized MDCK cell system to assay virus budding in the presence of peptide. Accordingly, cells were grown and infected on a supporting microporous membrane and treated with digitonin from the basolateral surface as described in Methods. The digitonin treatment rendered approximately 80% of MDCK cells permeable as measured by accessibility to trypan blue when applied from the basal side but not from the apical side (data not shown). The permeability after digitonin treatment was also assayed by determining the release of cytosolic marker LDH from cells into the medium. The results showed that 55 ± 5% of total LDH was released into the medium (data...
To determine the effect of CK2 peptide on virus budding, MDCK cells were grown on the membrane and infected with WSN virus at an m.o.i. of 1. At 13 h p.i., cells were permeabilized by digitonin and incubated with HeLa cytosol with CK2 substrate peptide or PKA inhibitor 6–22 amide or PKC inhibitor peptide 19–36. For studying virus budding in digitonin-permeabilized MDCK cells, we routinely used HeLa cytosol because it was easy to prepare. The HeLa cytosol was comparable with MDCK cytosol, since the virus budding efficiency was similar between these two cytosols (Table 2). The results showed that the 15 min virus budding rate after incubation with 20 or 100 µM CK2 substrate peptides in digitonin-permeabilized cells decreased to 65 ± 5% and 56 ± 4%, respectively, compared with 80 ± 5% after mock treatment (Fig. 5A, Table 2). Again, treatment with 20 or 200 nM PKA and 2 or 20 µM PKC inhibitor peptides did not significantly affect virus budding (79 ± 7%, 78 ± 11%, 83 ± 8% and 81 ± 6%, respectively; Fig. 5A, Table 2), supporting the results observed early (Table 1).

**Discussion**

Influenza virus binds to the cell surface sialic acid receptors, becomes internalized by receptor-mediated endocytosis and vRNP is released from the endosome in the acidic environment by membrane fusion and dissociation of M1 from vRNP. M1-free vRNP is then translocated into the host nucleus where mRNA transcription and vRNA (vRNP) replication occur. vRNPs are exported from the nucleus into the cytoplasm and subsequently all viral components, namely vRNP, M1 and the viral envelope containing selected host lipids and viral membrane proteins, are transported to the assembly site on the plasma membrane where assembly, budding and virus release.
into the outside environment occur (reviewed in Nayak, 1996, 2000). Consequently, various host components including PKs are intimately involved in regulating different aspects of the virus life-cycle (reviewed in Ludwig et al., 1999). For example, the influenza virus entry into the attached cells requires PKC (Root et al., 2000). PKC also plays a role in viral mRNA translation (Kurokawa et al., 1990). The transport of RNPs into the nucleus is mediated by the α class of the karyopherin import receptor protein (reviewed in Portela & Digard, 2002). RNP/M1 and NP in the cytoplasm associate with cytoskeletal components (Avalos et al., 1997). A recent microarray-based gene expression study (Geiss et al., 2001) listed the up- and down-regulation of expression of many genes during the early (4 h p.i.) and late (8 h p.i.) phases of infection.

However, relatively little information is available on the involvement of host components in the budding process of enveloped viruses in general and influenza viruses in particular. Recently, a number of elegant studies have shown that the regulatory role of host components in the release of virus by budding in retroviruses (HIV, Rous sarcoma virus and equine infectious anaemia virus), vesicular stomatitis virus (VSV), rabies virus and Ebola viruses. The AP-50 subunit (medium chain) of cellular AP-2 clathrin-associated adaptor protein complex, the Yes-associated protein (Yap), the Nedd4-like family of E3 ubiquitin ligase and Tsg101 have been shown to be actively involved in the release of virus by budding. The majority of these viruses contain specific late domains (L domains) in the matrix proteins, which interact with cellular components (reviewed in Luban, 2001; Freed, 2002).

However, similar information is not yet available for influenza virus budding. Towards this goal, we have initiated studies for defining the role of different host components in influenza virus budding. We have developed a system to reconstitute the budding process by permeabilizing the polarized MDCK cells from the basal side, exchanging and introducing the cytosolic components from the basal side and assaying virus budding from the intact apical side (Hui & Nayak, 2001).

Using such a system, we have recently shown that ATP is actively required for influenza virus budding and that ATP hydrolysis (and not just ATP binding) is required for the budding and release of virus particles.

In the present paper, we show that the G protein and CK2 also play a critical role in influenza virus budding. G proteins are known to play important roles in a number of cellular signals and the basic mechanism of G protein-mediated signal transduction is well understood. Different classes of G protein have been shown to be involved in apical versus basolateral transport in polarized epithelial cells. In virus-infected MDCK cells, the Gα class of G proteins has been shown to be involved in the apical transport of HA, whereas Gβ proteins affected the basolateral transport of VSV G proteins (Pimplikar & Simons, 1993). Our data show that GTP binding, which activates G protein signalling, is required for influenza virus budding. However, at present, the precise role of the G protein in influenza virus budding is unclear. Initiation of virus budding may involve processes similar to membrane ruffling, which is affected by cytoskeletal components and lipid rafts, as well as signal transduction by G proteins (see Ridley, 1994). Furthermore, it is likely that the M1 protein can directly or indirectly interact with G proteins in initiating the budding process or releasing virus particles, since the expression of M1 protein alone can lead to the formation and release of virus-like particles (Gómez-Puertas et al., 2000; Latham & Gularza, 2001).

Host PKs are also actively involved in different steps of influenza virus replication and many studies on the effect of PK inhibitors during influenza virus replication have been carried out (Kurokawa et al., 1990; Martin & Helenius, 1991; Vogel et al., 1994; Neumann et al., 1997; Bui et al., 2000; Root et al., 2000; Pleschka et al., 2001). It appears that six of the ten influenza A virus proteins can be phosphorylated. These are NP (Petri & Dimmock, 1981; Almond & Felsenreich, 1982; Kistner et al., 1985, 1989), NS1 (Petri et al., 1982), M1 (Gregoriades et al., 1984, 1990), NEP/NS2 (Richardson & Akkina, 1991), M2 (Holsinger et al., 1995) and PA (Sanchez-Esquerrro et al., 1998). Although phosphorylated M1 and NP have been found in virus particles (Gregoriades et al., 1984), there is no data showing a specific requirement for phosphorylation of any viral protein in the budding process. Among the viral proteins, M1 and PA have been shown to be phosphorylated by PKC (Reinhardt & Wolff, 2000) and CK2 (Perales et al., 2000), respectively. Kinases involved in the phosphorylation of other viral proteins are as yet uncharacterized. However, our studies show that neither PKA nor PKC, which have been shown to affect different phases of virus replication (Kurokawa et al., 1990; Neumann et al., 1997; Root et al., 2000), are involved in the budding process. CK2, on the other hand, appears to be actively involved in the budding process, since the CK2 activity was stimulated during the virus replication cycle, reaching a maximum during the budding phase, and specific inhibitors of CK2 decreased virus budding significantly.

CK2 is a ubiquitous eukaryotic Ser/Thr kinase present in the plasma membrane, cytoplasm, mitochondria and nucleus (reviewed in Faust & Montenegro, 2000). This enzyme is cyclic-nucleotide-independent and insensitive to calcium. CK2 is unusual among the PKs since it can use both ATP (Km 10 μM) and GTP (Km 20–30 μM) as phosphate donors. CK2 is known to phosphorylate more than a hundred cellular substrates, many of which are involved in regulating signal transduction pathways. Since virus budding is regulated by ATP, the membrane physical state, the G protein and PKs, as well as the cytoskeleton, the substrate(s) of CK2 during virus budding could be one or more of these components, including the cytoskeletal proteins, which interact with RNP/M1 complexes. Both membrane-bound and cytosolic CK2 phosphorylate many cytoskeletal proteins, such as spectrin, ankyrin, adducin, MAP-1B, tau and E-cadherin (reviewed in Allende & Allende, 1995; Faust & Montenegro, 2000). Moreover, CK2 is also...
known to play a role in the regulation of membrane proximal signalling events (for a review, see Allende & Allende, 1995). The presence of CK2 in the influenza virion (Tucker et al., 1990; reviewed in Hui, 2002) suggests the possibility of CK2 presence in the vicinity of the budding area of influenza virus and its active involvement in the budding process. Some viruses (such as HIV and herpes simplex virus) have been shown to encapsidate cellular PKs, which play an important role in virus infectivity and virulence (for a review, see Hui, 2002). However, the only known function of CK2 in influenza virus replication is to phosphorylate viral polymerase PA (Perales et al., 2000), which is likely to occur during the RNA replication phase of the influenza virus life-cycle. Furthermore, this function of CK2 should be restricted to cell nuclei, which contain high levels of CK2 and where the virus replication/transcription occurs. Therefore, the encapsidation of CK2 into influenza virus particles is not essential for PA phosphorylation and suggests a different role for CK2 function in the assembly and budding processes.

In summary, we have demonstrated that both G protein activity and CK2 function are critically involved in the budding process of influenza viruses. Influenza virus causes a serious worldwide health problem. Understanding more about virus-cell interactions during the budding process could provide new targets for therapy and intervention in the disease process.

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