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Short Communication

Influenza A virus proteins PB1 and NS1 are subject to functionally important phosphorylation by protein kinase C

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The virulence of influenza A viruses depends on the activity of the viral RNA polymerase complex and viral regulatory phosphoproteins. We identified that the protein kinase C (PKC) inhibitor Gö6976 had a post-entry anti-influenza viral effect, by using a polymerase activity-based reporter assay. This inhibitory effect was observed for influenza virus-infected cells as well as for cells transiently transfected with constructs for the RNA polymerase complex. Importantly, the *in vitro* analysis of viral protein phosphorylation identified PKCα as a kinase phosphorylating PB1 and NS1, but not PB2, PA or NP. Gö6976 was able to block PKC-specific phosphorylation *in vitro*. Thus, our data suggest that PKC contributes to the phosphorylation of influenza PB1 and NS1 proteins which appears to be functionally relevant for both viral RNA polymerase activity and efficient viral replication.

Influenza A viruses are pathogens that cause significant morbidity and mortality in humans and a variety of animal species (Palese & Shaw, 2007; Wright *et al.*, 2007). Influenza viruses possess a segmented negative-sense RNA genome which is replicated in the nucleus of infected cells. The virulence of individual virus strains is based on a complex co-action of determinants, including the viral haemagglutinin (HA), the RNA-dependent RNA polymerase (subunits PB1, PB2 and PA) and the nonstructural protein (NS1) (Geiss *et al.*, 2002; Tumpey *et al.*, 2005, 2007). The RNA-dependent RNA polymerase complex is responsible for both transcription and genomic replication (Torreira *et al.*, 2007; Palese & Shaw, 2007). PB1 represents the central catalytic subunit of the polymerase complex (Honda & Ishihama, 1997; Poch *et al.*, 1989; Torreira *et al.*, 2007), PB2 is responsible for recognition and binding of the cap structures of host mRNAs which are used for priming viral transcription (Fechter & Brownlee, 2005; Guilligay *et al.*, 2008), while PA combines essential activities required for genome replication and protein processing (Fodor & Smith, 2004; Maier *et al.*, 2008; Palese & Shaw, 2007). The viral nucleoprotein (NP) strictly associates with the RNA polymerase complex to form viral ribonucleoproteins (RNPs) (Noda *et al.*, 2006; Portela & Digard, 2002), it stimulates RNA polymerase activity and mediates nuclear RNP translocation (Neumann *et al.*, 1997; Bui *et al.*, 2002; Portela & Digard, 2002). The viral regulatory protein NS1 provides a number of functions required for efficient replication and gene expression (Hale *et al.*, 2008a). An interaction between NS1 and the viral transcription–replication complex was reported (Marion *et al.*, 1997) but awaits a confirmation in functional aspects. NS1 and the RNP components including NP are phosphoproteins in various types and strains of influenza viruses (Sanz-Ezquerro *et al.*, 1998; Bui *et al.*, 2002; Arrese & Portela, 1996; Skorko *et al.*, 1991; Marschall *et al.*, 1999; Neumann *et al.*, 1997; Privalsky & Penhoet, 1978, 1981). Several cellular protein kinases, including protein kinase C (PKC), casein kinase II, cyclin-dependent kinases (CDKs) and extracellular signal-regulated kinases (ERKs) were described as candidates for catalysing one or more of these important protein modifications (Anwar & Khan, 2007; Hale *et al.*, 2008b; Neumann *et al.*, 1997). PKC inhibitors were found to exert a block of influenza virus replication, mainly directed to viral entry (Sieczkarski *et al.*, 2003; Root *et al.*, 2000). Since PKC is considered to be a multifunctional effector within influenza virus-induced signalling cascades (Ludwig *et al.*, 2003), we investigated whether PKC activity is also critical for the later steps of viral replication, particularly the activity of the viral RNA polymerase and the phosphorylation-specific functional modulation of viral proteins.

We used the PKC inhibitor Gö6976 (Goekjian & Jirousek, 1999; Marschall *et al.*, 2001; Sieczkarski *et al.*, 2003) (Calbiochem) to study specific effects on influenza virus replication. 293T cells were cultivated in Dulbecco's

A supplementary table of primer sequences and a supplementary figure are available with the online version of this paper.

modified Eagle's medium containing 10 % fetal calf serum. Transient transfection of 293T cells was performed in 12-well plates at a confluence of 70–90 % (seeding cell number 2.5×10^5) by using Lipofectamine 2000 (Invitrogen). The influenza-specific green fluorescent protein (GFP) construct pHH21-NP-UTRhi-eGFP (Lutz *et al.*, 2005) served as a reporter of viral replication. One day post-transfection with the reporter construct, cells were infected with influenza A/WSN/33 virus and were subsequently cultivated in the presence or absence of Gö6976 (Fig. 1a). A dose-dependent inhibition of virus replication was noted, with 70 % inhibition at 5 μM Gö6976. To confirm the reporter assay, quantitative RT-PCR (qRT-PCR) was performed to analyse influenza virus RNA production in infected cells. Data showed that after Gö6976 treatment, there was a substantial reduction in viral RNA. At 2 days post-infection, there was a 4.5-fold inhibitory effect (Supplementary Fig. S1, available in JGV Online). As an additional control, cells were infected under treatment with cycloheximide (50 μg ml^{-1}) and cytoplasmic/nuclear viral particles in the post-entry phase (8 h) were visualized by immunofluorescence staining using monoclonal antibody (mAb)-M1 and mAb-NP. When Gö6976 was present during viral adsorption/entry, virus uptake was reduced, confirming the previous report that Gö6976 can block viral entry (Sieczkarski *et al.*, 2003). Furthermore, our data indicated that Gö6976 had almost no effect on virus uptake when added immediately after the adsorption/entry phase (Fig. 1a) or at later time points (data not shown). Gö6976 (at the concentrations tested) did not induce microscopically detectable alterations in cytotoxicity of treated 293T cells (data not shown). No increase in apoptosis signals was noted using a standard assay using highly apoptosis-sensitive Raji cells (Fig. 1b) or with 293T cells (which produced similar results, albeit with a lower quantitative level and uniformity of signal; data not shown).

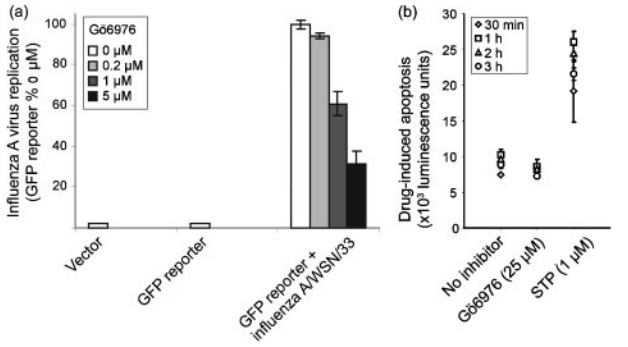

Fig. 1. Inhibition of influenza A virus replication by PKC inhibitor Gö6976. (a) 293T cells were grown in 12-well plates (seeding cell number 2.5×10^5) and transfected with GFP reporter pHH21-NP-UTRhi-eGFP or empty vector. One day post-transfection, cells were infected with influenza A/WSN/33 virus at an m.o.i. of 0.1 and treated with the indicated concentrations of Gö6976 (added after a 90 min period of virus adsorption/entry, when virus inoculum was replaced by fresh medium). Two days post-infection, cells were harvested and used to determine GFP reporter signals by automated GFP fluorometry (Victor 1420 Multilabel Counter, Wallac). (b) Raji cells were grown in 12-well plates and treated with the indicated inhibitors for 24 h. Cells were harvested and assayed for drug-induced apoptosis (Caspase-Glo Cytotoxicity assay, Promega) with staining intervals of 30 min, 1 h, 2 h and 3 h, as indicated. Staurosporine (STP; Calbiochem) was used as an apoptosis-inducing reference compound. All values were determined in quadruplicate; means ± SD are given.

Since the applied reporter construct reflected the conversion of negative- into positive-sense RNA (i.e. into GFP mRNA) in this polymerase activity-based GFP reporter assay, we investigated whether Gö6976 also had an inhibitory effect on a reconstituted RNA polymerase complex. For this purpose, the reporter construct was cotransfected with various combinations of influenza virus expression plasmids coding for PB1, PB2, PA, NP, HA, neuraminidase (NA), M or NS proteins (Hoffmann *et al.*, 2000). As shown in Fig. 2(a), the three RNA polymerase proteins PB1, PB2 and PA were poorly active in the absence of NP, while a strong GFP activity was measured for the combination of PB1, PB2, PA and NP constructs. The reporter signal could be enhanced by the addition of NS (Fig. 2a), whereas the addition of HA, NA or M had a negative effect. A control plasmid, pDsRed-N1 (Clontech), was used as an internal control in all experiments to assure a reliable efficiency of transfection. Thus, the optimal set of constructs to drive the influenza-specific GFP reporter was PB1, PB2, PA, NP and NS. It should be noted that besides NS1, NEP might also have been expressed from the NS construct; however, no NEP production was detected by Western blot (Wb), suggesting that this has very minor relevance for our experimental system. We next analysed the potential inhibitory effect of Gö6976 in this system by comparing the influenza-dependent GFP reporter with a control constitutively expressing GFP (Fig. 2b). It was striking that the GFP reporter was sensitive to Gö6976 while the GFP control was merely influenced by it. The activity of the influenza RNA polymerase complex was inhibited by Gö6976 in a concentration-dependent manner. Hence, the extended influenza RNA polymerase complex (PB1 + PB2 + PA + NP + NS) showed a more stringent concentration-dependent Gö6976 sensitivity than the complex without NS (Fig. 2b). This was compatible with the concept that PKC had a dual impact on the polymerase complex and the NS1 protein. Other protein kinase inhibitors, such as the CDK inhibitor roscovitine (Calbiochem) (Fig. 2c) or the tyrosine kinase inhibitor AG490 (tyrphostin) or an inhibitor of MAPK-p38 (Ax9930) (data not shown) did not produce an inhibitory effect.

The phosphorylation of influenza viral proteins was analysed by using *in vitro* kinase assays (IVKAs). FLAG (F)-tagged versions of influenza viral proteins were produced by transient transfection of expression plasmids generated by standard PCR amplification (Schregel *et al.*, 2007) of the PB1, PB2, PA, NP, M and NS open reading

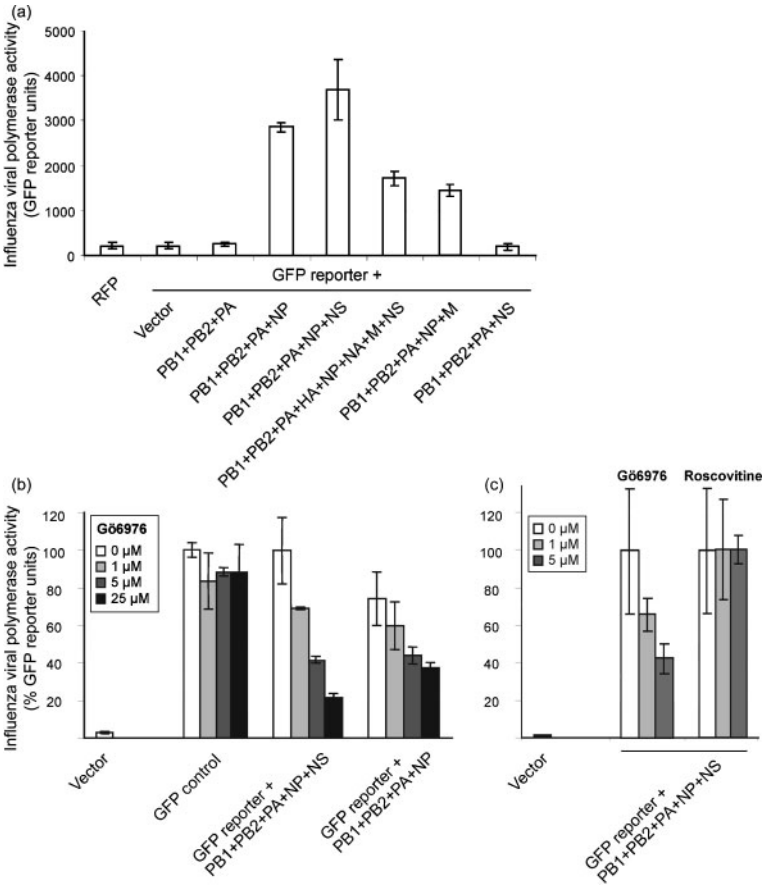

Fig. 2. Inhibition of influenza A viral RNA polymerase activity by PKC inhibitor Gö6976. (a) 293T cells were transiently cotransfected in 12-well plates with GFP reporter pHH21-NP-UTRhi-eGFP together with variable combinations of influenza virus expression constructs (pHW181-PB2, pHW182-PB1, pHW183-PA, pHW184-HA, pHW185-NP, pHW186-NA, pHW187-M, pHW188-NS; Hoffmann *et al.*, 2000). Two days post-transfection, cells were harvested and used to quantify GFP. A red fluorescent protein (RFP) expression construct was used as a transfection control (pDsRed1-N1). (b and c) Cells were transfected as in (a). For comparison with the GFP reporter pHH21-NP-UTRhi-eGFP, pEGFP-N1 (Clontech), which constitutively expresses GFP, was used as a GFP control. Gö6976 and roscovitine were added to the culture media after transfection and incubated for 2 days. The activity of all kinase inhibitors used in this study was ascertained by control IVKA analysis as described by Schleiss *et al.* (2008).

frames. pHW181-PB2, pHW182-PB1, pHW183-PA, pHW185-NP, pHW187 M and pHW188-NS were used as PCR templates (Hoffmann *et al.*, 2000); the PCR primers are given in Supplementary Table S1 (available in JGV Online); and pcDNA3.1 (Invitrogen) was used as the expression vector. PKC constructs were made as described by Milbradt *et al.* (2007, 2009). Transfected 293T cells were used as a source of recombinant proteins recovered by immunoprecipitation (mAb-FLAG; Sigma) and analysed by IVKA as described previously (Milbradt *et al.*, 2007) using 2.5 μCi (92.5 kBq) [γ-^{33}P]ATP. Phosphorylated proteins were detected by SDS-PAGE followed by Wb and exposure on phosphoimager plates. The integrity of recombinant expression products was monitored by a subsequent Wb staining of the IVKA blot (mAb-FLAG; data not shown). The data revealed the phosphorylation of FLAG-tagged F-PB1 and F-NS1, but not F-NP, by recombinant PKCα-F (Fig. 3a, upper panel). Controls with the influenza viral proteins alone, in the absence of PKCα-F, indicated that contamination by other protein kinases was not responsible for the reaction. A Wb expression control ascertained that sufficient amounts of proteins were produced (Fig. 3a, lower panel). A second experiment demonstrated that phosphorylation by PKC was detectable for neither influenza polymerase proteins F-PB2 and F-PA nor for matrix protein F-M1 (Fig. 3b). We cannot exclude, however, that phosphorylation of further influenza proteins may be detectable under more sensitive experimental conditions, as Reinhardt & Wolff (2000) provided evidence for a PKC-specific phosphorylation of M1. With respect to this, we labelled transiently expressed proteins *in vitro* with γ-^{33}P-labelled orthophosphate (150 μCi) and confirmed, by SDS-PAGE analysis, that F-NS1 was strongly phosphorylated (data not shown). However, not all influenza viral phosphoproteins described in the literature could be confirmed in this way, leading to the assumption that phosphorylation of some proteins may only be detectable under virus replication conditions. A fusion construct for PKCα-GFP was additionally used to confirm that the higher molecular mass of this kinase construct allowed a clear distinction from the polymerase proteins on the IVKA blot (Fig. 3b). This showed that there was a distinct phosphorylation of F-PB1, but not F-PB2 and F-PA, by PKCα-GFP (Fig. 3b, upper panel). Finally, an analysis of protein kinase inhibitors was performed and the data demonstrated that *in vitro* phosphorylation of NS1 was inhibited by four PKC inhibitors: Gö6976, rottlerin, bisindolylmaleimide I and calphostin C (Fig. 3c). Combined, these data strongly suggest that PKC is responsible for the phosphorylation of NS1 and PB1 and that these modifications are relevant for the activity of the viral RNA polymerase complex.

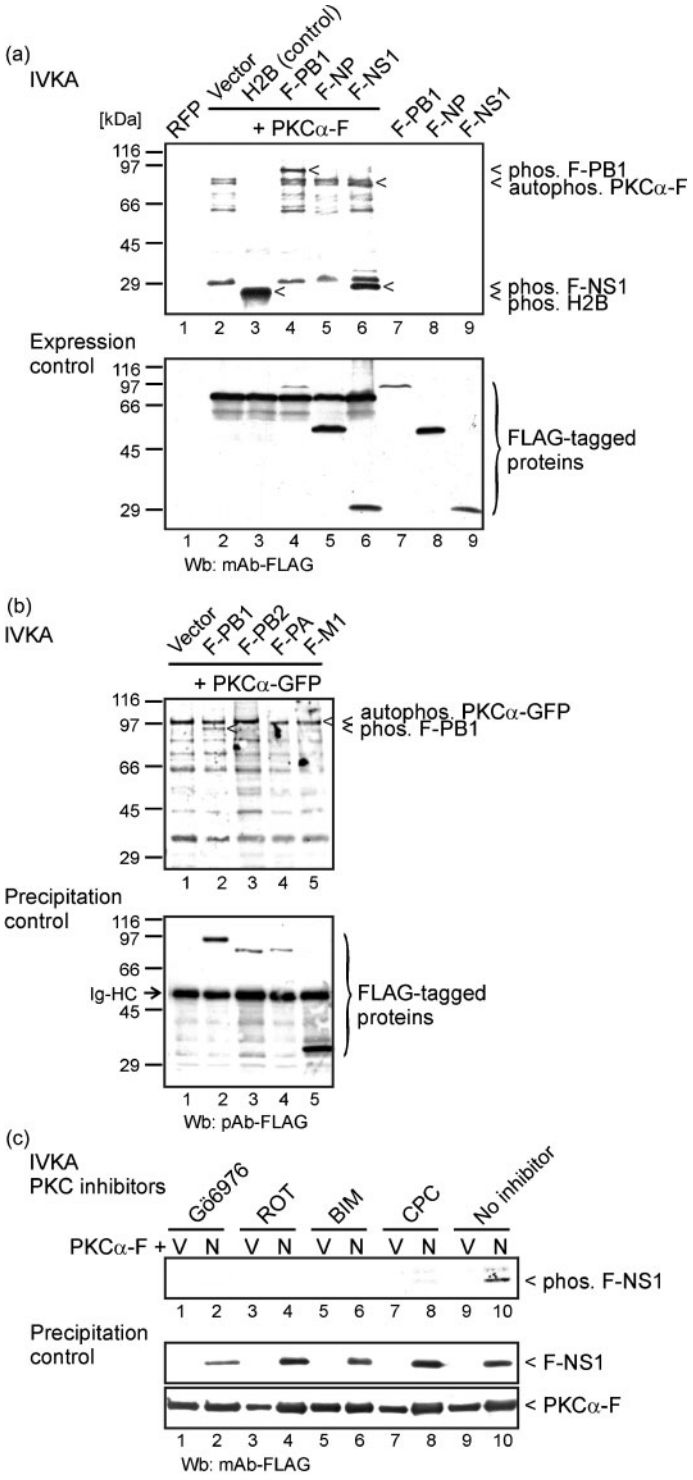

Fig. 3. *In vitro* phosphorylation of PB1 and NS1 proteins by PKCα. 293T cells were cotransfected in 10 cm dishes (5×10^6 cells) with expression constructs for the FLAG (F)-tagged proteins, as indicated. Two days post-transfection, cells were lysed and proteins were immunoprecipitated by the use of mAb-FLAG (a and c) or mAb-FLAG plus mAb-GFP (b) for subsequent analysis in a PKC-specific IVKA. Histone 2B (H2B; Sigma) was exogenously added to specific samples as a standard phosphorylation substrate. Expression control, Wb using aliquots of cell lysates taken prior to immunoprecipitation using mAb-FLAG (Sigma); precipitation control, Wb staining of the IVKA blot using pAb-FLAG (Sigma). Ig-HC indicates cross-reactivity with immunoglobulin heavy chain. (c) Cotransfections were performed with constructs for PKCα-F in combination with F-NS1 (N) or empty vector (V). PKC inhibitors were added to the IVKA reactions: Gö6976 (1 μM; Calbiochem), rottlerin (ROT; 5 μM), bisindolylmaleimide I (BIM; 1 μM) or calphostin C (CPC; 5 μM) (all ACC Corporation). Phosphorylated (phos.) and autophosphorylated (autophos.) proteins are marked.

The main conclusions of this study are: (i) inhibition of PKC activity has a negative effect on influenza A virus RNA production and replication, (ii) in a reporter system, PKC inhibitors reduce the activity of the viral RNA polymerase complex and/or associated factors, (iii) NS1 is required for optimal activity of the transiently expressed polymerase complex, (iv) PKCα can phosphorylate NS1 and PB1 *in vitro* and (v) *in vitro* phosphorylation is sensitive to various PKC inhibitors.

We used a set of constructs previously generated by Lutz *et al.* (2005) and Hoffmann *et al.* (2000) to perform

reporter assays in transiently transfected 293T cells. A similar approach was described very recently by Hoffmann *et al.* (2008). The assay system allowed us to quantify on the one hand the replication efficiency of influenza A virus and on the other hand the intracellular activity of a reconstituted viral RNA polymerase complex. Previous reports have demonstrated that PKC activity is required for viral entry and that PKC inhibitors induced an accumulation of virus in late endosomes (Root *et al.*, 2000; Sieczkarski *et al.*, 2003). However, it was a novel finding that Gö6976 exerted a post-entry inhibitory effect, as indicated by the RNA polymerase-based reporter assay. Although this approach could not strictly differentiate between potential underlying mechanisms, our data clearly demonstrate an inhibitory effect by PKC inhibitor Gö6976 but not by the other analysed protein kinase inhibitors. It is noteworthy that nuclear translocation of NP is one of the viral replicative steps dependent on PKC activity (Bui *et al.*, 2002; Neumann *et al.*, 1997; Root *et al.*, 2000). However, this may not fully explain the PKC dependence of viral RNA production and replication, particularly when taking into account the phosphorylation data from the present study. We detected the phosphorylation of PB1 and NS1 by PKCα *in vitro* but did not detect phosphorylation of other viral proteins such as NP. This strongly suggests that the phosphorylation state of PB1 and NS1 contributes to functionality. Hale *et al.* (2008b) described the phosphorylation of NS1 at threonine 215 (by CDKs and ERK2), concluding that this modification is important for NS1 function and efficient virus replication. Moreover, it was reported that PKC inhibitors such as rottlerin were very effective in reducing viral replication and that activation of PKC led to enhanced virus production (Hoffmann *et al.*, 2008). In combination, these reports underline the high functional importance of PKC for several steps of the influenza A virus replication cycle. Our study provides new insight into the PKC–virus interaction and highlights the multiple roles that cellular kinases have in influenza virus biology.

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