Influenza virus and redox mediated cell signaling: a complex network of virus/host interaction

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SUMMARY

Several viruses, including influenza, induce an imbalance of intracellular redox state toward pro-oxidant conditions. Through different mechanisms these alterations contribute both to influenza virus replication and to the pathogenesis of virus-induced disease. At the same time, influenza virus activates several intracellular signaling pathways involved in important physiological functions of the cell. Interestingly, many of these pathways are finely regulated by small changes in intracellular redox state, and the virus-induced redox imbalance might also control viral replication through this mechanism.
Here we review the main intracellular redox-sensitive pathways activated upon influenza infection and involved in regulating viral replication.

KEY WORDS: Influenza virus, MAPK, Redox state, GSH

INTRODUCTION

The influenza A virus, a widespread human pathogen, is an enveloped virus belonging to the *Orthomyxoviridae* family, characterized by a segmented single-stranded RNA genome that encodes eleven proteins. Within the envelope, the eight viral RNA segments, associated with the nucleoprotein (NP) and the three polymerases (PB1, PB2 and PA), form helical ribonucleoprotein capsids ($vRNP_S$). After infection, the $vRNP_S$ are transported to the host-cell nucleus, where they undergo transcription and replication. The viral polymerase complex (P), the NP, and the non-

structural protein 1 (NS1) are synthesized immediately after infection. The two major external glycoproteins, hemagglutinin (HA) and neuraminidase (NA), are late gene products, as are the matrix (M1), transmembrane (M2), and second non-structural (NS2) proteins. Within host cells, HA can be found in an uncleaved precursor form (HA_0) or in a cleaved form consisting of two disulfide-linked chains (HA_1 and HA_2) (Lamb and Krug, 2001). In the late phase of the replication cycle, viral RNAs are packaged into helical vRNP complexes with P and NP in the host-cell nucleus and subsequently exported into the cytosol to be assembled with the other structural proteins and packaged into progeny virions (Lamb and Krug, 2001; Cros and Palese, 2003). Nuclear RNP export and many other steps of the influenza virus life-cycle are strictly controlled by a complex network of host cell signaling pathways activated by the virus. (Bui *et al.*, 2000; Pleschka *et al.*, 2001; Nencioni *et al.*, 2003; Palamara *et al.*, 2005).

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Influenza, like other viruses uses several strategies to manipulate host cell machinery to its advantage. Among these, the imbalance of intracellular redox state caused by many viruses could play an important role in modulating the activity of several signaling pathways. In particular, a mild oxidative imbalance like that caused by viral infections (Fraternale *et al.*, 2006), ligand/receptor binding (Akhand *et al.*, 2002), cytokines (Nakamura *et al.*, 1997), etc. could result in localized oxidation of reactive cysteine residues of "redox sensitive" proteins, thus representing a molecular switch able to reversibly activate/deactivate protein function.

This review discusses some of main redox regulated intracellular pathways activated during influenza A virus infection and involved in regulating viral replication.

INTRACELLULAR REDOX STATE AND INFLUENZA VIRUS REPLICATION

The intracellular redox state is maintained in a physiologically-reduced condition by several molecules, principally by glutathione (GSH), the most prevalent intracellular thiol. GSH, a cysteine containing tripeptide (γ-glutamyl-cysteinyl-glycine), is found in eukaryotic cells at millimolar concentrations, and it takes part directly or indirectly in many important biological events including protein synthesis, enzyme activity, metabolism and cell protection. GSH is the most powerful intracellular antioxidant, and the GSH/oxidized glutathione ratio (GSSG) serves as a representative marker of the antioxidative capacity of the cell (Meister *et al.*, 1983). Remarkably, in the past decade new roles of GSH have been discovered in signal transduction, gene expression, apoptosis, protein glutathionylation, and nitric oxide (NO) metabolism. An imbalance of GSH has been observed in a wide range of diseases, including cancer, neurodegenerative disorders, cystic fibrosis, hepatitis, diabetes, Parkinson's disease, and as a natural part of the aging process (Townsend *et al.*, 2003).

Numerous studies have reported that viral infection is often associated with redox changes characteristic of oxidative stress (Peterhans *et al.*, 1997; Beck *et al.*, 2000; Kaul *et al.*, 2000). Indeed, a shift towards a pro-oxidant state has been observed in the cells and body fluids of patients infected with human immunodeficiency virus (HIV) (Eck *et al.*, 1989; Buhl *et al.*, 1989; Staal *et al.*, 1990; Herzenberg *et al.*, 1997; Banki *et al.*, 1998; Elbim *et al.*, 1999) and the hepatitis C virus (Barbaro *et al.*, 1996; Boya *et al.*, 1999; Gong *et al.*, 2001).

In particular, an alteration of the endogenous levels of GSH has been found *in vitro* in experimental infections with Herpes simplex virus type 1 (HSV-1) (Palamara *et al.*, 1995), Sendai virus (Garaci *et al.*, 1992), and HIV (Palamara *et al.*, 1996; Garaci *et al.*, 1997), as well as *in vivo* in HSV-1 and HIV infection (Nucci *et al.*, 2000; Mihm *et al.*, 1995; Choi *et al.*, 2000). A decrease in GSH levels and general oxidative stress have also been demonstrated during influenza virus infection in both *in vivo* and *in vitro* experimental models (Hennet *et al.*, 1992; Mileva *et al.*, 2000; Nencioni *et al.*, 2003; Cai *et al.*, 2003).

In particular, the bronchoalveolar lavage fluid from infected mice showed increased production of superoxide (Buffinton *et al.*, 1992), increased activity of the O_2^- generating enzyme xanthine oxidase (Oda *et al.*, 1989; Suliman *et al.*, 2001), decreased concentrations of GSH and increased levels of GSSG and malondialdehyde, an indicator of lipid peroxidation (Suliman *et al.*, 2001). Moreover, nutritional deficiency of antioxidants like selenium, results in greater lung pathology and altered immune function in mice infected with influenza virus (Beck *et al.*, 2004).

We previously demonstrated that different cell populations display differential permissiveness to influenza virus infection, depending on their intracellular GSH content and Bcl-2 expression (Nencioni *et al.*, 2003). In particular, we found that cells efficiently expressing Bcl-2 had consistently higher intracellular GSH contents than Bcl-2 negative cells, and that the higher reducing conditions within Bcl-2⁺ cells could interfere with the expression and maturation of late viral proteins like HA and M1.

However, this effect cannot fully account for the marked reduction in influenza virus replication observed in Bcl-2 expressing cells. Indeed, we demonstrated that at least two different steps in the influenza virus life-cycle are involved in Bcl-2/GSH mediated viral inhibition: the expression of late viral proteins and nuclear-cytoplasmic translocation of vRNPs (Nencioni *et al.*, 2003). This suggests that both Bcl-2 expression and GSH

content contribute to the host cell's ability to down regulate virus replication.

The involvement of GSH in controlling viral replication has been well demonstrated. Exogenous administration of several molecules (i.e. GSH, GSH derivatives, GSH precursors such as glutamine or cysteine, α-lipoic acid) able to increase cellular GSH concentration inhibits the replication of several viruses, including the influenza virus, through different mechanisms (Fraternale *et al.*, 2006). Cai *et al.* (2003) demonstrated that, when added extracellularly, GSH had a dose-dependent anti-influenza effect in cultured cells. He suggested that such an effect was probably due to an inhibition of apoptosis and subsequent release of the active virus from dead cells, but it is likely that other mechanisms are also involved. The anti-influenza activity of GSH has also been demonstrated in an in vivo experimental model. In particular, the addition of GSH to the drinking water of influenza infected mice inhibited viral titer in the trachea and lungs (Cai *et al.*, 2003).

Our group has demonstrated that exogenous GSH inhibits replication of Sendai, HSV-1 and HIV-1 viruses by directly affecting their envelope glycoproteins (Garaci *et al.*, 1992; Palamara *et al.*, 1995; Palamara *et al.*, 1996). One of the characteristics shared by these proteins is that their assembly into oligomers depends on the formation of disulfide bonds, which are strongly affected by reducing agents such as GSH (Lukacs *et al.*, 1985; Vidal *et al.*, 1989). The influenza A virus glycoprotein HA is organized as a homotrimer, and each monomer consists of two disulfide-linked subunits, HA1 and HA2 (Lamb and Krug, 2001). We previously demonstrated that HA expression was significantly reduced in cells containing high levels of GSH (Nencioni *et al.*, 2003). In the same paper, we found that buthionine sulfoximine (B-SO) a specific inhibitor of GSH neo-synthesis strongly increased HA expression. Together, these data strongly suggest that reducing conditions within the host cell could interfere with disulfide bond formation, thus preventing the correct folding and maturation of HA and consequently its transport and insertion into the cell membrane (Braakman *et al.*, 1992). We are currently characterizing the mechanisms underlying the GSH inhibition of HA folding by using different GSH compounds, including a GSH derivative GSH-C4, which is able to enter cells more easily than GSH,

and inhibit replication of Sendai and HSV-1 viruses more efficiently than GSH (Palamara *et al.*, 2004; Fraternale *et al.*, 2006).

MAP KINASE SIGNALING AND INFLUENZA VIRUS REPLICATION

A complex network of host-cell pathways is involved in controlling influenza virus replication and the fate of infected cells (Figure 1). Indeed, influenza virus activates several signaling cascades, including the Mitogen Activated Protein Kinase (MAPK) pathways (Ludwig *et al.*, 2006). MAP kinases are members of discrete signaling cascades, and serve as focal points in response to a variety of extracellular stimuli. They are involved in controlling embryogenesis, cell differentiation, cell proliferation, cell death/survival, inflammatory response, and the activation of several transcription factors [i.e. Nuclear Factor-kB (NF-kB), Activator-protein 1 (AP-1) and Interferon regulatory factors (IRFs)]. Four distinct subgroups within the MAP kinase family have been described:

1) extracellular signal-regulated kinases (ERKs);
2) c-jun N-terminal or stress-activated protein kinases (JNK/SAPK);
3) ERK5/big MAP kinase 1 (BMK1);
4) the p38 MAPK.

Different isoforms are known for each kinase (Pearson *et al.*, 2001; Chang *et al.*, 2001; Zarubin *et al.*, 2005).

The activation of JNK and p38 MAPK after influenza virus infection is known to play a role in the inflammatory and apoptotic responses to the infection (Kujime *et al.*, 2000; Ludwig *et al.*, 2001; Mizumura *et al.*, 2003; Maruoka *et al.*, 2003; Lee *et al.*, 2005).

Several studies have investigated the role of phosphorylation in controlling the influenza virus lifecycle. The influenza A virus has six phosphorylated proteins, including NP (Kistner *et al.*, 1989), and phosphorylative events (Bui *et al.*, 2002) are involved in nuclear-cytoplasmic translocation of vRNPs and other steps of the influenza virus lifecycle, such as cell penetration or budding (Root *et al.*, 2000; Hui and Nayak, 2002).

Particularly, it has been postulated that intracellular vRNP traffic is mediated by phosphorylation (Pleschka *et al.*, 2001; Bui *et al.*, 2002), al-
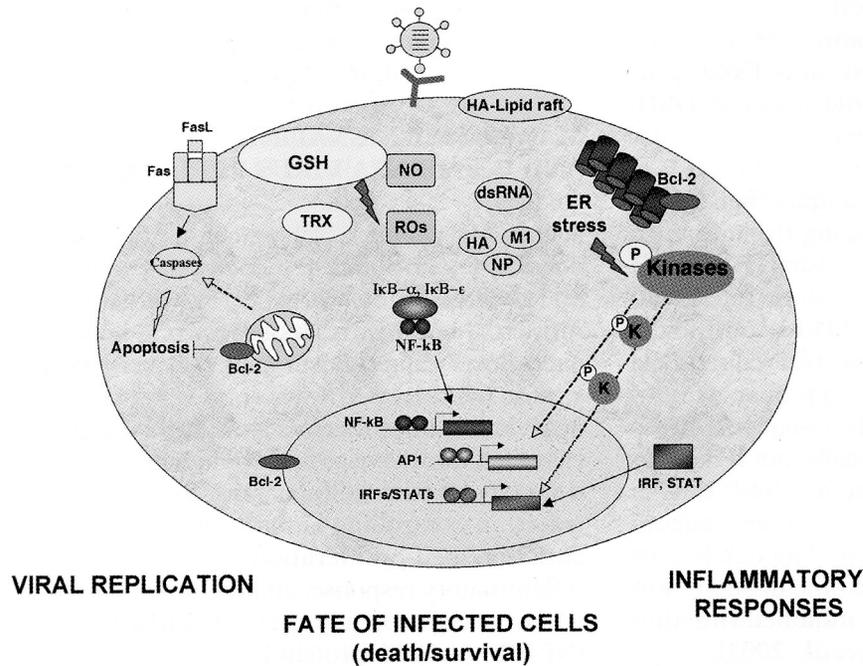
VIRAL REPLICATION

**FATE OF INFECTED CELLS
(death/survival)**

**INFLAMMATORY
RESPONSES**

FIGURE 1 - *Schematic representation of the main interactions between influenza virus and its host-cell.*

though the cellular kinases responsible for these events have yet to be identified.

Recently, it has been demonstrated that the accumulation of viral HA on the cell membrane and its close association with lipid-raft domains leads, by yet unknown mechanisms, to the activation of Raf/MEK/ERK cascade which in turn regulates vRNP export (Marjuki *et al.*, 2006). Other protein kinase inhibitors, such as H7 (Garland *et al.*, 1987 Bui *et al.*, 2000), and the ERK inhibitor U0126 (Pleschka *et al.*, 2001), also inhibit influenza replication by nuclear retention of vRNPs Our group has recently demonstrated that the natural polyphenol resveratrol (Frémont *et al.*, 2000) blocks vRNP traffic and influenza virus replication and that such an effect is related to a strong inhibition of PKC activity and its dependent pathways p38 MAPK and JNK (Palamara *et al.*, 2005). vRNP traffic has also been inhibited by adding SB203580, a specific inhibitor of p38 MAPK, suggesting that more than one cellular kinase is involved in controlling vRNP traffic (Nencioni *et al.*, submitted).

Other intracellular factors are involved in regulating viral replication and vRNP traffic. Indeed, we have recently shown that the expression of

Bcl-2 protein caused a strong decrease in vRNP nucleo-cytoplasmic translocation that in turn caused a decrease in influenza virus replication in Bcl-2 expressing cells (Nencioni *et al.*, 2003). Bcl-2 is a transmembrane protein well known for its involvement in the regulation of cell death/survival (Reed, 1998), and its expression by different cell types (e.g., lymphoid cells, neurons, epithelial lung cells) varies widely (Levine *et al.*, 1993; Tesfaigzi *et al.*, 1998; Nencioni *et al.*, 2003). The phosphorylation on several serine and threonine residues in the loop region between the Bcl-2 homology (BH) domains, BH4 and BH3, can profoundly alter the protein's anti-apoptotic potential in several cell contexts and in response to various apoptotic stimuli (Reed, 1998; Rosini *et al.*, 2000; Blagosklonny, 2001; Torcia *et al.*, 2001; De Chiara *et al.*, 2006). On the basis of all these data it seems reasonable to hypothesize that the decrease in vRNP traffic found in Bcl-2 expressing cells could be related to a protein's interaction with a cellular phosphorylative pathway involved in vRNP phosphorylation, but the mechanisms underlying this phenomenon have yet to be fully identified.

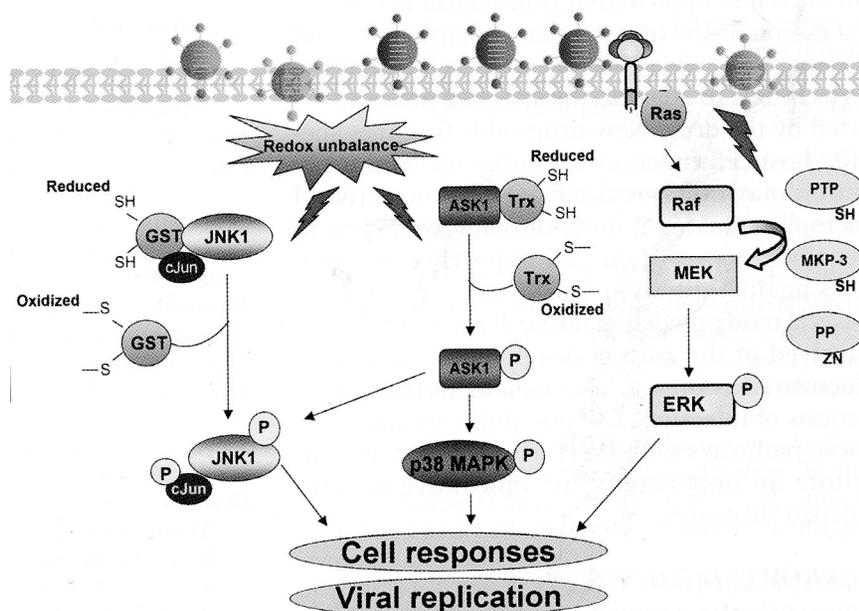The mechanisms through which the influenza

FIGURE 2 - *Redox regulation of MAP kinases activated during influenza virus infection. PTP: Protein tyrosine phosphatase; MKP-3: MAPK-selective phosphatases 3.*

virus activates intracellular kinases are still being actively investigated.

It is known that several kinases that are activated during influenza virus infection, such as the MAP kinases and the PKC family, are redox-sensitive (Torres and Forman, 2003). In particular, the addition of exogenous H_2O_2, and exposure to radiation or to drugs known to induce production of H_2O_2, such as menadione, activate the MAPKs (Guyton *et al.*, 1996; Lo *et al.*, 1996; Wang *et al.*, 1998; Kamata *et al.*, 1999). The modulation of GSH levels also plays a role in activating JNK and p38 MAPK, as shown after treatment with alkylating agents (Wilhelm *et al.*, 1997). ERK5 is activated by H_2O_2 in PC12 cells (Suzaki *et al.*, 2002). Furthermore, many studies have suggested an involvement of ROS in MAPK activation after cell stimulation with various agents (Hashimoto *et al.*, 2001). The mechanisms by which exogenously or endogenously produced ROS activate the MAPKs are not well understood. Several mechanisms have been proposed for activating JNK and p38 MAPK that involve ROS-dependent dissociation of a signalosome that maintains the pathway in an inactive state. ASK1, a MAPKKK for JNK and p38 MAPK are associated with reduced thioredoxin (Trx) in non-stressed cells.

A more oxidizing environment has been suggested to cause disulfide bridge formation on the Trx moiety thus destabilizing the dimer. As a result, ASK1 can escape from Trx binding and undergo multimerization, which corresponds to the active form of the enzyme (Saitoh *et al.*, 1998; Liu *et al.*, 2002). The dissociation of Trx from ASK1 leads to downstream activation of the phosphorylative cascade that ultimately induces the transcription of several genes involved in regulating cell cycle and apoptosis (Filomeni *et al.*, 2005). Another system that depends on a redox imbalance is the glutathione S-transferase Pi (GSTp)/JNK complex. Indeed, JNK associates with the monomeric form of GSTp and is inactive in non-stressed cells. JNK activation by ROS may result from oligomerization of GSTp and release of JNK (Adler *et al.*, 1999). The fact that several kinases responsible for efficient influenza virus replication are activated by a redox imbalance suggests that the pro-oxidant state induced by viral infection could play a key role in their modulation during infection. Further studies are currently in progress to verify this hypothesis.

CONCLUSIONS

All currently approved anti-influenza drugs target essential viral functions and/or structures, and the major drawback of this approach is that the virus is able to adapt to the selective pressure exerted by the drug. New drugs able to target specific host cell functions essential for viral replication may offer great advantages for antiviral therapies: making it more difficult for a virus to become drug resistant and improving effectiveness against different virus types and strains. Several redox-mediated host cell mechanisms are involved in the correct development of the influenza virus life-cycle as well as in the pathogenesis of infection. The possibility to inactivate these pathways with GSH derivatives may constitute an opportunity for innovative anti-influenza strategies.

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