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The nuclear protein Sam68 is redistributed to the cytoplasm and is involved in PI3K/Akt activation during EV71 infection

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ABSTRACT

Nuclear proteins can be triggered to be redistributed to the cytoplasm to assist with EV71 virus replication. This process is frequently involved in cellular signal transduction upon virus infection. In this study, we have demonstrated that a new nuclear protein, 68-kDa Src-associated in mitosis protein (Sam68), was translocated to the cytoplasm and was co-localized with EV71 during virus infection. Confocal microscopy and subcellular fractionation assay confirmed that virus 3C protease triggered the redistribution of Sam68 to the cytoplasm. Knockdown of Sam68 expression using ShRNA significantly inhibited virus replication, suggesting that Sam68 may be a host factor involved in EV71 life cycle. In addition, EV71-induced Akt phosphorylation involved a PI3K-dependent mechanism. Sam68 is known to be an upstream regulator of PI3K and our immunoprecipitation studies confirmed that Sam68 interacted directly with the p85 regulatory subunit of PI3K and mediated PI3K/Akt activation during EV71 infection. On the contrary, silencing of Sam68 dramatically abrogated Akt phosphorylation. These data, plus the fact that Sam68 is known to be a signaling adaptor protein, indicated that Sam68 is a signal molecule with a functional role in the PI3K/Akt signal pathway during EV71 infection.

1. Introduction

Enterovirus 71 (EV71) is one of the most important pathogens causing emerging infectious disease in humans and representing a major public health concern, particularly in the Asia-Pacific region (McMinn, 2002). EV71 has been reported to be associated with sporadic cases and outbreaks of a wide spectrum of diseases, including hand, foot and mouth disease (HFMD), herpangina, aseptic meningitis, encephalitis, poliomyelitis-like syndrome, and even fatal diseases (Bible et al., 2007). EV71 is a single, positive-stranded RNA virus that belongs to the *Enterovirus* genus of the *Picornaviridae* Family. During infection, the 7.4-kb genome of EV71 encodes a single polyprotein that is proteolytically cleaved into four structural proteins (VP1, VP2, VP3 and VP4) and seven nonstructural proteins (2A, 2B, 2C, 3A, 3B, 3C and 3D). VP1–VP4 constitute the virus capsid and the others participate in virus replication (Palacios and Oberste, 2005). To complete its life cycle, EV71 utilizes proteins of their host cell for virus replication (Lin et al., 2008, 2009b). At the same time, signaling cascades, such as the phosphatidylinositol 3-kinase (PI3K)-Akt, are activated during infection to prevent early apoptosis of the infected cells, thus increasing productive infection (Wang et al., 2012; Wong et al., 2005).

The PI3K/Akt signaling pathway plays important roles in cell survival, apoptosis, proliferation, migration and differentiation, as well as in metabolic regulation. PI3K consists of a regulatory subunit (p85) and a catalytic subunit (p110) and exhibits both protein kinase and lipid kinase activities (Dhand et al., 1994). Studies in mammalian cells have demonstrated that PI3K is activated by many different mitogenic signals (Stoyanov et al., 1995). PI3K phosphorylates phosphatidylinositol at the D-3 inositol ring. Akt, which is downstream of PI3K, is activated by the binding of 3-phosphoinositides arising from the action of PI3K. Phosphorylated Akt plays a central role in modulating the diverse downstream signaling pathways associated with many cellular processes (Datta et al., 1999; Yao and Cooper, 1995). PI3K interacts via its Src homology 2 (SH2) and SH3 domains with various proteins that contain a tyrosine phosphorylation-specific motif and a proline-rich domain. One of these proteins is the 68-kDa Src-associated in mitosis protein (Sam68), a member of the signal transduction and activation RNA (STAR) proteins (Fusaki et al., 1997). Sam68 is capable of both binding to RNA and interacting with signaling proteins containing SH2 and SH3 domains via its KH (heteronuclear ribonucleoprotein particle K homology) domain binding motifs and multiple tyrosine phosphorylation sites, respectively (Richard et al., 1995;

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Wong et al., 1992). It interacts with several signaling proteins, such as Grb2, phospholipase C-γ, p85-α, $p47^{phox}$, Cbl, SHP-1, p120GAP, $p21^{ras}$ and Nck (Fusaki et al., 1997; Huber et al., 1999; Jabado et al., 1998; Trub et al., 1997) and is a target of several tyrosine kinases, such as Src, Fyn, Lck, Tec, Jak3, Brk, Zap70 and Btk (Derry et al., 2000; Fusaki et al., 1997; Lang et al., 1997; Sanchez-Margalet and Najib, 1999).

It was previously demonstrated that Sam68 is relocalized to the cytoplasm after insulin or leptin engagement and is tyrosine-phosphorylated by the activated insulin receptor (IR) or leptin receptor (LP). The tyrosine-phosphorylated Sam68, like the insulin receptor substrate, then interacts with the p85 subunit of PI3K, thus activating the PI3K signaling pathway (Martin-Romero and Sanchez-Margalet, 2001; Sanchez-Jimenez et al., 2011; Sanchez-Margalet and Najib, 1999). Furthermore, during foot-and-mouse disease virus (FMDV) infection, the nuclear protein Sam68 is cleaved by the FMDV 3C protease and is redistributed to the cytoplasm to assist with virus replication (Lawrence et al., 2012). Similarly, EV71 replication takes place within protein-RNA complexes in the cytoplasm and EV71 infection induces an early activation of the PI3K/Akt signaling pathways (Wong et al., 2005). Thus, it is possible that the cellular protein Sam68, due to its protein- and RNA-binding properties, might act as an adapter protein that directs multiple cellular signaling proteins to the EV71 replication complex, both to support virus replication and to regulate signaling pathways. These interesting events prompted us to investigate a possible role for Sam68 in the activation of PI3K signaling pathway during EV71 infection. In this study, we present the evidences for such an interaction between Sam68 and the PI3K p85 subunit during EV71 infection, and show that Sam68 is redistributed into the cytoplasm and is involved in the activation of the PI3K/Akt signaling pathway to support EV71 replication.

2. Materials and methods

2.1. Cell and virus

HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) with 100 μg/ml penicillin and 100 μg/ml streptomycin. All cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2. EV71 (BrCr-Tr strain; GenBank accession number AB204852.1) was kindly provided by Prof Qi Jin (Institute of Pathogen Biology, Chinese Academy of Medical Science & Peking Union Medical College, Beijing, China). EV71 virus was adsorbed to confluent HeLa monolayer cells for 2 h at 37 °C with occasional shaking. Cells were then incubated in DMEM supplemented with 2.5% FBS until 80% cytopathic effect (CPE) was reached. After initial centrifugation at 2000 × *g* for 5 min, the cell pellet was subjected to three cycles of freezing and thawing followed by centrifugation to collect supernatants. Polyethylene glycol (*Mr*, 6000) is added to the final concentration of 8% for 2 h, followed by centrifugation at 12,000 × *g* for 2 h. The precipitate was dissolved in RNAase-free ddH_2O. Viral aliquots were stored at −80 °C (Wong et al., 2005).

2.2. Antibodies and PI3K inhibitor

Mouse EV71 VP1 monoclonal antibody (MAB979) was purchased from Millipore. Rabbit monoclonal anti-Sam68 (2981-1) antibody was purchased from Epitomics and mouse monoclonal anti-Sam68 (ab56836) antibody was purchased from Abcam. Mouse monoclonal anti-green fluorescent protein (GFP) (Sc-9996) antibody was purchased from Santa Cruz Biotech. Mouse anti-Flag monoclonal antibody (M2008) was purchased from Abmart. Rabbit monoclonal anti-phospho-Akt (Ser473) (4060), rabbit monoclonal anti-Akt (4685), rabbit monoclonal anti-phospho-PI3K p85 (Tyr458) (4228), rabbit monoclonal anti-PI3K p85 (4257), rabbit monoclonal anti-phospho-Src (Tyr416) (6943) and rabbit monoclonal anti-Src (2109) antibodies were purchased from Cell Signaling Technology (CST). Secondary antibodies conjugated to horseradish peroxidase (HRP) (goat anti-mouse [ZB-2305] and goat anti-rabbit [ZB-2301]) used for Western blot and secondary antibodies conjugated FITC (goat anti-rabbit [ZF-0311] and goat anti-mouse [ZF-0312]) and TRITC (goat anti-rabbit [ZF-0316] and goat anti-mouse [ZF-0313]) used for immunofluorescence were purchased from Beijing Zhongshan Biotech, China. The PI3K specific inhibitor wortmannin (CST) was freshly dissolved at 2 mM in DMSO and added to the culture medium at final concentrations of 0.2–l μM 1 h prior to virus infection and remained in the medium throughout the experiment.

2.3. Virus protein vectors, pEGFP-Sam68, ShRNA construction and transient transfection

To construct plasmids expressing the virus proteins 2A, 2B, 2C, 3A, 3B, 3C, and 3D, fragments of EV71 cDNA containing the coding region of each of these proteins were cloned into the *BamHI* and *EcoRI* sites of pcDNA3.0-Flag vector according to general methods. To construct pEGFP-Sam68, a Sam68 DNA fragment was amplified and cloned into the *EcoRI* and *SalI* sites of pEGFP, resulting in the EGFP fusion protein. For depletion of Sam68, two human RNAi targeting sequences were each cloned into the RNAi-Ready pSIREN-RetroQ to generate the corresponding RNAi-Ready pSIREN-RetroQ-Sam68 (Sam68 ShRNA). The RNAi targeting sequences were: Sam68 ShRNA#1, GGACCACAAGGGAATACAATC; and Sam68 ShRNA#2, GCATCCAGAGGATACCTTTGC (synthesized by Invitrogen) as described previously (Song et al., 2010). ShGFP (Invitrogen) was used as the negative control ShRNA. For plasmid transfections, the Lipofectamine 2000 (Invitrogen) was used according to the manufacturer's instructions.

2.4. Immunoprecipitation (IP) and Western blot analysis

Cells were washed two times with PBS and disrupted with lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl and 1% Triton X-100, supplemented with 1 tablet of Complete-Mini Protease Inhibitor Cocktail (Dhand et al.) per 50 ml buffer). Cell lysates were centrifugated at 12,000 × *g* for 10 min to harvest supernatants. Protein assays were performed on all supernatants using the Bradford method. For Western blot analysis of whole-cell lysates, samples, each containing 25–30 μg of protein equivalent, were dissociated in SDS-PAGE loading buffer and separated by SDS-PAGE as previously described (Lau et al., 2008). For immunoprecipitation, which was done at 4 °C, 0.5 mg of cell lysates were pre-cleared with 20 μl of protein A/G-Sepharose beads (Abmart) for 60 min. Non-specific complexes were pelleted by centrifugation at 10,000 × *g* at 4 °C for 10 min. The supernatants were removed and incubated with either 2.5 μg of anti-Sam68 antibody, anti-GFP antibody or the isotype control IgG for 60 min before the addition of 20 μl of protein A/G-Sepharose beads and incubated for another 60 min in an end-over-end rotor. Immunoprecipitates were pelleted and washed three times with RIPA buffer (150 mM NaCl, 50 mM Tris–HCl, pH 8.0, 1% Nonidet P-40 and 0.5% deoxycholate). After the final wash, the pellet was resuspended in 40 μl of 2 × SDS-PAGE loading buffer and was boiled for 10 min before being analyzed by Western blot. Western blot analysis was performed as described (Lau et al., 2008). Briefly, proteins were separated in 12% gradient SDS-PAGE and transferred to Hybond-P PVDF membranes (GE). Membranes were blocked for 2 h with 5% non-fat dry milk solution in Tris-buffered saline containing 0.5% Tween-20 (TBST). The membranes were then blotted with the required specific primary antibodies, followed by