Biochemical and Biophysical Research Communications 441 (2013) 953–957

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

NS1-binding protein abrogates the elevation of cell viability by the influenza A virus NS1 protein in association with CRKL

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ARTICLE INFO

Article history:
Received 18 October 2013
Available online 9 November 2013

Keywords:
NS1
CrkL
CrkII
NS1-BP
Cell proliferation
ERK

ABSTRACT

The influenza A virus non-structural protein 1 (NS1) is a multifunctional virulence factor consisting of an RNA binding domain and several Src-homology (SH) 2 and SH3 binding motifs, which promotes virus replication in the host cell and helps to evade antiviral immunity. NS1 modulates general host cell physiology in association with various cellular molecules including NS1-binding protein (NS1-BP) and signaling adapter protein CRK-like (CRKL), while the physiological role of NS1-BP during influenza A virus infection especially in association with NS1 remains unclear. In this study, we analyzed the intracellular association of NS1-BP, NS1 and CRKL to elucidate the physiological roles of these molecules in the host cell. In HEK293T cells, enforced expression of NS1 of A/Beijing (H1N1) and A/Indonesia (H5N1) significantly induced excessive phosphorylation of ERK and elevated cell viability, while the over-expression of NS1-BP and the abrogation of CRKL using siRNA abolished such survival effect of NS1. The pull-down assay using GST-fusion CRKL revealed the formation of intracellular complexes of NS1-BP, NS1 and CRKL. In addition, we identified that the N-terminus SH3 domain of CRKL was essential for binding to NS1-BP using GST-fusion CRKL-truncate mutants. This is the first report to elucidate the novel function of NS1-BP collaborating with viral protein NS1 in modulation of host cell physiology. In addition, an alternative role of adaptor protein CRKL in association with NS1 and NS1-BP during influenza A virus infection is demonstrated.

1. Introduction

Influenza A viruses belong to the family of Orthomyxoviridae, and the genome encodes up to 11 viral structural and nonstructural proteins. The non-structural protein 1 (NS1) of influenza A viruses is encoded by the smallest of the eight gene segments along with the nuclear export protein NS2 [1]. The major role ascribed to NS1 has been its inhibition of host immune responses, especially the limitation of both interferon (IFN) production and the antiviral effects of IFN-induced proteins, such as dsRNA-dependent protein kinase R and 2′5′-oligoadenylate synthetase/RNase L. In addition, NS1 also modulates other important aspects of the virus replication cycle, including viral RNA replication, viral protein synthesis, and general host-cell physiology [1,2].

NS1 was found to interact with a host protein termed NS1-binding protein (NS1-BP), a 70 kDa cellular protein that was shown to inhibit pre-mRNA splicing of a reporter gene in vitro [3]. Upon infection, NS1-BP was dispersed from speckles and redistributed throughout the nucleus, and NS1 was also found to alter the subcellular localization of splicing factors [3]. In addition, a recent report showed that NS1-BP interacted with the heterogenous nuclear ribonucleoprotein (hnRNP) K to promote splicing of M1 mRNA, which yields the viral M2 mRNA segment [4]. Thus, NS1-BP and hnRNP K were revealed as key mediators of influenza A viral gene expression and replication. However, the role of NS1-BP during influenza A virus infection especially associated with NS1 in host cell physiology remained unknown [1].

NS1 is a multifunctional protein consisting of an RNA binding domain and several protein–protein interaction motifs including

Abbreviations: NS1, non-structural protein 1; SH, Src-homology; NS1-BP, NS1-binding protein; HEK, human embryonic kidney; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; MTT, 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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http://dx.doi.org/10.1016/j.bbrc.2013.11.011

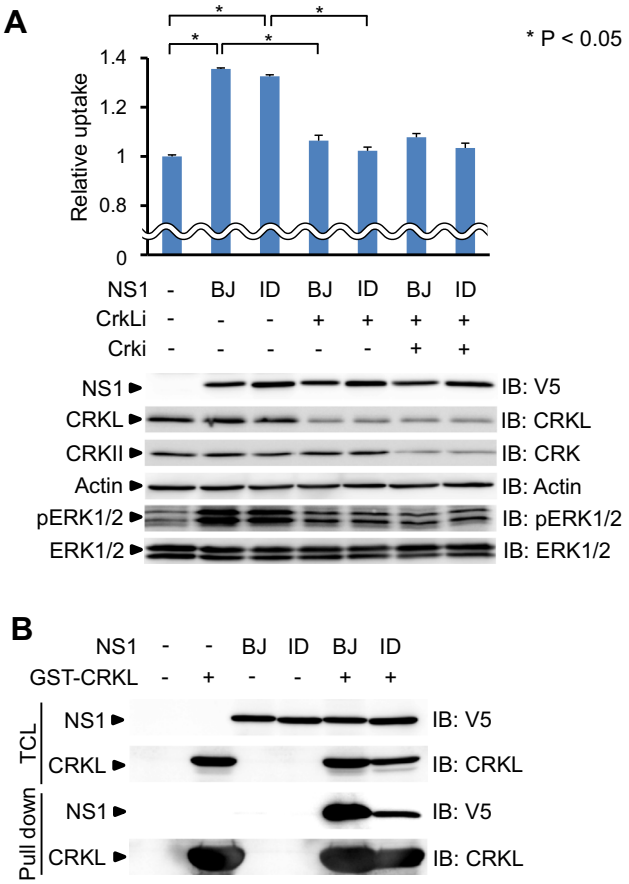

Fig. 1. NS1 elevated cell viability in a CRKL-dependent manner. (A) HEK293T cells were transfected with various combinations of expression plasmids for NS1, and/or siRNA-producing plasmid for CRKL (CrkLi) and CRK (Crki) as indicated. Cell growth activity was examined by MTT assay (upper panel), and the cell lysates were analyzed by Western blotting with indicated antibodies (lower panel). Error bars represent SD of three independent trials. (B) HEK293T cells were transfected with various combinations of expression plasmids for NS1 and GST–CRKL. After 36 h, cell lysates were subjected to GST pull-down assay. (BJ, Beijing; ID, Indonesia; TCL, total cell lysate.)

three Src-homology (SH) binding motifs, one SH2 binding motif and two SH3 binding motifs [5]. Recent studies have demonstrated that during influenza A virus infection NS1 protein activates the phosphatidylinositol 3-kinase (PI3K) signaling pathway, apparently via its association with the p85 regulatory subunit of PI3K [6–9]. Activation of the PI3K pathway seems to be important for influenza A virus replication, because in cell culture studies recombinant viruses with mutations that prevented binding of NS1 to p85 formed much smaller plaques and grew to 10-fold lower titers than the wild-type virus [9]. Moreover, compounds that inhibit PI3K can strongly suppress influenza A virus replication [7,9,10].

Recently, it was shown that NS1 proteins of avian virus strains associate with the adaptor proteins CRK and CRK-like (CRKL) [11]. Signaling adaptor protein CRK, which carries SH2 and SH3 domains, was originally identified as avian sarcoma virus CT10 (chicken tumor 10)-encoding oncogene product v-CRK [12], and this was followed by isolation of its mammalian homologs, CRKI, CRKII and CRKL [13,14]. The CRKL protein has high sequence identity within the SH2 and SH3 domains of CRKII which associates with $p130^{Cas}$ and paxillin through its SH2 domain and transmits signals to multiple downstream effectors by SH3 domain-binding proteins including C3G and DOCK180 [15–17]. A previous report showed that phosphorylated CRKL activates Ras and Jun kinase signaling pathways and transforms mouse fibroblasts in a BCR–ABL-dependent fashion [18]. In this study, we analyzed the intracellular association of NS1-BP, NS1 and CRKL to elucidate the physiological roles of these molecules in influenza A virus-infected cells.

2. Materials and methods

2.1. Plasmid

The cDNA fragment of human NS1-BP was obtained by RT-PCR using the HEK293T cells and subcloned into the mammalian expression vector pCXN2-Flag-V5, generating pCXN2-Flag-NS1-BP-V5. A/Beijing/262/95 (H1N1) and A/Indonesia/6/05 (H5N1) cDNA were cloned from total cellular RNA of virus-infected Madin–Darby canine kidney (MDCK) cells, and NS1 was subcloned into pcDNA3.1/V5-His vector (Invitrogen, Carlsbad, CA). pCXN2-Flag-CrkL, pLenti6.4-CrkLi1064 and pSUPER-Crki were described previously [19–21]. pEBG-CrkL, pEBG-CrkL-SH2SH3(N), pEBG-CrkL-SH2, pEBG-CrkL-SH3(N) and pEBG-CrkL-SH3(C) were generated by PCR using the CrkL cDNA as a template [22]. pEBG-CrkL-SH3(N)W160L was constructed by PCR from pEBG-CrkL-SH3(N).

2.2. Transfection and immunoblotting (antibodies)

HEK293T cells were maintained and cultured in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 U/ml penicillin and streptomycin. HEK293T cells were transiently transfected with expression plasmids using FuGENE HD (Roche Applied Science, Mannheim, Germany), according to the manufacturer's instructions. Cells were lysed with lysis buffer [0.5% Triton X-100, 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate (Na_3VO_4), and protease inhibitor mixture (Complete, EDTA-free®, Roche Molecular Biochemicals, Germany)] for 30 min on ice and centrifuged at 20,600*g* for 10 min at 4 °C. Antibodies were obtained from the following sources: anti-CRKL (C20) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-CRK (Transduction Laboratories, Lexington, KY, USA); anti-V5 (Invitrogen, Carlsbad, CA); anti-Actin (Millipore, Billerica, MA, USA); anti-Flag M2-peroxidase (HRP) (Sigma, St. Louis, MO, USA); anti-p44/42 MAP kinase (ERK1/2) (Cell Signaling Technologies, Beverly, MA, USA); and anti-phospho-p44/42 MAPK (T202/Y204) (Cell Signaling Technologies). The rabbit polyclonal antibody against GST was developed in our laboratory.

2.3. MTT assay

The MTT assay was performed using Cell Proliferation Kit I (Roche, Mannheim, Germany) according to the manufacturer's instructions. Briefly, 1.0×10^4 cells were seeded onto 96-well plates in 100 µl culture medium. 24 h later, MTT labeling reagent was added to the wells. The wavelength to measure absorbance of the formazan product was 550 nm, and the reference wavelength was 655 nm.

2.4. Pull-down assay

The cleared lysates were incubated with glutathione-Sepharose 4B beads (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) at 4 °C for 60 min. After washing with ice-cold lysis buffer, proteins were separated by SDS–polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride filter (Millipore, Billerica, MA, USA) by standard method.