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**HOKKAIDO UNIVERSITY**
Title page

Original Article

NS1-binding protein abrogates the elevation of cell viability by the influenza A virus

NS1 protein in association with CRKL

Masaya Miyazaki a, Hiroshi Nishihara b*, Hideki Hasegawa c, Masato Tashiro d, Lei Wang b, Taichi Kimura a, Mishie Tanino a, Masumi Tsuda a, and Shinya Tanaka a,b

a Department of Cancer Pathology and b Translational Pathology, Hokkaido University Graduate School of Medicine, N15W7, Kita-ku, Sapporo, 060-8638, Japan

Department of Pathology, and dInfluenza Virus Research Center, National Institute of Infectious Diseases, Sinjuku-ku, Tokyo, Japan

* Corresponding Author: Hiroshi Nishihara, M.D., Ph.D.

Department of Translational Pathology, Hokkaido University Graduate School of Medicine, N15W7, Kita-ku, Sapporo, 060-8638, Japan;

Tel: +81-11-706-5053, FAX: +81-11-706-5902, e-mail: hnishihara@med.hokudai.ac.jp

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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/Beijin (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.

(231 words)

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
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 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, 7, 8, 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 homologues, CRKI, CRKII and CRKL [13,14].

The CRKL protein has high sequence identity within the SH2 and SH3 domains of CRKII which associates with p130Cas and paxillin through its SH2 domain and transmits signals to multiple downstream effectors by SH3 domain-binding proteins including C3G and DOCK180 [15,16,17]. 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.
Materials and Methods

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 [19,20,21]. 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).

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$_3$VO$_4$), and protease inhibitor mixture (Complete, EDTA-free®, Roche Molecular Biochemicals, Germany)] for 30 min on ice and centrifuged at 20,600g 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.

**MTT assay**

The MTT assay was performed using Cell Proliferation Kit I (Roche, Mannheim, Germany) according to the manufacturer’s instructions. Briefly, $1.0 \times 10^4$ cells were seeded onto 96-well plates in 100 μl culture medium. 24 hours 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.

**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.

**Statistical analysis**

Data were presented as mean±s.e.m., and unpaired two-tailed Student’s t-test was used for comparisons, with P<0.05 considered significant.
Results

NS1 elevated cell viability in CRKL-dependent manner

The influenza A virus non-structural protein 1 (NS1) is a multifunctional virulence factor which modulates general host cell physiology in association with signaling adapter protein CRK family proteins [11,23]. In fact, we observed that enforced expression of NS1 derived from both A/Beijing/262/95 (H1N1) influenza virus (BJ) and A/Indonesia/6/05 (H5N1) influenza virus (ID) elevated cell viability and induced phosphorylation of ERK in HEK293T cells (Fig. 1A). To elucidate the involvement of CRK family proteins in this survival activity of NS1, we performed knockdown assay for CRKII and CRKL using a shRNA-expression vector. As a result, reduced expression of CRKL significantly suppressed cell viability in MTT assay and also phosphorylation of ERK, while additional knockdown for CRKII revealed no further inhibition (Fig. 1A), suggesting that CRKL plays a predominant role in the NS1-mediated survival mechanism. The intracellular binding of NS1 (BJ/ID) and CRKL was confirmed by using GST-fusion protein in HEK293T cells (Fig. 1B).

NS1-BP and CRKL form intracellular complex with NS1 and modulate the survival activity of NS1
NS1 modulates general host cell physiology in association with various cellular molecules including NS1-BP which promotes pre-mRNA splicing in host cells [3] and regulates influenza A virus RNA splicing in cooperation with hnRNP K [4], although the role of NS1-BP in the survival mechanism remains still to be investigated. We first confirmed the intracellular association of NS1 (BJ/ID), NS1-BP and CRKL in HEK293T cells (Fig. 2A). The pull-down assay using GST-fusion CRKL revealed that CRKL formed the complex with NS1-BP in the presence of NS1 (BJ/ID). Moreover, we found that enforced expression of NS1-BP canceled the elevation of cell viability and phosphorylation of ERK by NS1 (BJ/ID) regardless of CRKL overexpression (Fig. 2B).

**NS1-BP binds to N-terminus SH3 domain of CRKL**

CRKL consists of one SH2 domain and two SH3 domains, and the N-terminus SH3 domain was identified as the preferred binding partner for NS1 [11]. Interestingly, NS1-BP could bind to the full length of CRKL without the presence of NS1 (data not shown, and Fig. 3B), so we created the truncated mutants of GST-fusion CRKL as indicated in Fig. 3A, and co-transfected them with NS1-BP in HEK293T cells to confirm the responsible element of CRKL for formation of intracellular complex with
NS1-BP. The N-terminus SH3 domain (SH3(N)) of CRKL was essential for binding to NS1-BP, but the C-terminus SH3 (SH3(C)) was not involved in such binding (Fig. 3B). Contrary to our expectation, however, the mutated SH3(N) (W160L), in which the SH3-dependent protein interaction was abrogated [24], also bound to CRKL as well as wild-type SH3(N) (Fig. 3C), meaning that the PXXP-independent binding mechanism would be involved.
Discussion

NS1-BP was originally identified as an intracellular binding partner to interact with NS1 of influenza A viruses, and was previously shown to inhibit pre-mRNA splicing of a reporter gene in vitro [3]. In addition, a recent report showed that NS1-BP was one of the key mediators of influenza A viral gene expression and replication through the interaction with hnRNP K [4]. NS1-BP also regulates the concentration of functional aryl hydrocarbon receptor in mammalian cells, which is a ligand-activated transcription factor with important roles in metabolic adaptation, dioxin toxicology, and vascular development [25]. Moreover, NS1-BP enhances the inhibitory effect exerted by MBP-1 on c-Myc promoter, and the overexpression of both proteins resulted in an increased repression of basal c-Myc transcription and consistently affected the steady state levels of endogenous c-Myc mRNA, suggesting the distinct roles of alpha-enolase and its MBP-1 variant in maintaining mammalian cell homeostasis [26]. Here we revealed a novel function of NS1-BP to regulate cell viability through the inhibition of the ERK pathway which was stimulated by NS1 in association with CRKL, although enforced expression of NS1-BP alone demonstrated a negligible effect in survival activity (Fig. 2B). The N-terminus SH3 domain of CRKL was identified as a responsible element to associate with NS1-BP (Fig. 3) as well as with NS1 [11,23];
however, the W160L mutation of the N-terminal SH3 domain which specifically impaired the binding function of the SH3 domain [24] did not alter the affinity to NS1-BP. In fact, NS1-BP does not contain the consensus sequence (RKXXPXXP or PXXPXR) to bind the SH3 domain in its amino acid sequence [3], suggesting that the binding between NS1-BP and CRKL-SH3(N) could be in an SH3-independent manner.

The viral NS1 protein is widely regarded as the common factor by which all influenza A viruses antagonize host immune responses [1]. In addition, influenza A viruses have developed various means to inhibit apoptosis and sustain viability of host cells in order to avoid the cellular antiviral mechanisms that limit virus replication [8,27]. PI3K/Akt plays an important role in numerous host-cell processes, including anti-apoptosis, cell growth, proliferation and cytokine production/signaling [7,8,28,29]. Currently, the molecular mechanism of NS1-mediated PI3K activation is explained as follows; NS1 binds the inter-SH2 domain of p85b, thereby blocking normal inhibitory contacts between p85b and p110 [28,30]. In our study, no significant reduction of phosphorylation level of Akt by enforced expression of NS1-BP in HEK293T cells was observed (data not shown), and thus we concluded that NS1-BP would not be involved in NS1-mediated PI3K activation. Meanwhile the Ras-Raf-MEK-ERK signal transduction cascade participates in the regulation of a large variety of processes
including cell adhesion, cell cycle progression, cell migration, cell survival, differentiation, metabolism, proliferation, and transcription [31], and CRKL also participates in this signal transduction cascade and regulates various cell physiological conditions [14,32]. Besides the previous report regarding the ERK activation during the influenza A virus infection [33], our result proposes an alternative pathway of NS1-mediated ERK activation which collaborates with CRKL to avoid the cellular antiviral mechanisms in addition to PI3K/Akt activation (Fig. 4).

In summary, we explored the novel function of NS1-BP collaborating with viral protein NS1 in the modulation of host cell viability through the activation of ERK. In addition, an alternative role of adaptor protein CRKL in association with NS1 and NS1-BP during influenza A virus infection was elucidated.

**Acknowledgement**

Influenza virus A/Indonesia/6/05 (H5N1) was kindly provided by Dr. Pretty Multihartina Djoko Sasono.
Figure Legends

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 hs, cell lysates were subjected to GST pull-down assay. (BJ; Beijing, ID; Indonesia, TCL; total cell lysate)

Fig. 2

NS1-BP and CRKL form an intracellular complex with NS1 and modulate the survival activity of NS1. (A) HEK293T cells were transfected with various combinations of plasmids for NS1-BP, NS1 and GST-CRKL. After 36 hs, cell lysates were subjected to GST pull-down assay. (B) HEK293T cells were transfected with various combinations of expression plasmids for NS1, NS1-BP and CRKL. 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.

**Fig. 3**

NS1-BP binds to N-terminus SH3 domain of CRKL.  (A) Schematic structures of wild-type GST-CRKL and each of the truncated mutants.  (B) Association of NS1-BP and CRKL mutants was analyzed by GST pull-down assay.  HEK293T cells were transfected with NS1-BP and various types of GST-CRKL expression vector, and cell lysates were subjected to GST pull-down assay.  (C) The expression plasmid for wild type (WT) or W160L mutant (W160L) of CRKL-SH3(N) was co-transfected with the NS1-BP expression plasmid into HEK293T cells and cell lysates were subjected to GST pull-down assay.

**Fig. 4**

The proposed schematic model of protein interaction among CRKL, NS1 and NS1-BP in the influenza A virus-infected cell is shown. NS1 contributes proliferation of influenza A virus-infected cells in cooperation with CRKL through the phosphorylation
of ERK in addition to Akt (left), while NS1-BP interacts with CRKL and NS1, resulting in attenuation of ERK phosphorylation and cell proliferation activity.
References


Fig. 1.

A

NS1  -  BJ  ID  BJ  ID  BJ  ID
CrkLi - - +  +  +  +
Crki  -  -  -  -  +  +

Relative uptake

IB: V5
IB: CRKL
IB: CRK
IB: Actin
IB: pERK1/2
IB: ERK1/2

* P < 0.05

B

NS1  -  -  BJ  ID  BJ  ID
GST-CRKL - + - - + +

Pull down TCL

NS1  IB: V5
CRKL  IB: CRKL
NS1  IB: V5
CRKL  IB: CRKL
Fig. 2.

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* P < 0.05

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Fig. 3.

A

GST

SH2

SH3(N)

SH3(C)

GST

SH2

SH3(N)

B

NS1-BP

GST-CRKL

full

SH2

SH3(N)

SH3

(N)

SH3

(C)

TCL Pull down

IB: FLAG

IB: GST

IB: FLAG

IB: GST

Mut: W160L

C

NS1-BP

GST-CRKL

-GST-SH3(N)

WT

WT

Mut

Mut

TCL Pull down

IB: FLAG

IB: GST

IB: FLAG

IB: GST

Mut: W160L
Fig. 4.