International Journal of Multidisciplinary Research And Studies

Available online at https://ijmras.com/

INTERNATIONAL JOURNAL OF MULTIDISCIPLINARY RESEARCH AND STUDIES
ISSN: 2640 7272
Volume: 04.; **Issue**: 06/ 2021

Page no.- **10/10**

THE HIDDEN MYSTRY BEHIND ESTRICTION OF VIRAL REPLICATION BY MUTATION OF THE INFLUENZA VIRUS MATRIX PROTEIN

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ARTICLE INFO

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ABSTRACT

The matrix protein (M1) of the influenza virus plays a vital role in viral assembly and encompasses a form of functions, including association with influenza virus ribonucleoproteins (RNP). The RNA-binding domains of M1 are mapped to 2 independent regions: a zinc finger motif at organic compound positions 148 to 162 and a series of basic amino-alkanoic acids. To further understand the role of the RNP-binding domain of M1 in viral assembly and replication, mutations within the coding sequences of RKLKR and therefore the zinc finger motif of M1 were constructed employing a PCR technique and introduced into wild-type influenza virus by reverse genetics. These results indicate that the RKLKR domain of the M1 protein plays a crucial role in viral replication. The genome of influenza a pandemic consists of eight distinct segments of negative-sense RNA coding for a minimum of 10 viral proteins, of which 3 are known to function as polymerases. The viral RNA, nucleoprotein (NP), and polymerases are closely related to the ribonucleo protein (RNP) (11, 18, 22). Matrix protein (M1) is found between the RNP and also the inner surface of the lipid envelope within the intact virion (1, 3, 33). Two major external glycoproteins, hemagglutinin (HA) and neuraminidase (NA), and a little protein, M2, which is a trans membrane channel are anchored within the viral envelope (20, 36). M1 isn't only a necessary structural component of the virion but also participates in other steps during the replication of the influenza virus. During early infection, dissociation of M1 from RNP is required for entry of viral RNP into the cytoplasm of the host cell (4, 12, 21). Dissociation is triggered by the transport of H+ ions across the viral membrane by M2 (12, 19, 36). it's been shown also that M1 is transported from the cytoplasm into the nucleus during early viral replication (27). Later within the replication cycle, the buildup of M1 within the cytoplasm is concomitant with the nuclear export of RNP (4, 5, 13, 16, 35). within the maturation of viral particles, the M1/NP ratio of viral particles influences the virion's morphological features and

therefore the infectivity of the released viruses (29). The interactions of M1 with RNP are studied extensively (2, 6, 26, 31, 33, 41). Two domains in M1 are shown to affect its association with RNA (41, 42). One RNA-binding domain, containing a zinc finger motif (148C-C—-H-H162), has been shown to keep company with zinc ions (7) and to inhibit viral replication (23). the opposite domain, residing during a palindromic stretch of basic amino acids (101-RKLKR-105), has been shown to bind viral RNA (8, 37, 42), fulfilling a prediction supported by X-ray crystallographic studies (34). This domain also is a nuclear localization signal (NLS) for M1 (40, 43), but its role in viral replication is a smaller amount certain. Although it's been reported that under acidic conditions, M1 dissociates from RNP, leading to a discount in the transport of RNP from the nucleus to the cytosol (4), the biological significance Our recent studies demonstrate that viral RNP isn't assembled within the absence of M1 (15).

KEYWORDS: Influenza Virus**,** viral replication, crystallographic studies, RNA-binding domain.

INTRODUCTION

The communicable disease, influenza or "the flu" is probably the foremost familiar respiratory tract infection within the world. Within u. s. alone, approximately 25 to 50 million people contract influenza every year. The symptoms of the flu are just like those of respiratory illness but tend to be more severe. Fever, headache, fatigue, muscle weakness and pain, pharyngitis, dry cough, and a runny or stuffy nose are common and should develop rapidly. Gastrointestinal symptoms related to influenza are sometimes experienced by children, except for most adults, illnesses that manifest in diarrhea, nausea, and vomiting aren't caused by the influenza virus though they're often inaccurately cited because of the "stomach flu." Several complications, like the onset of bronchitis and pneumonia, may occur in association with influenza and are especially common among the elderly, young children, and anyone with a suppressed system. Influenza is extremely contagious and is more common during the colder months of the year. Contrary to traditional belief, however, the climate itself isn't to blame for the rise in incidence, but rather is as a result of the greater amount of your time spent indoors near other individuals during inclement weather,

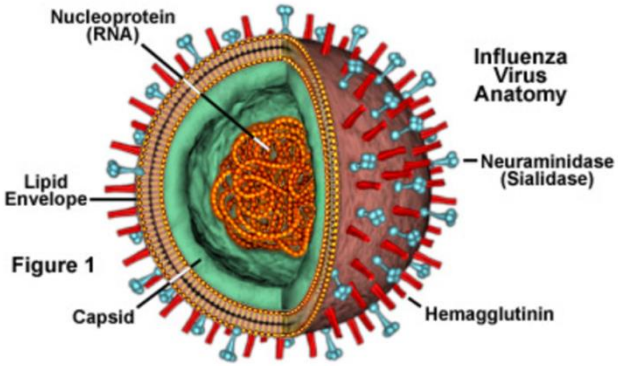

Figure 1

Incubation typically is from one to 2 days from the time of infection, and the majority begin to naturally get over symptoms within every week. The overwhelming majority of influenza-related deaths are caused by complications of the flu instead of the particular influenza virus. Three distinct varieties of influenza virus, dubbed A, B, and C, are identified. Together these viruses, which are antigenically distinct from each other, comprise their own viral family, Orthomyxoviridae. Most cases of the flu, especially people who occur in epidemics or pandemics, are caused by influenza a pestilence, which might affect a range of animal species, but the B virus, which normally is just found in humans, is chargeable for many localized outbreaks. The influenza C virus is morphologically and genetically different from the opposite two viruses and is usually non symptomatic, so is of little medical concern. Sometimes filamentous sorts of the virus occur also and are more

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common among some influenza strains than others. The influenza virion is an enveloped virus that derives its lipid bilayer from the cyto-membrane of a bunch cell. Two different kinds of glycoprotein spikes are embedded within the envelope. Approximately 80 percent of the spikes are hem-agglutinin, a trimeric protein that functions within the attachment of the virus to number cells. The remaining 20 percent just about of the glycoprotein spikes carries with it neuraminidase, which is assumed to be predominantly involved in facilitating the discharge of newly produced virus particles from the host cell. Within the envelope is that the influenza genome, which is organized into eight pieces of single-stranded RNA (A and B forms only; influenza C has 7 RNA segments). The RNA is packaged with nucleoprotein into a helical ribonucleo protein form, with three polymerase peptides for every RNA segment. Mutations within the antigenic structure of the influenza virus have resulted in several different influenza subtypes and strains. Specific forms of the virus are generally named in step with the actual antigenic determinants of hemagglutinin (13 major types) and neuraminidase (9 major types) surface proteins they possess, as in influenza A(H2N1) and A(H3N2). New strains of the nfluenza virus emerge because of a gradual process called antigenic drift, during which mutations within the virus antibody-binding sites accumulate over time. Through this mechanism, the virus can largely circumvent the body's system, which can not be able to recognize and confer immunity to a brand new influenza strain whether or not a private has already built up immunity to a special strain of the virus. Both A and B influenza viruses continually undergo antigenic drift, but the reformulation of influenza vaccines annually often enables scientists to require under consideration any new strains that have emerged. Influenza A also experiences another style of mutation called antigenic shift that ends up in a brand new subtype of the virus. Antigenic shift could be a sudden change in antigenicity caused by the recombination of the influenza genome, which may occur when a cell becomes simultaneously infected by two different strains of type influenza. The unusually broad range of hosts prone to influenza A appears to extend the likelihood that this event will occur. Particularly, the blending of strains that may infect birds, pigs, and humans is believed to be accountable for most antigenic shifts. Notably, in some parts of the globe, humans live near both swine and fowl, so human strains and bird strains, may readily infect a pig at the identical time, leading to a singular virus. New subtypes of influenza A develop abruptly and unpredictably so scientists are unable to arrange vaccines beforehand that are effective against them. Consequently, the emergence of a brand new subtype of the virus can cause a world pandemic during a very short amount of your time. In addition to vaccines, some other weapons are designed to combat the flu. The antiviral medications amantadine and rimantadine can help reduce the severity of illness in individuals with influenza that begin utilizing the drugs within two days of the onset of symptoms. These drugs work by hindering the change in pH that's necessary for the flu virion to release its contents into the cytosol of a number cells.

GENERATION OF M1 MUTANTS SIMILAR TO RNP- AND RNA-BINDING DOMAINS.

The RKLKR sequence is found between amino acids 101 and 105, and also the zinc finger motif is found between amino acids 148 and 162, of M1 (40, 41, 42). Figure Figure1B1B shows that plasmid Wt expressed the wild-type (wt) M gene; plasmid Del-101-RKLKR-105 contained an altered M gene expressing M1 with a deletion of RKLKR; plasmid M101-SNLNS-105 expressed an M protein with amino acids RKLKR replaced with SNLNS; plasmids M-R101S, M-K102N, M-K104N, and M-R105S each contained an altered M gene expressing one organic compound substitution of Arg with Ser or of Lys with Asn; and plasmid M-C148S contained a DNA sequence coding for an alteration predicted to disrupt the zinc finger motif (7). The wt and altered M genes were also subcloned into the pCR3.1 vector (Invitrogen, Carlsbad, Calif.), which contains a T7 promoter which will express its downstream protein in vitro. The mutated M1's were expressed in vitro by employing a coupled reticulocyte lysate system

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(Promega, Madison, Wis.) and were analyzed by autoradiographic densitometry after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Analysis of the proteins indicated that the migration of the mutated M1's loved that of WSN Wt M1 (Fig. (Fig.1C1C).

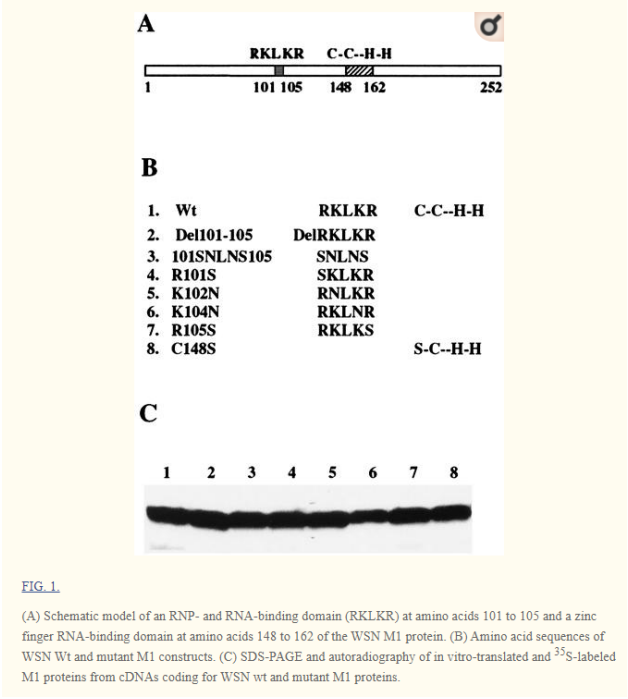

FIG. 1.

(A) Schematic model of an RNP- and RNA-binding domain (RKLKR) at amino acids 101 to 105 and a zinc finger RNA-binding domain at amino acids 148 to 162 of the WSN M1 protein. (B) Amino acid sequences of WSN Wt and mutant M1 constructs. (C) SDS-PAGE and autoradiography of in vitro-translated and ^{35}S-labeled M1 proteins from cDNAs coding for WSN wt and mutant M1 proteins.

Neumann et al. (24), with minor modifications. Briefly, 1 day before transfection, confluent 293T cells were subcultured into 12-well plates with a dilution of roughly 1:10 in Opti-MEM I medium (Invitrogen, Gaithersburg, Md.). a major structural protein, mutation of M1 might impair viral particle assembly. pCAGGS-WSN-M (a gift from Y. Kawaoka), coding for wt M1 protein, was also included within the transfection system, allowing the formation of complete virions expressing the mutant M1 proteins.

The DNA-Trans IT LT-1 mixture was incubated at a temperature for 45 min and added to the cells. day after transfection, 0.5 ml of Opti-MEM I containing 1 μg of tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin per ml was added to the cells. At different time points after post-transfection, samples were harvested. The titer of the virus resolve by HA units (HAU) or PFU assays. The virus particles generated by reverse-genetics were purified thrice by plaque formation in MDCK cells and amplified within the allantoic cavities of 9-day-old embryonated eggs. The altered M gene within the virus was confirmed by sequencing analysis (data not shown).

DELETION OF RKLKR OR SUBSTITUTION OF LYS WITH ASN IN RKLKR WAS A LETHAL MUTATION.

The seven-plasmid pol I-pol II system (carrying the PB2, PB1, PA, NP, HA, NA, and NS genes) plus plasmids expressing wt M1 protein and therefore the relevant individual M1 mutation. Virus titers within the supernatants, 48 h post transfection, were analyzed by measurement of HAU and PFU in MDCK cells (Table1). Approximately 16 HAU/ml and 6 × 106 PFU/ml were rescued from the transfection mixture containing the wt M gene. one aminoalkanoic acid substitution at position 102 (K102N) or position 104 (K104N) was also a lethal mutation for the M gene; there was no detectable infectious virus.

Prolonging the time period to 72 h after transfection failed to end in the recovery of infectious virus particles of the M1 mutants (data not shown).

TABLE 1.

Virus titers in the supernatants of 293T cells after plasmid transfection[a]

Transfectant virus	Amino acid	HAU/ml	PFU/ml
wt	101-RKLKR-105	16	6×10^6
Del101-105	DelRKLKR	ND[b]	ND
101SNLNS105	SNLNS	ND	ND
R101S	SKLKR	4	1×10^4
K102N	RNLKR	ND	ND
K104N	RKLNR	ND	ND
R105S	RKLKS	4	4×10^4
C148S	148-S-C-H-H-162	8	2×10^6

[a]293T cells were transfected with a modified reverse-genetics system to rescue M gene mutants, as described in Materials and Methods. The infectious particles in the supernatants of 293T cells were harvested 48 h posttranfection and titrated in MDCK cells.
[b]ND, none detectable.

At 48 h postinfection, the titer of infectious RIPs was approximately 103/ml for Del101-105, 101SNLNS105, K102N, or K104N.

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M1 PROTEINS WITH MUTATIONS IN ORGANIC COMPOUND POSITION 102 OR 104 REMAINED WITHIN THE CYTOPLASM, AND RNPS OF THE IDENTICAL MUTANTS REMAINED WITHIN THE NUCLEUS.

Replication-incompetent influenza viruses within the supernatants of 293T cells, transfected with plasmids expressing the Del101-105, 101SNLNS105, K102N, or K104N M1 mutant, were collected 48 h after transfection and transferred to MDCK cells. The cellular distributions of M1 and RNP in these cells were visualized by fluorescence microscopy staining with monoclonal antibodies to M1 and NP proteins.

Infected MDCK cells were fixed with freshly prepared 4% formaldehyde in phosphate-buffered saline (PBS) for 20 min at temperature. Background staining was blocked with 3% powdered milk in PBS for 1 h. Cells were then incubated at temperature for 40 min with a antibody to either M1 or NP. The cells were incubated with donkey anti-mouse immune serum globulin (IgG) conjugated with fluorescein and incubated for 40 min at temperature. Washed coverslips were mounted in 90% glycerol and 10% PBS in 3,4,5-trihydroxy carboxylic acid N-propyl ester to stop photobleaching. an contrast, newly synthesized NPs of those RIP-infected cells were retained within the nucleus, and no NP was detectable within the cytoplasm by fluorescence microscopy staining. MUTATION AT organic compound POSITION 101 OR 105 OF RKLKR OR within the ZINC FINGER MOTIF didn't AFFECT RNP NUCLEAR EXPORT. Because none of the R101S, R105S, and C148S substitutions resulted during a lethal mutation of the influenza virus (Table1), the cellular distributions of M1 and RNP within the resulting viruses

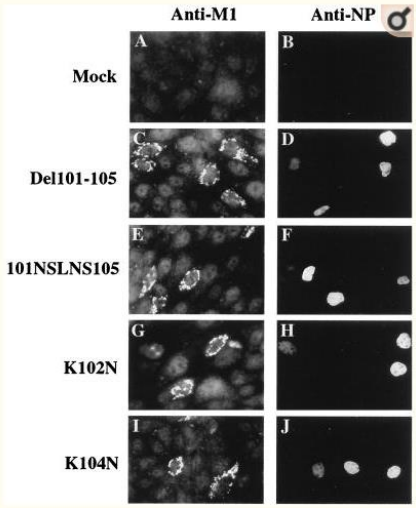

FIG. 2.

Immunofluorescence staining of MDCK cells infected with RIPs from the supernatants of transfected 293T cells. MDCK cells were infected for 16 h with defective M gene mutant particles in the supernatants of plasmid-transfected 293T cells. The cells were incubated with a mouse anti-M monoclonal antibody (A, C, E, G, and I) or a mouse anti-NP monoclonal antibody (B, D, F, H, and J) and with donkey anti-mouse IgG conjugated with fluorescein. The cellular distribution of immunofluorescence was determined with a transmission microscope under an epifluorescent UV light source. Magnification, ×472.

As shown in Fig. Fig.3, wt M1 resides mostly within the cytoplasm of infected cells. (Fig.3A). Figure 3A shows the nuclear localization of the M1 protein within the early replication stage of the virus, within which the bulk of M1 was within the nucleus of the infected cell. were basically identical as those in cells infected with wt virus (Fig.3A and B). These results indicate that substitution of the Arg residues at positions 101 and 105 of RKLKR didn't have a significant impact on nuclear export of RNPs which mutation of the zinc finger motif didn't affect nuclear export of NP the least bit. Substitution of organic compound Phe with Ser at position 79 has been identified because the only mutation within the M gene of the ts51 strain, a present temperature-sensitive mutant with a cutoff temperature of 39.5°C (38). viral growth at 33°C assayed by plaque titration of samples harvested at 2, 4, 8, 24, and 48 h post-infection. the info demonstrates that the M gene mutants grow at a rate kind of like that of the wt at 33°C, aside from the F79S transfectant virus, which had a rather lower titer at the identical temperature. Infectious particles were first detectable at 8 h post-infection, the height of the expansion was at 24 h post-infection, and virus titers of all mutants, including wt virus, declined 24 h post-infection. The slower

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growth of mutants at 39.5°C suggests that replication of the R105S, R101S, and C148S M1 mutants is also temperature labile at 39.5°C. Virus yields were also studied by measurement of HAU; growth rates were kind of like the expansion rate measured by PFU, except that the virus titers reached a plateau 48 h post-infection (data not shown).

THE PLAQUE SIZES OF M1 MUTANT VIRUSES ARE CORRELATED WITH THEIR GROWTH RATES.

Because mutated M gene transfectant viruses had different growth rates in MDCK cells, we determined the plaque formation of those viruses in MDCK cells. to review the plaque morphology of the mutant viruses, MDCK cells in 6-well-plates (diameter, 35 mm) were infected with serial 10-fold dilutions of plaque-purified transfectant viruses. The medium within the dish was replaced with 0.75% agarose in Eagle's MEM (E-MEM) containing 2 μg of trypsin/ml. Cells were fixed with 100% methanol and stained with 1% antibacterial. Figure55 shows the plaque formation of transfectant viruses in MDCK cells at 33°C. In contrast, the F79S transfectant virus, which had a lower rate of growth, formed small plaques (0.7 mm).

However, the ts51 virus, which had the identical M chromosomal mutation because the F79S virus, also formed large plaques (diameter, 2.3 mm). The plaque formation of the WSN and ts51 viruses has been studied extensively by Yasuda et al. (39). Their observations indicate that the ts51 virus, with one mutation at organic compound 79 (from F to S) in M1, forms large plaques. However, the substitution of Phe with Ser at organic compound 79 in M1 of the transfectant virus in our study showed that this substitution resulted in small plaques and a somewhat attenuated phenotype compared with those of present ts51. At this time, we don't have a satisfactory explanation for the difference between the transfectant F79S virus and therefore the present ts51 mutant virus.

The difference is also because of the gene constellation effect between transfectant virus and ts51. Although the zinc finger domain has inhibitory activity against viral replication when a peptide containing this domain is employed in cell culture (23), mutation of the zinc finger domain within the M gene didn't have a significant impact on viral replication supported our results However, the substitution of Arg with Ser in RKLKR in M1 had a minimal effect on viral replication. the various consequences of mutation at Arg versus Lys within the RKLKR sequence on viral replication could also be because mutation of Lys in RKLKR not only impairs RNP-binding activity but also disrupts the core consensus sequence of the NLS, K(R/K)X(R/K) (17). Table Table22 summarizes the comparative sequences of the NLS (amino acids 101 to 105) for influenza A viruses. the remainder of the organic compound sequences are strictly conserved.

The consensus sequence of the NLS of M1's in influenza A viruses, like H5N1, H3N2, and H1N1 viruses, is XKLKR. However, from our results, the substitution of Arg at organic compound position 105 had a minimal effect on viral replication, indicating that Arg at position 105 is additionally minor. Our previous research (41) indicated that the RKLKR sequence is involved not only within the RNA and RNP association but also within the nuclear translocation of M1, whereas the zinc the dearth of nuclear localization of M1 in RIP-infected cells (Del101-105, 101NSLNS105, K102N, and K104N mutants) and therefore the lack of cytoplasmic translocation of RNP might result from disruption. Nuclear localization and RNP-binding activities must be studied to substantiate whether one mutation of RKLKR like R101S or R105S will end in the reduction of nuclear localization of M1 and/or reduction of RNP-binding activities of M1. M1 protein plays a very important role in particle assembly and viral replication. Both the dissociation of M1 from RNP within the early phase of infection and also the association of M1 and RNP within the late phase of the infection are required for sufficient viral replication. it's believed that the association of M1with RNP results in translocation of RNP from the nucleus to the cytoplasm.

The essential organic compound sequence (RKLKR) of M1 is vital for the function of M1

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protein in viral replication by translocation of M1 from the cytoplasm into the nucleus and for interaction with RNP. Our recent studies (15) have demonstrated that viral RNA and M1 together promote the self-assembly of influenza virus NP into the quaternary helical structure almost like the everyday viral RNP.

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