Communication

Nuclear Import of Influenza Virus RNA Can Be Mediated by Viral Nucleoprotein and Transport Factors Required for Protein Import*

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We have fluorescently labeled one of the eight genomic segments of influenza virus RNA and a recombinant influenza viral protein, the nucleoprotein (NP), to investigate the requirement for their uptake into nuclei of digitonin-permeabilized cells. We found that the influenza viral NP behaves like a nuclear localization sequence (NLS) containing protein. Thus, at 0 °C it docks at the nuclear envelope only in the presence of the heterodimeric karyopherin (either karyopherin α/β or karyopherin α/α2), and docking is competitively inhibited by an unlabeled NLS containing substrate. Like other NLS-containing proteins, at 20 °C NP is imported into the nucleus after further addition of the GTPase Ran and of p10. In contrast, the fluorescently labeled, 890-nucleotide-long viral RNA segment does not dock to the nuclear envelope or enter the nucleus either in the presence of exogenous cytosol or of karyopherin heterodimer, Ran, and p10. However, in the presence of NP the RNA is able to dock and enter the nucleus with transport requirements indistinguishable from those for docking and entry of NP. These data indicate that uptake of the influenza virus RNA segment is not via a signal in the RNA but via an NLS of a viral protein such as NP.

The mechanism of entry of ribonucleoproteins into the nucleus is not known. Microinjection studies have suggested that import of some of the U small nuclear RNAs occurs by two biochemically distinct pathways that do not compete with the nuclear localization sequence (NLS)1-mediated route for nuclear import of proteins (1). However, all three pathways use the nuclear pore complex (NPC) as antibodies to some nucleoporins (a collective term for NPC proteins) or wheat germ agglutinin (some nucleoporins contain N-acetylglycosamine residues) block import of proteins as well as of RNPs (1) (for review see Ref. 2).

The genome of influenza virus is transcribed and replicated in the nucleus (3, 4). The entry of isolated influenza viral RNPs into the nucleus has been explored by microinjection and also been shown to proceed via the NPC (5, 6). However, it is not known by which pathway the viral RNP enters.

The influenza virus particle contains eight different negative strand RNAs varying in length from approximately 900 to 2,500 nucleotides (7). Each of the RNA molecules is coated by multiple copies of viral nucleoprotein (NP) at one NP per 20 nucleotides (8). A heterotrimeric RNA polymerase complex is located at one end of each of the viral RNAs (9). The viral matrix protein, M1, forms an inner shell around the RNPs and is bounded by a membrane containing the integral membrane proteins M2, hemagglutinin, and neuraminidase (10, 11). Virus entry into the cell is via hemagglutinin-mediated membrane fusion in a late endosomal compartment (12, 13). The M2 functions as an acid-activated channel for monovalent cations (14, 15) and appears to be required to divest the multimeric RNP complex of its M1, yielding individual viral RNPs. These RNPs that enter the nucleus both after infection (5) and after microinjection (6). Nuclear uptake is an ATP- and temperature-sensitive process (5, 6).

As each of the four viral RNA-associated proteins in the microinjected RNPs has been reported to contain an NLS (16–18), nuclear import might proceed via an NLS-mediated pathway. Consistent with this idea is the finding that in a yeast two-hybrid system, NP interacted with two proteins, termed NP1-1 (19) and NP1-3.2 These two proteins were subsequently shown to be the transport factors karyopherin α1 and karyopherin α2, respectively, that have been demonstrated to bind to NLS-containing proteins (20, 21).

Using digitonin-permeabilized mammalian cells and an NLS-containing protein, a number of transport factors required for import into nuclei have been isolated from cytosol. A heterodimeric protein complex, termed karyopherin (22), is required for targeting an NLS substrate to NPCs, and two proteins, the GTPase Ran (23, 24) and p10 (25), are required for transport into the nucleus. Either karyopherin α1 (20) (corresponding to NP1-1/hSRP-1/importin 60 (19, 26, 27)) or karyopherin α2 (21) (corresponding to NP1-3/Rch-1/hSRP-α1 (28, 29)) serves as NLS binding subunit (20, 21, 29) whereas karyopherin β (or importin 90 (30)) serves as an adapter subunit that mediates binding to peptide repeat-containing nucleoporins (21, 31).

To address the question of whether influenza viral RNA can enter the nucleus by itself or whether its entry is mediated by an associated NLS-containing protein, such as NP, we pre-

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References

1 The abbreviations used are: NLS, nuclear localization sequence; NP, nucleoprotein of influenza virus; NPC, nuclear pore complex; RNP, ribonucleoprotein; FITC, fluorescein isothiocyanate; BRL, Buffalo rat liver; vRNA, viral RNA; HSA, human serum albumin.

2 R. E. O’Neill and P. Palese, unpublished results.
pared unlabeled or fluorescently labeled components, either RNA (NS segment of influenza A/WSN/33 virus) or NP. These components were then incubated with digitonin-permeabilized cells in reactions assaying docking to the nuclear envelope and uptake into the nucleus using all recombinant transport factors. We found that NP by itself could be docked and imported into the nucleus in the presence of transport factors but that RNA by itself could not. However, RNA could be docked and imported in the presence of NP, demonstrating that viral RNA import into nuclei is by an NLS-mediated pathway.

EXPERIMENTAL PROCEDURES

Assays for Nuclear Docking and Import—Buffalo rat liver (BRL) cells were grown on coverslips and were extracted with digitonin (50 µg/ml for 5 min at room temperature) as described previously (20, 21). Docking of various fluorescently labeled substrates to the nuclear envelope of the digitonin-permeabilized cells was assayed by incubation at 0 °C in the presence of components indicated in the legends to Figs. 1 and 2 and was monitored by fluorescence microscopy (32). In contrast, import was assayed at room temperature (22 °C) and the fluorescent signal quantitated as described previously (32).

Recombinant Proteins—The expression and purification of recombinant karyopherin α1 (NPI-1/HRP) and karyopherin α2 (NPI-3/ Rch-2) (21), of recombinant karyopherin β (20), of recombinant human Ran loaded with GTP (33), and of recombinant human p10 were as described (21).

The NP of influenza A/PR/8/34 virus was subcloned between the EcoRI and Xhol restriction sites of pET28a (+) (Novagen, Inc.) for expression of a His 6-tagged recombinant protein. Bacterially expressed NP (containing 37 additional N-terminal residues and 6 N-terminal His) was purified by nickel-nitrotriacetic acid affinity chromatography. Fluorescein isothiocyanate (FITC) labeling of NP (260 µg/ml) was performed at room temperature in 0.1 M Na2CO3, pH 9.0, 50 mM NaCl, and 100 µg/ml FITC. FITC-NP was dialyzed against 20 mM Hepes, pH 7.3, 100 mM KCl.

Synthesis of Tetramethylrhodamine-labeled Viral RNA—pH2GNS (34) and pNP3-1 were digested with Hgial and Hincl restriction enzymes, respectively. pNP3-1 was constructed by inserting the Rch-1 (28) open reading frame between the EcoRI and Xhol restriction sites of pET28a (+) (Novagen, Inc.). Run-off transcription using T7 RNA polymerase generates an 890-nucleotide-long negative-sense RNA of the NS segment of influenza A/WSN/33 virus and a 790-nucleotide-long truncated NPI-3 transcript. 100-µl transcription reactions contained 40 mM Tris-HCl, pH 7.9, 6 mM MgCl2, 10 mM dithiothreitol, 2 mM spermidine, 10 mM NaCl, 400 mM each of GTP, CTP, and UTP, 200 µM ATP, 200 µM N6-(aminohexyl)ATP (Life Technologies, Inc.), 100 units of RNAse (Promega), 5 µg of phosphatase DNA, and 4 µl of TNT T7 RNA polymerase (Promega). Transcription reactions were incubated for 90 min at 37 °C. Template RNA was removed with RNase-free DNase I (Promega). RNA was purified by extraction with phenol-chloroform (1:1) twice, extraction with chloroform, and chromatography over a Sepharose G-50 column. Derivatization of RNA (150 µg/ml) was carried out in 0.2 M NaHCO3, pH 8.2, with 2 mg/ml tetramethylrhodamine isothiocyanate for 2 h at room temperature. Tetramethylrhodamine isothiocyanate-RNA was purified by chromatography over Sepharose G-50, extraction with phenol, and ethanol precipitation.

RESULTS

Import of Recombinant Protein NP of Influenza Virus into Nuclei of Digitonin-permeabilized Cells Requires Transport Factors—It was reported that influenza virus NP could enter into and accumulate in nuclei after microinjection into oocytes of NP itself or of the corresponding mRNA or cDNA (17). The sequence of NP that functions as an NLS to allow entry into the nucleus has not yet been mapped although a region encoding amino acids 327–345 has been reported to function in nuclear retention (17). To determine whether NP behaves like an NLS-containing substrate and is transported into nuclei of digitonin-permeabilized cells in a transport factor-dependent fashion, we prepared recombinant fluorescently labeled NP (NP*). As previously reported for a model import substrate (NLS conjugated to human serum albumin (NLS-HSA)) there was docking of NP* to the nuclear envelope only in the presence of karyopherin α1 and β (Fig. 1b) or karyopherin α2 and β (Fig. 1c). Neither buffer alone (Fig. 1a) nor karyopherin α alone (not shown) nor karyopherin β alone (not shown) yielded docking.

Docking could be competitively inhibited by NLS-HSA (not shown), indicating that recombinant NP* contains a functional NLS that can be recognized and docked by either karyopherin heterodimer, α1β or α2β. Moreover, as previously reported for NLS-HSA (20, 21), NP* can be imported into the nucleus in the presence of either one of the two karyopherin heterodimers and of Ran and p10 (Figs. 1, e and f).

Nuclear Import of a Fluorescently Labeled Influenza Virus RNA Occurs in the Presence of Viral NP—We fluorescently labeled an 890-nucleotide negative-sense RNA of the NS segment of influenza virus (see “Experimental Procedures”) and incubated the labeled viral RNA (vRNA*) with digitonin-permeabilized cells in the presence of Xenopus cytosol or of Xenopus cytosol fraction A (32) (not shown) or of karyopherin α1 and β (Fig. 2a) or karyopherin α2 and β (Fig. 2c). Neither of these incubations yielded docking of the vRNA* at the nuclear envelope. However, when NP (not fluorescently labeled) was included in the docking reaction there was docking of the vRNA* at the nuclear envelope with either karyopherin α1β (Fig. 2b) or karyopherin α2β (Fig. 2d) and no docking when either α1 and α2 or β were omitted (not shown). These results indicate that viral RNA itself was unable to dock at the nuclear envelope and that docking occurred only in the presence of NP in an NLS- and karyopherin-mediated fashion.

Likewise, there was no import of the vRNA* alone in the presence of the karyopherin heterodimer, Ran and p10 (Fig. 3a). However, there was import when NP was present in the reaction (Fig. 3b). Import was competitively inhibited by NLS-HSA (data not shown). Thus, we conclude that import of vRNA* into nuclei of digitonin-permeabilized cells could be mediated by NP in an NLS-dependent pathway using all the transport factors that are required for import of an NLS-containing protein.

We have also tested whether influenza virus NP can mediate nuclear import of non-viral RNA. A 790-nucleotide-long, fluorescently labeled NPI-3 RNA by itself was not transported into the nucleus, but it was when NP was included into the assay (data not shown). Thus, NP can also bind to a non-virus-derived RNA and mediate its transport into the nucleus of digitonin-permeabilized cells in the presence of the karyopherin...
We investigated the time course of import of NP* (Fig. 4A) or vRNA* (Fig. 4B). In both cases import proceeded with similar kinetics with maximal import at about 20 min.

The concentrations of vRNA (200 ng/assay) and NP (200 ng/assay) that were used in the experiments shown in Figs. 2–4 amounted to a molar ratio of 4.8 NP/vRNA, i.e. far below the molar ratio of 45 NP/vRNA if the vRNA were fully coated by NP (8). To determine the effect of NP concentrations on the rate of vRNA* import, we varied the concentration of NP at a constant concentration of vRNA* (200 ng/assay). We found maximal import at NP concentrations of 100–200 ng/assay (Fig. 5), i.e. the import of vRNA* in Figs. 2–4 was indeed at maximal rates. However, we do not know the total amount of vRNA*-NP that was imported nor what the actual molar NP/vRNA* ratio of the imported vRNA*-NP was. Interestingly, at very high molar NP/vRNA* ratios there was inhibition of vRNA*-NP import. For example at 10 μg of NP and 200 ng of vRNA*/assay amounting to an NP/vRNA* ratio of 240 the rate of import of vRNA*-NP dropped to 70% of maximum (data not shown), probably as a result of competition between NP and vRNA*-NP.

**DISCUSSION**

Our data here suggest that influenza virus RNA does not contain its own ("cis-acting") signal for nuclear import but that import is via RNA-associated protein(s) containing an NLS and therefore requires all the transport factors (karyopherin, Ran, and p10) obligatory for uptake of NLS-containing proteins into nuclei of digitonin-permeabilized cells. There are potentially four such NLS-containing proteins associated with the genomic viral RNA: the heterotrimeric RNA polymerase complex and the NP (see the Introduction). It is therefore conceivable that the heterotrimeric RNA polymerase complex might mediate entry of the influenza virus RNA as well.

Unlike specific viral RNA binding proteins (35–37) NP can bind to any RNA provided that it is longer than 15 nucleotides (38). NP binding is to the sugar phosphate backbone leaving the bases exposed and melting the secondary structure of the RNA (39). These binding properties explain NP’s ability to mediate import into the nuclei of digitonin-permeabilized cells even of a non-viral RNA. Whether these promiscuous binding properties of NP could affect synthesis, transport, function, and metabolism of endogenous RNAs of virally infected cells is not known. It should be kept in mind, however, that there are numerous cellular RNA-binding proteins with which NP would have to compete in the infected cell.

The binding site of NP for RNA has been mapped to amino acid residues 91–188 (40). The recombinant NP that we tested...
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...here contained an additional 37 N-terminal residues and a C-terminal His tag. Neither of these two NP modifications seemed to interfere with nuclear import of NP+ or with the association of NP with RNA as RNA+ could be imported in an NP-dependent fashion.

It remains to be seen whether nuclear entry of nucleic acids via specifically or nonspecifically associated proteins and an NLS pathway is unique to genomic influenza viral RNAs or whether it is common to other viral or cellular nucleic acids. DNA viruses such as herpesvirus or adenovirus have been reported to dock at the NPC and are thought to inject their DNA across the NPC into the nucleus (41–43). Based on our studies it is more likely that associated, NLS-containing proteins mediate nuclear entry of the viral genomes of the DNA viruses as well. Lentiviruses, of which HIV-1 is a member, are unique among retroviruses in that they can infect non-dividing cells. The HIV-1 matrix protein contains an NLS that is required for this function (44, 45). It is likely that the matrix protein functions in importing the HIV-1 preintegration complex into the nucleus.

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