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Original article

Interaction between the yellow fever virus nonstructural protein NS3 and the host protein Alix contributes to the release of infectious particles

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Abstract

The ESCRT (endosomal sorting complex required for transport) machinery normally executes cargo sorting and internalization during multivesicular body biogenesis, but is also utilized by several enveloped viruses to facilitate their budding from cellular membranes. Although the mechanisms of flavivirus infectious particle assembly and release are poorly understood, the nonstructural protein NS3 has been reported to have an essential role via an undescribed mechanism. Here, we shed light on the role of NS3 by connecting it to the host factor Alix, a protein intimately connected with the ESCRT machinery. We demonstrate that NS3 and Alix interact and show that dominant negative versions of Alix inhibit YFV release. Furthermore, we show that NS3 supplied in *trans* rescues this effect. We propose that the interaction between NS3 and Alix contributes to YFV release.
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Keywords: Yellow fever virus; *Flavivirus*; Alix protein; Viral nonstructural proteins

1. Introduction

Yellow fever virus (YFV) is an enveloped RNA virus belonging to the Flaviviridae family and the *Flavivirus* genus. The YFV genome, a single-stranded positive sense RNA molecule of ~11 kb, is translated by the host cell into a single polyprotein which is cleaved by host and viral proteases, thereby generating the mature viral proteins [1]. The structural proteins (C, prM, and E) are incorporated into the virion, while the nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) are important for RNA replication [1]. Flavivirus NS3 is a large (~70 kDa) multifunctional protein possessing helicase, protease, and RNA triphosphatase activities [2–4] and essential for viral replication [5]. In addition to these activities, a surprising role for NS3 in virus assembly/release was also demonstrated [6,7]; indeed, growing evidence suggests that nonstructural proteins are involved in particle morphogenesis (for review, see [8]). Although the mechanism of flaviviral particle morphogenesis is poorly understood, it is believed that the capsid protein associates with genomic RNA, acquires its host-derived envelope by budding into the lumen of the endoplasmic reticulum (ER), and exits via the secretory pathway [1]. Of particular note is the direction in which flavivirus particles are thought to be formed: by budding into the lumen of the ER and away from the cytosol, a physiologically unusual direction [1].

A topologically similar process occurs at the late endosomal membrane to sort membrane proteins bound for degradation into vesicles which invaginate into the endosomal lumen (and away from the cytosol), thereby generating a multivesicular body (MVB). This process is executed by the ESCRT proteins [9,10]. ESCRT proteins are recruited from the cytosol as complexes which act sequentially (ESCRT-0, -I, -II, and -III) and are required for MVB formation: ESCRT-0 and ESCRT-I recognize ubiquitinated proteins destined for degradation and also recruit downstream ESCRT machinery

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[11–13]. Likewise, the ESCRT-II complex interacts with components of ESCRT-III [14]. ESCRT-III subunits are recruited as soluble monomers and oligomerize on membranes [15], a process that has been proposed to promote the negative membrane curvature required for membrane budding [16]. These proteins are dissociated from the membrane by Vps4, an AAA-type ATPase [17]. Several other proteins are also intimately connected with the ESCRT machinery, including AIP1/Alix, a multifunctional protein that interacts with components of both ESCRT-I and ESCRT-III [18–20]. Altogether, the current model of ESCRT protein function proposes that they are involved in both initial cargo sorting events and the subsequent inward vesiculation/scission to generate the internal luminal vesicles of the MVB [10].

It is well documented that many viruses co-opt ESCRT machinery to facilitate their egress from cellular membranes. ESCRT proteins are recruited via conserved motifs which act as docking sites for cellular proteins to viral proteins; these motifs are termed late domains [21] because mutation of these motifs arrests viral budding at a late stage (i.e., scission of the virion from the host membrane). Three late domain consensus sequences within retroviral Gag proteins have been identified: PPxY, PT/SAP, and YPxL [21]. In addition to inhibiting viral budding, mutations in late domains also disrupt interaction with ESCRT proteins in a correlating manner [19,22]. Viruses also seem to have evolved multiple exit routes: Although human immunodeficiency virus type 1 (HIV-1) preferentially utilizes Tsg101 for budding [22], overproduction of Alix can rescue the release of HIV-1 when the Gag binding site for Tsg101 is mutated [23]. Therefore, it appears that multiple ESCRT and ESCRT-associated proteins can be used by the same virus.

Most work studying the role of ESCRT proteins in viral budding has focused on retroviruses which generally bud from the plasma membrane [21], and it is largely unknown whether the same machinery is used by viruses that bud from intracellular membranes, such as YFV. Recently, however, Alix was reported to be involved in the release of hepatitis B virus [24], a DNA virus which buds from intracellular membranes. It therefore seems likely that ESCRT-associated proteins may be broadly used by many viruses, not just retroviruses. In this study, we aimed to clarify the role of NS3 in YFV assembly by investigating its relationship with Alix. We show that NS3 associates with Alix using confocal microscopy and biochemical techniques. We also demonstrate that dominant negative versions of Alix inhibit the release of infectious YFV particles; furthermore, we show that NS3 supplied in *trans* can rescue this dominant negative effect. Our data therefore generate insight into the mechanism of YFV infectious particle production and lend further support to the idea that nonstructural proteins are involved in this process.

2. Materials and methods

2.1. Plasmids

The sequence encoding YFV NS3 (nt 4571-6439; Gen Bank accession no. X03700) was PCR amplified from pT3 [25] using oligos LC10 (5′ – **GCGGCCGCGC**CACC**ATG***GCC*A-GTGGGGATGTCTTGTG – 3′; ***Not*I** Kozak sequence (**start codon**), *alanine to remain in frame*) and LC8 (5′ – **GTCGAC**CCTCCTACCTTCAGCAAAC – 3′; ***Sal*I**). The *Sal*I site in the reverse primer was designed to insert the NS3 fragment in-frame with the C-terminal FLAG tag provided by the pFLAG-CMV-5a vector (Sigma). The 1.7 kb PCR product was subcloned first into pGEM-Easy (Promega) and then as a *Not*I/*Sal*I fragment into pFLAG-CMV-5a cut with the same enzymes. The S138A and R461Q mutations were introduced using the QuikChange mutagenesis kit (Stratagene) with oligos LC16/LC17 (5′ – CTTGACTATCCGAGTGGCACT**GCA**G-GATCTCCTATTGTTAAC – 3′/5′ – GTTAACAATAGGA-GATCC**TGC**AGTGCCACTCGGATAGTCAAG – 3′; **138A**) and LC14/LC15 (5′ – TCCGCATCCTCTGCTGCTCAA**CA-G**AGGGGGCGCATTGGGAGAAAT – 3′/5′ – ATTTCTC-CCAATGCGCCCCCT**CTG**TTGAGCAGCAGAGGATGCG-GA – 3′; **R461Q**), respectively. All mutations were verified by DNA sequencing.

For recombinant expression in *Escherichia coli*, the sequence encoding the helicase domain of NS3 (nt 5082-6439; Gen Bank accession no. X03700) was amplified from plasmid pT3 [25] using oligos RG113 (5′ – CG**GAATTC**ACTGAGGTGAAG-GAAGAAGG – 3′; ***Eco*RI**) and LC9 (5′ – **GTCGAC***TTAT-TA*CCTCCTACCTTCAGCAAAC – 3′; ***Sal*I**, *stop codons*). The ~1400 bp fragment was subcloned into pGEM-Easy (Promega) and further subcloned as an *Eco*RI/*Sal*I fragment into pET-28a (Novagen) cut with the same enzymes, generating pNS3-hel. The resulting construct produced the helicase domain of NS3 (residues 170–623) as an N-terminally His(6)-tagged fusion protein. The YPTI->APTA mutations were introduced as described above with oligos LC5 (5′–CAGGAAAACCTTTG AGAGAGAA**GCC**CCCACG**GCA**AAGCAGAAGAAACCTG AC – 3′; **399A, 402A**) and LC6 (reverse complement of LC5).

Constructs driving the expression of N-terminally EGFP-tagged Alix, N-terminally tagged HA-Alix and HA-Bro1V, and the N- and C-terminal domains of EGFP-Alix were generously provided by Prof. Wes Sundquist (University of Utah), Dr. Fadila Bouamr (National Institutes of Health) and Prof. Rémy Sadoul (Grenoble Institut des Neurosciences), respectively.

2.2. Antibodies

To raise polyclonal antibodies against NS3, a construct expressing N-terminally $His_{(6)}$-tagged NS3 was created. A fragment corresponding to nt 4571-6439 of the YFV cDNA was PCR amplified from pYFM5.2 [26] using oligos RG93 (5′ – CG**GAATTC**AGTGGGGATGTCTTGTG – 3′; ***Eco*RI**) and RG94 (5′– CCAATGCATTGG**CTGCAG**CCTCCTACCTT-CAGCA – 3′; ***Pst*I**) and subcloned into pDS56 [27]. NS3 was then produced recombinantly in *E. coli* and purified under denaturing conditions using Ni-NTA affinity chromatography. The resulting insoluble inclusion bodies (60 μg protein/ml) were resuspended in 2% SDS, 140 mM NaCl, and 2 mg/ml polyA–U and used to immunize rabbits. Anti-NS3 antibodies were concentrated through ammonium sulfate precipitation of

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