BRIEF REVIEW

The molecular biology of nairoviruses, an emerging group of tick-borne arboviruses

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Abstract The nairoviruses are a rapidly emerging group of tick-borne bunyaviruses that includes pathogens of humans (Crimean-Congo hemorrhagic fever virus [CCHFV]) and livestock (Nairobi sheep disease virus [NSDV], also known as Ganjam virus), as well as a large number of viruses for which the normal vertebrate host has not been established. Studies on this group of viruses have been fairly limited, not least because CCHFV is a BSL4 human pathogen, restricting the number of labs able to study the live virus, while NSDV, although highly pathogenic in naive animals, is not seen as a threat in developed countries, making it a low priority. Nevertheless, recent years have seen significant progress in our understanding of the biology of these viruses, particularly that of CCHFV, and this article seeks to draw together our existing knowledge to generate an overall picture of their molecular biology, underlining areas of particular ignorance for future studies.

Introduction

New viral diseases appear with increasing frequency. Just in the last two years we have seen the appearance of a new livestock virus (Schmallenberg) and a new human pathogen (MERS coronavirus). In some cases these viruses appear to be completely new, in others they have 'emerged' into our awareness as human use of different habitats changes, leading to increased contact with carriers of disease, whether those carriers are "bush meat" or the

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many insect and tick species that can act as vectors of disease. One such group of viruses that is rapidly becoming more important is the genus *Nairovirus* in the family *Bunyaviridae*. This genus includes a number of human and livestock pathogens, as well as a collection of other viruses about which little is known, not even the host in which they naturally circulate. The nairoviruses show a number of unique features, and the purpose of this article is to summarise our current knowledge of the molecular biology of these viruses and highlight areas where it is to be expected that research will soon bear fruit.

A primary characteristic of the viruses of the genus *Nairovirus*, distinguishing them from most of the other members of the family *Bunyaviridae*, is that they are all transmitted by ticks [109]. Based on antibody cross-reactivity, nairoviruses are classified into seven serogroups (Table 1) [32, 39, 190], of which the most important are the Crimean-Congo haemorrhagic fever (CCHF) group, which includes the human pathogen Crimean-Congo haemorrhagic fever virus (CCHFV), and the Nairobi sheep disease (NSD) group, to which belong Nairobi sheep disease virus (NSDV) Dugbe virus (DUGV) and Kupe virus (KUPV). KUPV [43] and Finch Creek virus [102] are the most recently discovered viruses of this genus. CCHFV is arguably the most important from a human perspective, causing haemorrhagic fever in humans, with mortality up to 30 % (reviewed in refs. [58] and [182]). After dengue virus, this is the second most widespread of the arboviruses that are pathogenic to humans; cases of CCHF have been reported in sub-Saharan Africa, the former Soviet Union, Bulgaria, Turkey, China, Pakistan, India, the Arabian Peninsula, northern Greece, Iraq and Iran [26, 53–55, 115, 121–123, 183]. Although CCHFV can infect several mammalian species, it appears to cause disease only in man [124, 158]. Because of its importance as a human pathogen,

Table 1 Viruses of the genus *Nairovirus*: the serogroup to which they belong and any known association with disease

Serogroup	Viruses	Known association with disease
Nairobi sheep disease group	Nairobi sheep disease virus (NSDV)=Ganjam virus (GV)	Haemorrhagic gastroenteritis in sheep and goats [106, 113]. Antibodies to NSDV/GV have been reported in humans, but otherwise only laboratory-acquired infections have been seen [13, 45, 133]
	Dugbe virus (DUGV)	Frequently isolated from ticks infesting livestock in which DUGV appears to be apathogenic [27, 46, 146]. While one case of human infection has been described [27], the link to DUGV was circumstantial
	Kupe virus (KUPV)	
Crimean-Congo haemorrhagic fever group	Crimean-Congo haemorrhagic fever virus (CCHFV)	Haemorrhagic fever in man [58, 179, 182]
	Hazara virus (HAZV)	
	Khazan virus (KHAV)	
Hughes group	Farallon virus (FARV)	Humans bitten by infected ticks may experience pruritus (i.e. itching), fever and headache [41, 42, 76]
	Fraser Point virus (FPV)	
	Great Saltee virus (GRSV)	
	Hughes virus (HUGV)	
	Puffin Island virus (PIV)	
	Punta Salinas virus (PSV)	
	Raza virus (RAZAV)	
	Sapphire II virus (SAPV)	
	Soldado virus (SOLV)	
	Zirqa virus ZIRV)	
Dera Ghazi Khan group	Abu Hammad virus (AHV)	
	Abu Mina virus (ABMV)	
	Dera Ghazi Khan virus (DGKV)	
	Kao Shuan virus (KSV)	
	Pathum Thani virus (PTHV)	
	Pretoria virus (PREV)	
Qalyub group	Bakel virus (BAKV)	
	Bandia virus (BDAV)	
	Omo virus (OMOV)	
	Qalyub virus (QYBV)	
Sakhalin group [102]	Avalon virus (AVAV)	
	(=Paramushir virus)	
	Clo Mor virus	
	Finch creek virus	
	Kachemak Bay virus (KBV)	
	Sakhalin virus (SAKV)	
	Taggert virus (TAGV)	
	Tillamook virus (TILLV)	
Thiafora group	Erve virus (ERVEV)	Erve virus may cause severe headache in man and neurological disorders [165, 184]. Mode of transmission into humans is currently unknown
	Thiafora virus (TFAV)	

most of the available data on nairoviruses come from studies on this virus, though studies on other members of the group have also contributed, notably on NSDV, a virus first identified nearly a hundred years ago as a tick-borne virus that causes severe haemorrhagic gastroenteritis in sheep and goats, with mortality rates of up to 90 % in susceptible populations [113, 167, 181]. Interestingly, an Asian virus causing a similar disease, Ganjam virus (GV), was recently identified, based on genetic and serological studies, as being the same virus as that causing NSD in East

Africa [47, 106, 187]. It is not possible to say yet whether GV is a variant of NSDV or vice versa, but it is clear that this virus also has a wide distribution.

Molecular characteristics of nairoviruses

Nairoviruses, like the members of the other genera of the family *Bunyaviridae*, are enveloped viruses that appear spherical in the electron microscope, with a diameter of approximately 100 nm [21, 46, 142]. As with all bunyaviruses, the genome consists of three segments of negative-sense RNA [39] (reviewed in refs. [109] and [175]). These are termed the small (S) segment, encoding the nucleocapsid (N) protein, which forms a complex with each RNA segment [24, 39]; the medium (M) segment, which encodes a polyprotein that is processed into two mature glycoproteins (Gn and Gc) as well as one or more non-structural proteins [3, 17, 39, 107, 144]; and the large (L) segment, which encodes the viral RNA-dependent RNA polymerase (RdRp) [85, 108].

Viral replication takes place in the cytoplasm, and viral budding occurs in the Golgi [57, 142] (Fig. 1). During viral replication, the genome segments are used as the template
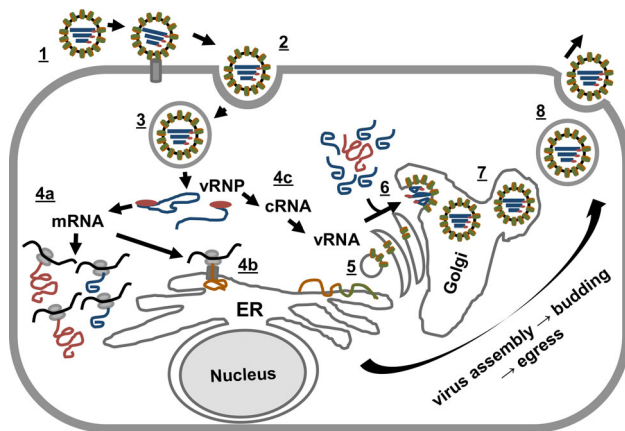

Fig. 1 Schematic representation of the replication cycle of nairoviruses (family *Bunyaviridae*). (1) Virus attaches to an unknown cellular receptor (2), after which the virus is internalised into the host cell in an endocytosis-mediated manner. (3) Upon reaching the low-pH environment of the endosome, viral glycoproteins probably undergo a conformational change that releases ribonucleocapsids (RNPs) and viral polymerase (L) into the cytoplasm. (4a) vRNA is transcribed into mRNA, giving rise to synthesis of viral proteins; nucleoprotein (N) and L are synthesised on cytoplasmic ribosomes while (4b) the viral glycoproteins are synthesised as a single polyprotein on ER-associated ribosomes. (4c) Viral RNA (vRNA) is transcribed into complementary RNA (cRNA), which is used as the template for replication of vRNA. (5) The glycoprotein polyprotein is further processed in the ER and Golgi into mature glycoproteins. (6) Once viral proteins and vRNA are synthesised, new virions assemble at the Golgi. (7) The new virus buds into the Golgi and (8) exits the host cell, probably in Golgi-derived vesicles

for synthesis of both mRNA (transcription) and complementary RNA (cRNA) (replication), where cRNAs are then used as the template for the synthesis of progeny genomic viral RNA (vRNA) [16]. The 5' and 3' untranslated regions (UTRs) of the S, M and L segments contain the minimum *cis*-acting elements necessary for transcription, replication and packaging [16, 67]. The terminal 5' and 3' non-coding regions of each segment are complementary to each other and highly conserved among different nairoviruses [37]. The first nine nucleotides are usually the same in different segments, which suggests that this sequence is recognised by the viral polymerase to initiate viral transcription/replication [107]. It is not clear whether the complementarity between 5' and 3' ends reflects an interaction between the ends of segments or the requirement for the same 3' sequence in genome and anti-genome RNAs to act as the attachment site for the viral polymerase [14, 66] (reviewed in refs. [109] and [147]). As with other bunyaviruses, the nairoviruses utilise capped primers snatched from host mRNA for initiation of their mRNA transcription [90]. The initiation of cRNA and vRNA synthesis occurs by a different mechanism, which has not yet been fully worked out. The fact that polymerase slippage occurs during mRNA synthesis [90], coupled with the observation that the RNA segments of CCHFV, DUGV and NSDV contain a 5'-terminal pyrimidine [90, 144, 187], while viral RNA polymerases can initiate RNA synthesis only by attaching purines [12], suggests that initiation of RNA replication occurs in a prime-and-realign manner.

Entry and exit

The two membrane glycoproteins (Gn and Gc) are believed to determine cell tropism and the ability of the viruses to infect susceptible cells via recognition and binding of one or more cellular receptors. The specific cellular receptor(s) used by nairoviruses are currently unknown, but the Gc protein is thought to be involved in virus attachment to the target cell receptor, as antibodies against Gc, but not Gn, appear to protect cells from CCHFV infection in plaque reduction neutralisation assays [1, 17]. The ectodomain of CCHFV Gc contains an epitope that is highly conserved among strains [1]. This fragment is probably exposed in the virus, as antibodies against this epitope are neutralising for different strains of CCHFV and protect mice from challenge with CCHFV [1]. Similarly, the Gc of DUGV was also demonstrated to be targeted by neutralising antibodies [107]. Antibodies to the Gn protein were found to be non-neutralising *in vitro*, though could still be protective *in vivo* [17].

Based on the finding that an independently folding section of this Gc protein bound to CCHFV-susceptible cells, and using this part of Gc as a probe [185], nucleolin

has been suggested as a putative CCHFV receptor. Nucleolin is a cellular protein that is abundant in the nucleus but can also be expressed on the cell surface of several cell types [36, 83, 148, 149, 151], including mononuclear phagocytes, endothelial cells and hepatocytes which are known to be targets for CCHFV [28, 185]. Although nucleolin has also been suggested to act as a receptor for several other viruses, such as parainfluenza virus type 3 [22], human immunodeficiency virus (HIV) [118], coxsackie B virus [49] and respiratory syncytial virus (RSV) [162], further studies are required to determine whether nucleolin acts as the primary receptor for CCHFV; in particular, infection studies with wild-type (not cell-culture-adapted) viruses are required to confirm the biological relevance of any potential receptor candidates.

After binding to their receptor, viruses fuse with the plasma membrane or internalise through one of several endocytosis pathways to gain entry into the intracellular environment (reviewed in refs. [40] and [153]). CCHFV entry is dependent on the low pH of the endosomal compartment, which appears to be required in the first steps of the virus entry post-internalisation [74, 156], and the virus has been shown to use clathrin-dependent endocytosis, while caveolin-1 is dispensable for its entry [74, 156]. Studies using dominant negative Rab5 and Rab7 indicate that CCHFV fuses with early, but not late, endosomes to gain access to the cytoplasm [74]. Cholesterol also appears to be important for CCHFV replication, especially at early stages after binding and internalisation; it is possible that depletion of cholesterol from cells traps viral particles in endosomes [88] or interferes with clathrin-mediated endocytosis [140].

The internalisation of CCHFV virions was shown to be dependent on intact microtubules [155]. Further investigation of CCHFV infection showed that disruption of the cell microtubules inhibited viral RNA replication [155], and both intact actin and microtubules are essential for correct distribution of the N protein to the perinuclear area [5, 155]. As nairoviruses enter via endocytosis and bud in the Golgi compartment, the observation of a redistribution of viral proteins and suppression of CCHFV assembly/egress by microtubule modification (depolarisation and stabilisation) [155] is not surprising, and this might be caused by interference with the endogenous secretory pathway, which also occurs along microtubules [101] (reviewed in ref. [87]). Disruption of actin filaments in CCHFV-infected cells also drastically reduced replication of the virus [5]. While the N protein is often located in close proximity to the Golgi apparatus, where generation of new viral particles occurs [16], N does not interact directly with Golgi membranes [5, 134]. The N protein appears to interact with the L protein, even in the absence of viral RNA, as expressed N protein seems to redistribute most of the L protein into the perinuclear area containing N in transfected cells [16].

The N protein

The N protein of nairoviruses, at approximately 53 kDa, is almost twice of size of the N proteins of other bunyaviruses, with the exception of those of hantaviruses, which, at approx. 48 kDa, are similar in size [39, 110]. The N protein is the most abundant protein in the virion and encapsidates newly synthesised vRNA and cRNA; this process is necessary for completion of the replication cycle and packaging of the genome into virions [16, 65, 100]. Viruses of different genera in the family *Bunyaviridae* appear to adopt different mechanisms of RNA encapsidation, e.g. some bunyavirus N proteins recognise generic ssRNA, while others recognise specific structures in the vRNA [110, 120, 135, 137, 150]. There is a need to understand the mechanism by which nairoviruses encapsidate genomic and antigenomic RNA; inhibition of vRNA encapsidation could be a target for potential antiviral drugs, e.g., using an RNA decoy to bind the viral N protein, as has been suggested as an antiviral treatment for hepatitis B virus (HBV) [62], or selecting RNA-binding proteins that target specific packaging sequences in the viral genome [193].

The recently determined crystal structure of the N protein of CCHFV shows that it is built from two major domains: a globular head and an extended stalk [31, 77, 174]. The globular domain is the larger and is formed from both N-terminal and C-terminal helices, while the stalk domain is formed by a group of internal helices (the exact numbering of the helices varies between the several papers publishing the structure of this protein). Three potential RNA-binding regions were identified in the N protein structure. Two positively charged grooves are located in the globular domain: the smaller one is under the stalk domain, and the larger one is in the opposite site of the globular domain. The third positive groove is located in the stalk domain. The positively charged residues forming the RNA-binding site appear to be well conserved across the genus *Nairovirus*, and several residues were identified as crucial for RNA binding and virus replication, e.g., K132 and Q300 in the smaller groove and K411 and H456 in the larger groove of the head domain [31, 77].

The N protein of CCHFV was also crystallised as a linear oligomer, where three monomeric N subunits were organised in head-to-tail manner, with the stalk domain of one monomer interacting with an oligomeric groove located at the base of the head domain of an adjacent molecule [174]. In addition, the linear oligomers of N were predicted to interact in a dimeric manner to form an antiparallel double superhelix [31, 174]. Further predictions suggest that that there are nine N molecules per turn of the superhelix, which is 210 Å in diameter [174]. Additionally, a positively charged crevice located on the outside of the double superhelix is predicted to serve as an additional RNA binding cleft [174].

Studies performed by Guo and collaborators [77] suggest that the CCHFV N protein, when expressed without RNA, predominantly exists as a monomer, and the nairovirus N protein is only a weak binder of nonspecific RNA in this monomeric state [77, 80, 111, 135]. This suggests that nairovirus N binds RNA only in the oligomeric state and/or that the N recognises specific structures of viral RNA, as was shown for viruses of the genus *Hantavirus* [110, 120]. However the fact that the nairovirus N protein does not form oligomers without RNA suggests that its oligomerisation is RNA-stabilised, where monomeric N protein requires binding to RNA to form an oligomer, a mechanism that was previously suggested for influenza A virus [160, 161]. In a similar fashion, the N protein of Rift Valley fever virus (RVFV) can form only a short oligomeric form in the absence of RNA [64].

Superimposing the structures obtained for the CCHFV N protein by two different groups, using two CCHFV isolates, revealed the head and stalk with very similar folds, but with a transposition of the stalk domain when comparing these two molecules; results which suggest a flexibility of the stalk domain and the possibility of different conformations of N for different functions or different states of the N-RNA complexes (e.g., transcription vs replication) [31]. Additionally, comparison of the crystal structure of the N protein in the monomeric and oligomeric forms suggests that the stalk domain changes its conformation upon oligomerisation, probably by binding to the oligomerisation groove on the head domain of the adjacent molecule [174]. Such flexibility of the stalk domain has also been shown for the N proteins of RVFV and LASV, which, during binding to an oligomerisation groove on an adjacent N molecule, undergo conformational changes exposing an RNA-binding groove [64, 80]. These structural data have led to suggestions of possible models for the initiation of transcription and replication. Transcription of viral mRNAs by nairoviruses utilises short capped RNA fragments (10-20 nt in length) derived from host mRNAs as primers [90]. Incubation of the CCHFV N protein with primer-length ssRNA resulted in a conformational change of the stalk domain, which resulted in disruption of the oligomeric interactions and release of the monomeric N protein from the antiparallel double superhelix [174]. As discussed by Wang *et al.*, presentation of the capped primers to the ribonucleoprotein (RNP) may initiate conformational changes in the stalk domain, leading to the release of monomeric N and exposing vRNA to the primer and the viral polymerase. Given that head-to-stalk interactions between two adjacent N molecules have also been observed for RVFV, LASV, and Bunyamwera virus (BUNV) [9, 64, 80], it seems likely that the model proposed for CCHFV may be biologically valid.

The N protein of CCHFV has also been shown to have nuclease activity specific for dsDNA and ssDNA (but not for RNA); the importance of this DNase activity and its function in the virus life cycle are unknown [77]. The residues involved in the nuclease activity are located in the globular head domain of the N protein and are conserved in all nairoviruses [77]. In contrast, the N protein of LASV, the head domain of which exhibits high structural homology with that of CCHFV, has RNA-specific nuclease activity [79, 80, 130].

The N protein of nairoviruses also contains a conserved sequence signature specific for catalytic motif II (CMII) of N-6 adenine-specific DNA methylases (Lasecka and Baron, unpublished) (Fig. 2), where the conserved motif NPPW could be involved in substrate binding or in catalytic activity [97, 164]. The motif is located on an exposed loop of the stalk domain, and the function and potential importance of this motif still need to be determined. The methylation of DNA is used for regulation of gene expression, but so far there is no indication that the N protein of nairoviruses travels to the nucleus; the protein does not contain a classical nuclear localisation signal (NLS), nor does it accumulate in the nucleus of infected or transfected cells. N6 methylation is also used as a post-transcriptional modification of mRNA, and RNA methyltransferases appear to contain motifs very similar to the CMI and CMII motifs identified in DNA methyltransferases (reviewed in ref. [119]); further studies are required to determine if the nairovirus N protein can modify its own or host cell RNAs in this way.

Viral glycoproteins

Like those of members of the other genera of the family *Bunyaviridae*, the M segment of nairoviruses contains a single open reading frame (ORF) encoding a polyprotein that is co- and post-translationally cleaved into the mature viral glycoproteins [39]. The glycoproteins of most nairoviruses are still poorly characterised, and most of the available data come from studies on CCHFV, largely through studies on proteins expressed from plasmids. The processing of the CCHFV M polyprotein to generate the mature glycoproteins appears to be more complex than that of other bunyaviruses, as it involves first the generation of glycoprotein precursors through the action of the signal protease in the endoplasmic reticulum (ER) followed by further cleavages to give rise to the full set of mature glycoproteins, a process that employs other cellular proteases [15, 144, 145, 172] (Fig. 3).

The virions of most of the nairoviruses contain two mature glycoproteins, Gn and Gc [15, 33, 38, 107, 144, 145, 172]; however, two nairoviruses, Hazara virus and Clo
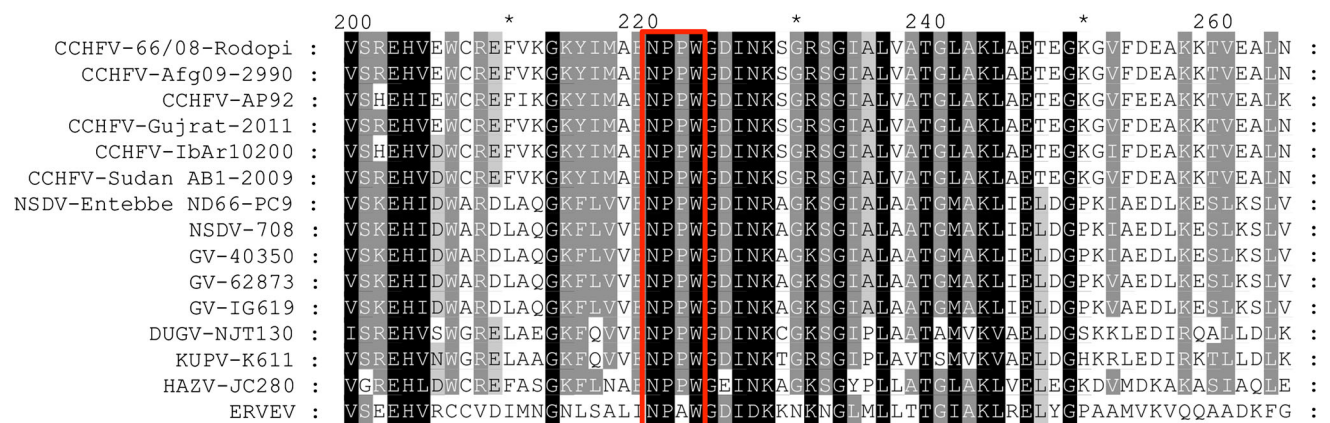
Fig. 2 Conserved CMII motif in the N protein of nairoviruses. N protein sequences, either available from NCBI (National Centre for Biotechnology Information) or as sequenced in our laboratory, were aligned using ClustalX2. Viruses and their strain/isolate designation are indicated on the left. CCHFV, Crimean-Congo haemorrhagic fever virus; NSDV, Nairobi sheep disease virus; GV, Ganjam virus; DUGV, Dugbe virus; KUPV, Kupe virus; HAZV, Hazara virus; ERVEV, Erve virus. The numbering at the top indicates the relative position in the alignment. The conserved NPPW motif is marked in red. Protein sequence accession numbers are as follows: CCHFV-66/08-Rodopi (ACF93431.2); CCHFV-Afg09-2990 (ADQ57288.1); CCHFV-AP92 (AAA50177.1); CCHFV-Gujrat-2011 (AFY97403.1); CCHFV-IbAr10200 (P89522.1); CCHFV-Sudan AB1-2009 (AEI70581.1); NSDV-708 (AAM33323.1); GV-40350 (AED88236.1); GV-62873 (AED88237.1); DUGV-NJT130 (ACL68470.2); KUPV-K611 (ABY82500.1); HAZV-JC280 (P27318.1); ERVEV (AFH89034.1)
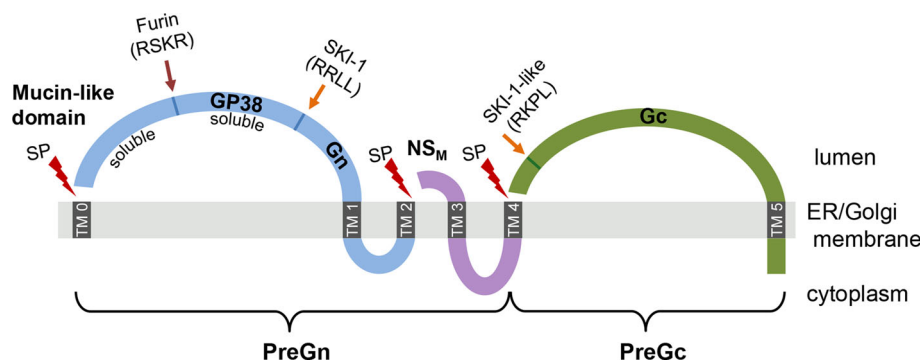

Fig. 3 Schematic representation of CCHFV M polyprotein processing. A schematic model of the M-encoded polyprotein is shown with the glycoprotein precursors PreGn and PreGc labelled and the approximate positions of the mature proteins mucin-like domain, GP38, Gn, NS_M and Gc indicated. The black bars represent transmembrane helices (TM_0-TM_5). The initial signal protease (SP) cleavage sites are indicated, and arrows indicate further cleavage sites during polyprotein processing. See text for the details of CCHFV M polyprotein processing. Schematic diagram not drawn to scale and adapted from refs. [4, 161, 162]

Mor virus, have been shown to contain three structural glycoproteins [68, 175]. The full nairovirus M-encoded polyprotein appears to have six hydrophobic regions (TM_0-TM_5), which could function as transmembrane helices [3, 144] and act either as classic secretory signal peptides (TM_0), membrane anchors ($TM_{1, 3}$ and $_5$), or a combination of both ($TM_{2,4}$) (Fig. 3). Signal cleavage motifs are found after TM_0 (releasing the amino terminus of the Gn precursor, PreGn) and TM_4 (releasing the Gc precursor PreGc). Sequence inspection revealed a signal cleavage signal immediately after TM_2, suggesting that there might be a separate protein released, consisting of the sequence between the distal ends of TM_2 and TM_4; such a protein has been shown to be produced by CCHFV and has been termed NS_M [3]. Further non-structural glycoproteins have been identified in CCHFV-infected cells, referred to as the mucin-like domain (a highly O-glycosylated peptide), GP38, GP85 and GP160, all of which are released from infected cells; GP85 and GP160 contain both the GP38 protein and the mucin-like domain [145].

A model for the generation of the CCHFV glycoproteins is summarised in Figure 3 based on a number of studies [3, 144, 145, 172]. Co-translational cleavage in the ER by cellular signalase generates the 140-kDa PreGn (containing the mucin-like domain, GP38, and Gn), the predominantly cytoplasmically oriented NS_M, and the 85-kDa PreGc. The PreGn is further cleaved, either in the ER or the Golgi compartment, to separate mucin-like domain/GP38 from

Gn. This cleavage occurs at a conserved RRLL↓ motif and is effected by the host's subtilisin kexin isozyme-1/site-1 protease (SKI-1/S1P) [145, 172]. This cleavage has been shown to be critical for virus replication and subsequent infectivity, and lack of the cleavage prevents incorporation of the glycoproteins into viral particles [15]. A similar tetrapeptide (RKPL) is found 41 amino acids downstream of the signalase cleavage site in PreGc, which, although it is not processed also by SKI-1/S1P, is predicted to be utilised by a related subtilisin-like protease in the ER/cis-Golgi to generate the mature Gc (75 kDa) [144, 172]. The mature Gn interacts (directly or indirectly) with the Gc, and both are translocated to the virus-assembly sites in the Golgi [17, 59, 145]. The interaction of mature Gn with Gc is essential for the Gc to travel from ER to Golgi, as Gn, but not Gc, contains a Golgi localisation signal [17]. The ectodomains of Gn and Gc appear to be sufficient for heterodimer formation and transport to the Golgi, indicating that at least partial Golgi-targeting information is located in the Gn ectodomain [17].

The final processing of the glycoproteins takes place in the trans-Golgi, where the mucin-like domain and GP38 are separated from each other by a furin-like protein convertase at the RSKR↓ cleavage site [116, 145]. This cleavage is not required for CCHFV Gn maturation [145], and it appears that not the entire pool of mucin-like domain/GP38 polyprotein is being cleaved, as the non-structural proteins termed GP85 and GP160 contain both the mucin-like domain and GP38 and are resistant to resolution into smaller proteins by denaturation in SDS and urea [145]. Resistance to denaturation also suggests that GP160 is probably not a dimer of GP85 [145]. Interestingly, the mucin-like domain/GP38 and GP38 can fold independently of the rest of Gn and are secreted even when expressed on their own in transfected cells [145]. No specific biological function has been assigned to the mucin-like domain, GP38, GP85 or GP160. The mucin-like domain of the Ebola virus glycoprotein has been shown to play a major role in pathogenesis, including involvement in the observed increase of endothelium permeability [154, 188], and it will be important to see if this is also true of CCHFV, especially given the changes visible in the endothelial cells of CCHFV patients.

The NS_M protein, when expressed in transfected cells on its own, is transported to the Golgi [3]. While the function(s) of the nairovirus NS_M have still to be determined, the fact that Gn requires NS_M for maturation may mean that NS_M is necessary for virus replication [3].

Glycosylation is an important post-translational modification of secreted and membrane proteins that can influence protein folding, transport and function. N-linked glycosylation in particular is known to regulate protein folding, association with chaperones [112], transport,

cellular localisation [81, 82], and even virus infectivity (reviewed in ref. [171]). The N-terminal part of the nairovirus M polyprotein, which contains the mucin-like domain, is heavily O-glycosylated, while the adjacent GP38 domain appears to have few O-glycosylation sites [144, 145]. Both domains are also N-glycosylated, the mucin-like domain containing five potential sites and GP38 two [145]. Of two predicted N-glycosylation sites in each of the mature Gn and Gc proteins, only one is functional in Gn (N557), while both sites in Gc (N1054 and N1563) are glycosylated [59]. However, only the glycosylation of the Gn is essential for Gn maturation, correct localisation, and transport of itself and other CCHFV glycoproteins [59]. Given that the Gn glycosylation sites are conserved among CCHFV strains [51], it is likely that correct glycosylation of the CCHFV Gn is critical for virus viability.

Recently, NMR has been used to determine a solution structure for the C-terminal (cytoplasmic) tail of CCHFV Gn, showing that this region contains a dual zinc-finger domain, the sequence of which is highly conserved among nairoviruses [60, 61]. Classical ββα-zinc finger domains have been shown to take part in protein-protein interactions (reviewed in ref. [71]), and it is possible that the dual zinc-finger domain in the C-terminus of the nairovirus Gn protein is involved in interaction with RNPs, which would help drive assembly/budding of the virus.

Analysis of the sequences of M segments of other nairoviruses suggests that the general model proposed for processing of the M polyprotein described above for CCHFV holds true for the other nairoviruses; M polyproteins show similar membrane topology, with six transmembrane regions and conserved signalase cleavage sites after the transmembrane domains TM_0, TM_2 and TM_4 (Fig. 3). Glycoprotein maturation from precursor proteins has also been observed for DUGV, where PreGn is around 70 kDa and PreGc around 85 kDa [107]. An exception to this general similarity is Erve virus (ERVEV), which does not contain TM_3 and TM_4, its M polyprotein lacking the entire amino acid sequence between TM_2 and the Gc ectodomain. This suggests that ERVEV lacks an NS_M protein. Most of the nairoviruses for which we have sequence data appear to have a PreGn domain that is about 120 amino acids shorter than that seen in CCHFV, due to a much shorter mucin-like domain, as was previously described for DUGV [144]. Another difference is that other nairoviruses do not contain a furin-cleavage site (RSKR) following the O-glycosylated mucin-like domain. The SKI-1/S1P-like protease cleavage tetrapeptides in many nairoviruses appear to be different to those proposed for CCHFV; however, all appear to fit the consensus subtilisin cleavage sequence (R/K)X(hydrophobic)Z↓ (where X is any amino acid and Z is preferably F, K, L or T but not V, P, E, D, C) [56]. This may reflect differences in adaptation
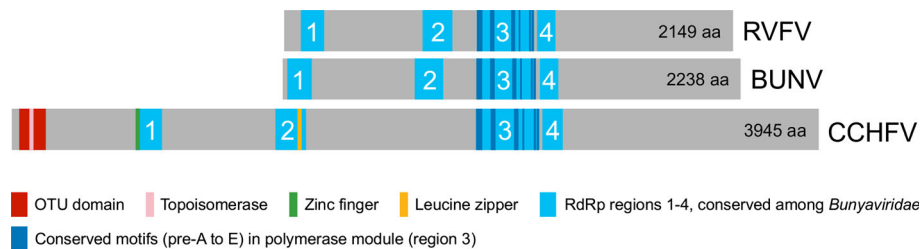
Fig. 4 Schematic representation of the proteins encoded on the L RNA segments of viruses of the family *Bunyaviridae*. Diagrammatic representation of the L proteins of Rift Valley fever virus (RVFV), Bunyamwera virus (BUNV) and Crimean-Congo haemorrhagic fever virus (CCHFV) in which all proteins were aligned to the polymerase module (i.e., region 3). All proteins contain four (1-4) conserved regions of the RNA-dependent RNA polymerase domain (RdRp), and the conserved motifs (pre-A, A, B, C, D and E) in the polymerase module are also highlighted. However, only the nairovirus L contains the zinc-finger domain and leucine zipper motif. Additionally, the L protein of nairoviruses contains an OTU-like domain (OTU) located at the amino terminus, which contains a topoisomerase-like motif. The approximate size, expressed as the number of amino acids (aa), is indicated for each protein, and the diagram is drawn approximately to scale. See text for details of the various motifs. Adapted from ref. [91]

to specific tick or mammalian hosts, and further studies on the importance of these various stages in the maturation of the glycoproteins for different hosts are clearly required.

The L protein

The L segment of nairoviruses, which contains a single open reading frame (ORF) of approximately 12 kb, encoding a protein of approximately 450 kDa, is almost twice as long as the L proteins of most other bunyaviruses, with the exception of the tospoviruses, in which the L segment is approximately 9 kb in length [48, 85, 94, 108] (Fig. 4). Despite this difference in length and sequence, nairovirus L proteins still show the four conserved functional regions previously described for other bunyavirus L proteins [94, 108, 131, 139, 177]. The bunyavirus L proteins contain the RNA-dependent RNA polymerase (RdRp), and the most conserved region of the L segment among nairoviruses is the region corresponding to the coding sequence for the core catalytic domains of the RdRp [8, 85, 108]. Within this polymerase module (also called region 3) can be distinguished six conserved motifs [94, 108] (Fig. 4). Motifs A through D are conserved among all RNA-dependent polymerases [50, 129]; motif pre-A, upstream of motif A, is present in all RNA-dependent RNA polymerases and reverse transcriptases, and motif E, which is downstream of motif D, is conserved in segmented negative-strand RNA viruses [114, 129, 186]. Motifs A, C and D are predicted to bind nucleoside triphosphates (NTPs) and are therefore likely to be involved in the catalytic functions of the polymerase [50, 114], while motifs B and E are predicted to take part in template and/or primer positioning [114]. Motif pre-A is also predicted to be involved in template positioning [94, 114]. The fact that the inter-motif distances are more or less constant suggests that the polymerase module functions in a structurally dependent manner [94]. Upstream of the

polymerase module are regions 1 and 2 (Fig. 4) which are conserved in bunyaviruses and arenaviruses [94, 114], while downstream of the polymerase module is region 4 (Fig. 4), which, although originally suggested to be specific for bunyaviruses [8], appears to be conserved in other segmented negative-strand RNA viruses [94]. Protein sequence analysis shows that the distances between regions appear to be conserved among bunyaviruses, with the exception of the interval between regions 2 and 3, where nairoviruses appear to have much longer amino acid sequences than other bunyaviruses (Fig. 4). Region 1, based on sequence similarity with other viruses, appears to be responsible for cap-snatching endonuclease activity [52, 136, 189]; however, this needs to be confirmed experimentally.

In the case of nairoviruses, the RdRp accounts for only one-third of the entire L protein (Fig. 4), with regions of unidentified function in both the amino (N) and carboxy (C) termini [94]. All bunyaviruses have a significant C-terminal section after the RdRp, although the function of this region is so far unknown in any of the viruses of this family, and there appear to be no cross-genera-conserved motifs [136]. N-terminal to the RdRp motifs, the L proteins of nairoviruses contain several additional domains that are unique to this genus, of which the ovarian-tumour (OTU)-like protease domain is the most studied [2, 29, 85, 89, 94]. This domain belongs to a larger papain-like cysteine protease family also found in other viruses (e.g., blueberry scorch virus (BlScV) of the genus *Carlavirus*, and equine arteritis virus (EAV) and porcine respiratory and reproductive syndrome virus (PRRSV) of the genus *Arterivirus*), in *Saccharomyces cerevisiae*, in *Drosophila melanogaster* and in mammalian cells [11, 103]. Curiously, the region containing the OTU domain also contains a sequence resembling a topoisomerase-like domain, located at amino acids 85-9, and therefore lying between amino acids that form part of the of the OTU catalytic site – cysteine 40 and histidine 151 [85, 94]. The consensus topoisomerase motif

(SKXXY) is not conserved across nairoviruses, being mostly SLXXY in CCHFV and NSDV/GV, but the active-site tyrosine is conserved across all nairoviruses so far sequenced. Given the structural similarities between topoisomerases and strand-specific recombinases [35], this motif may indicate a role for this region of L in RNA strand manipulation as well as its function as a protease. Alternatively, nairovirus L proteins may include their own topoisomerase activity, rather than having to recruit the host-cell topoisomerase I, as has been shown for at least one non-segmented RNA virus [159].

Downstream of the OTU domain, the L protein of nairoviruses contains a C2H2-type zinc finger domain and a leucine zipper motif [85, 94], both of which are highly conserved among nairoviruses, but the function of which in the viral replication cycle is still to be determined. Interestingly, the zinc-finger domain and leucine zipper motif are located in region 1 and region 2, respectively, of the nairoviral L proteins but do not appear to be present in these regions in the L proteins of other bunyaviruses.

The OTU-domain protease activity is dispensable for virus genome replication [16], and most recent studies have focussed on the effects of this enzymatic activity on host proteins. Mammalian OTU-domain proteins are primarily deubiquitinating enzymes (DUBs), responsible for cleaving the modified peptide bond that links ubiquitin (Ub) to host-cell proteins or to other Ub molecules. Most mammalian DUBs are only able to deubiquitinate and usually have a limited set of targets that they de-conjugate in this way (reviewed in ref. [96]). The OTU domains of nairoviruses, in contrast, are capable of de-conjugating not only Ub but also other ubiquitin-like peptides, notably the interferon-stimulated gene 15 protein (ISG15), removing these peptides from a variety of protein targets [10, 69, 84]. It has been shown that amino acids 1 to 169 of the L proteins of CCHFV, NSDV or DUGV are sufficient for enzymatic activity, and conserved active site residues that are critical for its catalytic activity have been identified [10, 69, 84]. In the last few years, the crystal structures of the OTU domain with and without a ubiquitin molecule have been determined [2, 29, 89]. The CCHFV OTU showed an overall similar structure to yeast Otu1, but with an additional domain formed by two antiparallel β-strands that allow the viral OTUs to bind both Ub and ISG15 [2, 89].

Specific cleavage targets, other than host ubiquitinated or ISGylated proteins, for the viral OTU-like protease have not yet been described. As several potential cysteine-protease-like cleavage sites have been identified in the L protein sequence of nairoviruses [94] and some viral proteins containing an OTU-like protease domain have also been shown to undergo autoproteolytic cleavage to generate multiple mature proteins, e.g., the replicase of BlScV [98], it has been suggested that the L proteins of

nairoviruses may also be autoproteolytically cleaved into an active RNA polymerase and protein(s) with additional function [85]. We have raised specific antibodies to the N- and C-termini of the NSDV L protein and shown that such cleavage does not occur in infected cells (Lasecka and Baron, unpublished).

Controlling host innate immune responses

Early studies on nairoviruses showed that, unlike other bunyaviruses, they do not shut off host protein synthesis [33, 176], so the viruses must have a different way of controlling the immediate host cell responses to infection, such as the innate immune response and apoptosis.

CCHFV infection in cultured cells induced apoptosis, albeit at late stages of infection [91, 141]. One of the ways in which nairoviruses might induce apoptosis has been suggested to be via the induction of ER stress [141]. Certainly CCHFV and DUGV have both been shown to induce ER stress [141], and we have observed the same in cells infected with NSDV (Lasecka, unpublished data). During replication, nairoviruses synthesise large amounts of their glycoproteins, which mature in the ER and Golgi [17, 21, 142] and are likely to overload the normal ER protein synthesis machinery. As long as the apoptosis occurs after the virus has completed the assembly and release of progeny virus, apoptosis is not a major problem, but it is unclear as yet whether nairoviruses take active steps to inhibit apoptotic pathways. There is the possibility that apoptosis may be delayed or inhibited in CCHFV infections by the presence of a highly conserved caspase-3 cleavage site (DEVD) in the N protein [31, 91], which may act as a decoy substrate for caspase-3. Although this motif has been suggested to be involved in control of apoptosis [31, 91], the cleavage of N protein by caspase-3 is not required for replication/transcription of a CCHFV minigenome [31]. In addition, this DEVD motif appears to be inaccessible to caspase-3 in the oligomeric form of CCHFV N, suggesting that only N monomers are caspase-3 sensitive [174]. CCHFV also appears to be the only nairovirus that contains the DEVD motif [174]; other nairoviruses, such as NSDV/GV, do not have the motif, yet are still highly pathogenic.

The innate immune response to viral infection has been described in a number of recent reviews [72, 105, 132, 143]. Cells detect viral pathogen-associated molecular patterns (PAMPs) (e.g., double-stranded RNA (dsRNA) or DNA with unmethylated CpG motifs) using pattern recognition receptors (PRRs), of which the most well-known are the Toll-like receptors (TLRs), which scan extra-cytoplasmic spaces including the interior of endosomes and lysosomes, and the cytosolic proteins melanoma-associated

differentiation gene-5 protein (MDA-5) and retinoic acid-inducible gene-I protein (RIG-I), which detect virus-associated PAMPS in the cytoplasm. Recognition of a PAMP leads to activation of the PRR, followed by an intracellular signalling cascade leading to the transcription of interferon β (IFNβ) mRNA. Secreted IFNβ works in an autocrine and paracrine manner by binding to the IFNα/β receptor of both infected and uninfected cells. This in turn activates a signalling pathway, which up-regulates interferon-stimulated genes (ISGs), including IFNα, which increases the stimulus to other ISGs. Finally, the products of ISGs (directly or indirectly) lead to the cell entering the so-called antiviral state, in which many proteins are present that can inhibit different viruses. Interferon and its actions have strong inhibitory effects on the replication of nairoviruses. For instance, treatment of human endothelial cells with IFNα significantly reduced the yield of CCHFV [6], while the replication of CCHFV and DUGV is impaired in Vero cells stably expressing the human Mx (MxA) protein; MxA is the product of an ISG and appears to act through sequestration of the N protein of these viruses in a perinuclear region [4, 25].

However, as with other viruses, nairoviruses have developed mechanisms to evade this innate antiviral response. Some of these mechanisms appear to be unique to one virus; others appear to be common to all the viruses in the genus that have been studied. For example, CCHFV can delay IFNα/β production in infected cells; Andersson *et al.* showed that, in CCHFV-infected cells, an increase in IFNβ mRNA could only be detected 48 h after infection, which leaves the virus a replication window with no antiviral state [7]. This delay is related to a delay in translocation of the transcription factor interferon regulatory factor-3 (IRF-3) to the nucleus [7] and hence a delay in the induction of ISG56, one of the genes whose transcription is regulated by IRF-3. The ISG56 protein is a cytoplasmic protein (p56) that is involved in the global inhibition of translation in response to infection (reviewed in ref. [63]). The delay in ISG56 expression correlates with earlier findings that nairoviruses do not appear to shut off cellular protein synthesis [33, 176]. In contrast to the findings with CCHFV, IFNβ induction was seen in NSDV/GV-infected cells after about 16 hours; however, during those 16 hours, the virus actively blocked induction of IFNβ [84]. NSDV/GV infection also blocked the signalling pathways activated by external IFNα or IFNγ, blocking the activation of transcription from promoters normally activated by one or the other of these cytokines by directly inhibiting the phosphorylation of the transcription factors STAT1 and STAT2 [84].

Of the cytoplasmic PRRs, RIG-I is activated by RNAs with a 5' triphosphate group, such as viral vRNAs and cRNAs, while MDA-5 is activated by binding dsRNAs [44, 86, 92, 128]. Like other negative-sense RNA viruses, nairoviruses do not produce detectable amounts of dsRNA during replication [178] and hence avoid activation of a number of dsRNA-sensitive PRRs (e.g., MDA-5, TLR3) and dsRNA-dependent enzymes (e.g., dsRNA-activated protein kinase R [PKR] and 2'5'-oligoadenylate synthetase [2'5' OAS]). The newly synthesised vRNA and cRNA of nairoviruses, as for other bunyaviruses, is cotranscriptionally encapsidated by the N protein, minimising the exposure of vRNAs and preventing formation of dsRNA intermediates [127, 135, 173]. It is thought that the major sensor for bunyavirus infection is therefore RIG-I. 5'triphosphate groups are present on RNA molecules generated during viral replication but are absent on cellular RNAs, as cellular mRNA contains a cap structure at the 5' end, and the transcription products of other cellular RNA polymerases (RNA polymerases I and III) contain a monophosphate at the 5' end. By utilising capped, short nucleotide sequences snatched from host mRNA to initiate viral mRNA transcription [90], nairoviruses prevent recognition of viral mRNA by PRRs. CCHFV further avoids activation of RIG-I as its vRNA and cRNA have a monophosphate group rather than the triphosphate group found at the 5' end of most viral RNAs [78], though the mechanism of 5' monophosphate generation during replication of CCHFV remains unknown. Strategies to generate 5'monophosphates on viral RNA have been shown for other viruses: HTNV utilises a prime-and-realign mechanism to generate 5' and 3' complementary ends, where a viral endonuclease (which is probably also involved in cap-snatching) is proposed to remove 5'-terminal extensions, leading to a 5' monophosphate on the final RNA [73]. Interestingly, this mechanism is not common to all bunyaviruses, as RVFV was shown to contain triphosphate groups at the 5' ends of its vRNAs, which can activate IFNβ via the RIG-I pathway [78]. It will be interesting to see if other nairoviruses adopt the RNA processing seen for CCHFV. It is possible that bunyaviruses that have developed methods of blocking the RIG-I-activated pathway (e.g., by the activities of a nonstructural protein such as the phlebovirus NSs protein) can have triphosphates at the 5' end of their vRNAs while viruses that do not have an NSs activity must generate monophosphates to avoid activating the RIG-I pathway in the first place [18, 19, 23, 73].

Ubiquitin and ubiquitin-like molecules play important roles in the initiation and maintenance of immune response. For example, they are essential for the action of cytokines such as IFNα/β and tumour necrosis factor alpha (TNFα) (reviewed in ref. [95]). Ubiquitination allows for the activation of nuclear factor kappa B (NF-κB) by targeting the inhibitor of NF-κB (I-κB) for degradation [163]. K63-linked ubiquitination activates several molecules of the IFNβ induction pathway, including RIG-I, mitochondrial antiviral

signalling protein (MAVS), TANK-binding kinase-1 (TBK1), IκB kinase-ε (IKK-ε), tumour necrosis factor receptor-associated factor 3 (TRAF3) and TRAF6 [70, 125, 126, 166]. In addition to modulation of the innate immune signalling, ubiquitination also plays an important role in antigen presentation by major histocompatibility complex (MHC) class I and II proteins [152]. ISG15 is an IFN-induced 15-kDa ubiquitin-like molecule that is composed of two-ubiquitin-like domains [20, 117]. Although the precise role of ISG15 in modulation of protein function is unknown, conjugation by ISG15 is also known to modify hundreds of cellular proteins, including several of those involved in the antiviral response, e.g., PKR, MxA, STAT1, RIG-I, Janus kinase 1 (JAK1), and IRF-3 [75, 104, 138, 192].

Both Ub and ISG15 are synthesised as precursors, which are cleaved in order to expose the conjugation sequence (LRLRGG) by which they are attached to other proteins. The conjugation is mediated by a sequence involving activating enzymes (E1), conjugating enzymes (E2) and protein ligases (E3) [157, 180, 191] (reviewed in ref. [169]). Removal of these conjugated proteins is carried out by cellular deubiquitinating enzymes and deISGylating enzymes, which have roles, as expected, in negative feedback regulation of IFN induction and action [93, 99]. As described in the discussion of the nairovirus L protein, the N-terminus of these proteins contains an OTU protease-like domain [85] similar to that often found in mammalian DUBs, and experimental evidence showed that this protease domain in the viral protein indeed deconjugated Ub and ISG15. Global ubiquitination and ISGylation levels were greatly reduced in NSDV/GV-infected cells [84], and overexpression of the amino-terminal end of the L proteins of CCHFV, NSDV or DUGV in cell culture resulted in a similar reduction in ubiquitin- and ISG15-conjugated proteins [10, 30, 69, 84]. The L protein OTU domains of several nairoviruses have been shown to block IFNβ induction and the actions of type I and type II IFNs [10, 69, 84]. Interestingly, at high enough concentrations, even catalytically inactive OTUs are capable of blocking IFNα-induced transcription [10, 69]. This suggests that catalytically inactive OTU domain proteins are still capable of sequestering specific ubiquitinated or ISG15ylated targets by binding to them.

The nsp2 protein of EAV and PRRSV also contain OTU-like domains [69]. Recently, it was shown that both the nsp2 of EAV and the OTU domain of CCHFV L protein are able to deubiquitinate RIG-I and hence block RIG-I-mediated activation of IFNβ [168]. Interestingly, the RNA of neither CCHFV nor EAV appears to activate RIG-I [78, 168], so the fact that these viruses have evolved specific mechanisms to inhibit the RIG-I-mediated induction of IFNβ suggests that RIG-I still has a function in the antiviral response to these viruses. However, unlike CCHFV, the vRNA of NSDV/GV

does activate transcription from the IFNβ promoter (Lasecka and Baron, unpublished observations). The ability to directly block the RIG-I pathway would therefore be particularly important for this virus.

Comparison of the OTU domains of different nairoviruses revealed some differences between their affinity for different types of poly-Ub and ISG15 [30]. For instance, CCHFV shows a higher affinity for K63-poly-Ub than K48-poly-Ub, and the CCHFV OTU was more active in the deubiquitination of host proteins than the OTUs of either NSDV or DUGV [10, 30, 84]. On the other hand, while the ERVEV OTU appears to bind any poly-Ub weakly (when compared to those of CCHFV and DUGV), it had higher affinity for ISG15 [30]. This may indicate that different nairoviruses have adopted slightly different ways of utilising their core deubiquitinating and deISGylating activities, which might reflect the wide range of pathogenicity caused by these viruses, or differences in the requirements imposed on these viruses by their arthropod hosts, about which we know very little. ISG15 is not as strongly conserved as ubiquitin among different species, even mammals, so there can be real effects of species preference in ISG15 binding/cleavage, e.g., the CCHFV OTU appears to show a preference for ISG15 of human origin over that of mouse origin, while ERVEV appears to recognise both human and mouse ISG15s equally [30]. The better binding of the murine ISG15 by the ERVEV OTU may be associated with the homology between the ISG15 of mouse and the white-toothed shrew from which ERVEV is commonly isolated [34, 184].

Final remarks

Nairoviruses share many of their features with other bunyaviruses, e.g., replication in the cytoplasm, budding in the Golgi, and their coding and RNA replication strategy. From phylogenetic studies, the members of the genus *Nairovirus* appear to be most closely related to those of the genus *Phlebovirus* of all bunyaviruses [108, 131, 139, 177]. However, nairoviruses possess many features not found in other bunyaviruses. Nairoviruses appear to have complex processing of their glycoproteins, which involves the actions of cellular proteases such as SKI-1/S1P-like proteases and furin. The proteins of nairoviruses also contain domains that have not been observed in other bunyaviruses, such as the L protein OTU domain. Nairoviruses express a secreted mucin-like domain, which may play an essential role in the pathogenicity of the virus [154, 188]. Structural similarity between the nairovirus N protein globular domain or the RdRp region of its L protein and equivalent proteins of arenaviruses has been taken to suggest that the nairoviruses are more closely related to arenaviruses than

to members other genera of the family *Bunyaviridae* [31, 170], with some authors even suggesting that the current classification of the *Nairoviridae* might need re-evaluation in the future [31].

Several areas for future study stand out. Given that the nairoviruses are, in general, tick-borne, while most other bunyaviruses are insect-borne, it is to be expected that the interaction of these viruses with their arthropod hosts will be specific to the virus genus and need specific study. Fortunately, expertise with handling ixodid ticks and tick cell lines is rapidly increasing, and it is to be hoped that our understanding of the replication of these viruses in their tick hosts will catch up with our knowledge of what is happening in mammals. The nairoviruses have been more resistant to the development of successful reverse genetics than e.g. the orthobunyaviruses or the phleboviruses, and development of such a system for nairoviruses will be invaluable in helping us understand the roles of various nairovirus-specific domains such as the topoisomerase-like domain, C2H2-zinc finger domain, leucine zipper motif, and OTU domain of the L protein, or the NS_M and mucin-like domain from the M segment, both in mammalian and arthropod hosts. The ability to create targeted mutations will also enable us to more rapidly develop stably attenuated viruses that could act as vaccines.

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