

# Chapter 3

## Cholesterol-Binding Viral Proteins in Virus Entry and Morphogenesis

Cornelia Schroeder

**Abstract** Up to now less than a handful of viral cholesterol-binding proteins have been characterized, in HIV, influenza virus and Semliki Forest virus. These are proteins with roles in virus entry or morphogenesis. In the case of the HIV fusion protein gp41 cholesterol binding is attributed to a cholesterol recognition consensus (CRAC) motif in a flexible domain of the ectodomain preceding the trans-membrane segment. This specific CRAC sequence mediates gp41 binding to a cholesterol affinity column. Mutations in this motif arrest virus fusion at the hemifusion stage and modify the ability of the isolated CRAC peptide to induce segregation of cholesterol in artificial membranes.

Influenza A virus M2 protein co-purifies with cholesterol. Its proton translocation activity, responsible for virus uncoating, is not cholesterol-dependent, and the transmembrane channel appears too short for integral raft insertion. Cholesterol binding may be mediated by CRAC motifs in the flexible post-TM domain, which harbours three determinants of binding to membrane rafts. Mutation of the CRAC motif of the WSN strain attenuates virulence for mice. Its affinity to the raft–non-raft interface is predicted to target M2 protein to the periphery of lipid raft microdomains, the sites of virus assembly. Its influence on the morphology of budding virus implicates M2 as factor in virus fission at the raft boundary. Moreover, M2 is an essential factor in sorting the segmented genome into virus particles, indicating that M2 also has a role in priming the outgrowth of virus buds.

SFV E1 protein is the first viral type-II fusion protein demonstrated to directly bind cholesterol when the fusion peptide loop locks into the target membrane. Cholesterol binding is modulated by another, proximal loop, which is also important during virus budding and as a host range determinant, as shown by mutational studies.

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C. Schroeder (✉)  
Max Planck Institute for Molecular Cell Biology and Genetics, Pflotenhauerstr. 108, D-01307,  
Dresden, Germany  
e-mail: cornelia.schroeder@mpi-cbg.de

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### Abbreviation

aa	amino acid
CBPPA	cholesterol-protein binding blot assay
CCM	cholesterol consensus motif
CHS	cholesterol hemisuccinate
CRAC	cholesterol recognition amino acid consensus
DHSM	dihydrosphingomyelin
DRM	detergent-resistant membrane
DSC	differential scanning calorimetry
DV	dengue virus
FP	fusion peptide
FPLC	fast performance liquid chromatography
gp41	glycoprotein 41 (refers to molecular weight 41 kD)
GPCR	G protein-coupled receptor
HA	hemagglutinin
HIV	human immunodeficiency virus
K-D	Kyte-Doolittle (scale of hydrophobicity)
ld	liquid-disordered
LLP	lentivirus lytic peptide
lo	liquid-ordered
LUV	large unilamellar vesicles
Mab	monoclonal antibody
MAS	magic angle spinning
MBP	maltose binding protein
MLV	murine leukaemia virus
MPR	membrane proximal region
m $\beta$ CD	methyl- $\beta$ -cyclodextrin
NA	neuraminidase
NMR	nuclear magnetic resonance
pHtrans	pH of conformational transition
PIP3	phosphatidylinositol-3,4,5-triphosphate
PIP4,5P2	phosphatidylinositol-4,5-bisphosphate
PM	plasma membrane
POPC	palmitoyl oleyl phosphatidylcholine
pre-TM	pre-transmembrane
pre-TMp	pre-transmembrane peptide
RNP	ribonucleoprotein
S protein	spike protein
SARS	severe acute respiratory syndrome
SFV	Semliki Forest virus

SIN	Sindbis virus
SIV	Simian immunodeficiency virus
SM	sphingomyelin
SOPC	stearoyl oleyl phosphatidylcholine
$\beta$ 2AR	human $\beta$ 2-adrenergic receptor
TBE	tick-borne encephalitis virus
TM	transmembrane
TSPO	outer mitochondrial membrane translocator protein
TX-100	Triton X-100
Udorn	influenza A/Udorn/307/72
VSV	Vesicular stomatitis virus
WSN	influenza A/WSN/33
W-W	White-Wimley (scale of hydrophobicity)
XIP	exchanger inhibitory peptide

### 3.1 Introduction

Viruses cross membrane barriers in the process of infection and again during assembly and release through membranous compartments. The role of membrane lipid composition and protein-lipid binding for specific biological functions of viruses and organelles is an area of intense investigation. Proteins with functions in membrane domain organization, trafficking, fusion and fission often possess specific lipid binding sites. X-ray crystallography revealed the first cholesterol-binding site in the 3D-structure of a signalling protein (Cherezov et al., 2007, Hanson et al., 2008). The discovery of another cholesterol binding motif 10 years earlier in a mitochondrial protein (Li and Papadopoulos, 1998) has been influential for a number of studies of viral proteins carrying this motif.

Cholesterol is a class apart from the other membrane lipids. Unlike these it cannot form membranes on its own, and being the most compact of the lipids, it penetrates less deeply into the hydrophobic layer of membrane leaflets, while its miniature hydroxyl head-group barely projects into the interfacial zone. Cholesterol is also the most diffusible lipid, and the one most akin to small-molecule drugs in structure and function. Modulation of membrane cholesterol levels – physiological or induced by drug therapies or viral infection itself – can have significant consequences for virus replication. Cholesterol anchors on antiviral drugs and other inhibitors make these raftophilic and target and concentrate them into membrane rafts and to membrane trafficking pathways where they interfere most effectively with pathogenic processes (reviewed by Rajendran et al., 2010).

Membrane rafts are implicated in the entry and egress of many virus species (Nayak and Hui, 2004; Ono and Freed, 2005). Rafts are nanoscale dynamic, lateral membrane domains with a specific lipid and protein composition, enriched in cholesterol and sphingolipids, that form a liquid ordered-like phase separated from bulk membrane (Simons and Ikonen, 1997; Hancock, 2006). Signalling cycles at the plasma membrane involve reversible coalescence and disassembly of rafts driven by

activation states of the raft proteins (Rajendran and Simons, 2005), whereas during virus morphogenesis rafts merge irreversibly into microscale platforms of assembly and budding (for reviews see Schmitt and Lamb, 2005; Waheed and Freed, 2009). The affinity of virus envelope proteins to rafts is not attributed to specific lipid binding sites but to a combination of acylation and long transmembrane (TM) segments (Scheiffele et al., 1997; Melkonian et al., 1999; Rousso et al., 2000). In addition, certain viral envelope proteins exhibit distinct affinity to cholesterol. It has proved challenging to correlate this property to specific cholesterol binding sites on the one hand and to biological function on the other. These issues are at the center of this review. Since viral cholesterol-binding proteins usually are components of the virion, the review begins with virus lipidomics.

### ***3.1.1 Virus Lipidomics***

The composition of influenza virus (IFV) and human immunodeficiency virus (HIV) envelopes was determined by liquid chromatography (Aloia et al., 1993; Zhang et al., 2000), while recent data for HIV and other viruses has been generated by mass spectroscopy (Brügger et al., 2006, 2007; Chan et al., 2008; Kalvodova et al., 2009; Lorizate et al., 2009).

The lipid compositions of the envelopes of ‘non-raft’ enveloped viruses (Semliki Forest virus, SFV, family *Togaviridae*, genus *Alphavirus*) and vesicular stomatitis virus – VSV, family *Rhabdoviridae*, genus *Vesiculovirus*) are remarkably similar and closely resemble that of the host cell plasma membrane (PM) from which they bud (Kalvodova et al., 2009). No significant differences between SFV and VSV were seen at the lipid class level, but saturated and mono-unsaturated glycerophospholipids were enriched in SFV as compared to VSV and differences in fatty acid chain length were seen. Compared to PM the viruses showed some selectivity for sphingomyelin (SM), especially, long chain and dihydrosphingomyelin – DHSM, and depletion of GM3 (Kalvodova et al., 2009).

IFV buds from apical PM with an envelope of cholesterol and sphingolipid-rich raft membrane (Scheiffele et al. 1999, Zhang et al., 2000). Raft association is intrinsically encoded in HA (Scheiffele et al., 1997), the most abundant envelope glycoprotein. The ability of viral envelope proteins to select a cognate lipid environment is uncovered in IFV mutants defective in raft association and budding. Thus, the envelope of a double mutant lacking the cytoplasmic tails of hemagglutinin (HA) and neuraminidase (NA) (HA<sup>-</sup>/NA<sup>-</sup>) incorporated three times more triglycerides and proportionally less raft lipids, cholesterol and SM (Zhang et al., 2000). Membrane rafts and specifically cholesterol are also involved in IFV entry. Sun and Whittaker (2003) demonstrated that depleting IFV envelope cholesterol by methyl- $\beta$ -cyclodextrin (m $\beta$ CD) extraction specifically blocked the fusion of infectious virus with the PM of pH 5-treated host cells, a process mimicking virus infection by fusion with the endosomal membrane. Similarly, the requirement of cholesterol in the HIV envelope for infection (Campbell et al., 2002; Guyader et al., 2002) correlates with the integrity of viral envelope rafts (Campbell et al., 2004).

Brügger et al. (2006) presented the first comprehensive HIV-1 lipidomics, extended by Chan et al. (2008) who added analysis of phosphoinositides and compared retroviruses, HIV-1 and 2 and murine leukaemia virus (MLV), as well as the PMs of three host cell lines. The lipid compositions of HIV-1 and 2 vary with that of the plasma membrane raft domain of their host cells (Chan et al., 2008). For example, the content of DHSM in HIV-1 from a macrophage cell line (MDM) is twice that of the T cell line H9. Similar differences in lipid composition were observed between HIV budding from MT4 versus 293T cells and correlated to membrane order of the virus envelope reported by the dye laurdan (Lorizate et al., 2009).

The HIV envelope protein gp160 seems to exert little influence on the envelope's lipid composition (Chan et al. 2008). Nevertheless, there are ways in which HIV proteins modify the lipid composition of the viral envelope. HIV accessory protein Nef enforces the raft character of the plasma membrane by significantly reducing polyunsaturated PC species and enriching SM (Brügger et al., 2007). Although Nef also boosts cholesterol synthesis (Zheng et al., 2003), cholesterol levels of the PM and the virus envelope are not altered in HIV infection (Brügger et al., 2007). Nef is a raft protein (Wang et al., 2000) but cholesterol binding by Nef itself (Zheng et al., 2003) has been contested (Brügger et al., 2007).

Gag is the main determinant of HIV raft association (Bhattacharya et al., 2006) and the driving force of particle formation and budding (Morita and Sundquist, 2004).

Phosphatidylinositol phosphates are the one raft lipid class preferentially incorporated into retroviral envelope over PM. Phosphatidylinositol-4,5-bisphosphate (PIP4,5P2) enrichment disappears upon deletion of the polybasic stretch at the head of the Gag protein MA domain (Chan et al. 2008), confirming its essential binding to PIP4,5P2 and via PIP4,5P2 to membrane (Ono et al., 2004; Murray et al., 2005; Chukkapalli et al., 2008). Enzymatic degradation of PIP4,5P2 also interferes with HIV budding (Chan et al., 2008). Similarly, PIP4,5P2 and PIP3 levels are increased during Respiratory syncytial virus (RSV) infection, and inhibiting their synthesis impaired formation of virus progeny (Yeo et al., 2009).

### ***3.1.2 Cholesterol Binding Sites***

In 1998 Papadopoulos and colleagues described a cholesterol recognition site VLNYYVWR in the outer mitochondrial membrane translocator protein TSPO, formerly known as peripheral-type benzodiazepine binding protein. Based on homology searches of other cholesterol binding proteins they proposed a cholesterol recognition amino acid consensus  $L/V-(X)_{1-5}-Y-(X)_{1-5}-R/K$  (CRAC; Li and Papadopoulos, 1998). TSPO is involved in cholesterol transport to cytochrome P450 which catalyzes the first steroidogenic reaction (Papadopoulos et al., 2007) (see also Chapter 15). The cholesterol-binding groove in a hydrophobic  $\alpha$ -helix near the cytosolic C-terminus was confirmed by mutational studies and modelled (Li and Papadopoulos, 1998, Li et al., 2001, Jamin et al., 2005). Contributions from other TSPO  $\alpha$ -helices to the binding site were predicted (Jamin et al., 2005; Murail et al.,

2008) and presented in a 3D homology model based on apolipoprotein III, with the five TM  $\alpha$ -helices surrounding one cholesterol molecule docked to the CRAC domain (Rone et al., 2009).

The crystal structure of a human  $\beta$ 2-adrenergic receptor ( $\beta$ 2AR), a G protein-coupled receptor (GPCR), revealed a cholesterol binding site formed by amino acids of  $\alpha$ -helices IV and II (Cherezov et al., 2007; Hanson et al., 2008). These define a cholesterol consensus motif, CCM, conserved among human class A GPCRs (Hanson et al., 2008). For the purpose of comparison to CRAC, CCM is written as R/K-(X)<sub>7-10</sub>-W/Y-(X)<sub>4</sub>-I/V/L on one  $\alpha$ -helix, and F/Y on the other. The motifs CCM and CRAC are obviously related by inversion. In three dimensions both motifs may determine similar binding grooves, however,  $\beta$ 2AR in contrast to TSPO appears to bind two stacked molecules of cholesterol. Since CRAC and CCM are both quite degenerate, they occur frequently; not every occurrence will be a cholesterol recognition site. A common feature of CRAC and CCM is the  $\alpha$ -helical secondary structure. It is reasonable to muster additional criteria for a cholesterol recognition site of this type, i.e. inclusion in or proximity to an  $\alpha$ -helical amphiphilic or trans-membrane (TM) domain. Table 3.1 cites examples of CRAC motifs in viral proteins reviewed here, in comparison to CRAC motifs of cellular proteins. Also shown are CRAC motifs currently not implicated in cholesterol binding. For example, the influenza A M1 protein exhibits three such motifs, one of which is shown. It is part of the helix six domain which has affinity to membrane and to RNP (Ruigrok et al., 2000). Other short sequence motifs proposed in cholesterol binding (cf. Politowska et al., 2001; Yao and Papadopoulos, 2002) have not been analysed in virus proteins.

### ***3.1.3 Methods Demonstrating Protein–Cholesterol Binding***

Since cholesterol adheres to hydrophobic surfaces, evidence of binding specificity collected with independent methods is desirable. Table 3.2 lists approaches for probing the physical association of proteins with cholesterol, as reported for selected viral and cellular proteins. A comprehensive discussion of such methods is the subject of Chapter 1 of this book (Gimpl, 2010). The upper half of Table 3.2 lists ways of analysing cholesterol bound to proteins or peptides, purified or in membrane fractions, the lower part addresses binding-site mapping. The  $\beta$ 2-adrenergic receptor, where X-ray crystallography revealed the cholesterol binding site belongs to the GPCR superfamily; previous studies on various GPCR have indicated a function of cholesterol in receptor activity (reviewed by Hanson et al., 2008; Paila et al., 2009). The natural variation of the cholesterol-binding site CCM in GPCRs will enable structure-function analysis. In *Drosophila* metabotropic glutamate receptor, ligand affinity increases with raft association; labelling with <sup>3</sup>H-photocholesterol (Thiele et al., 2000) demonstrated the sterol affinity of this particular GPCR (Eroglu et al., 2003). Semliki Forest Virus (SFV) E1 fusion protein was also labelled with photocholesterol (Umashankar et al., 2008).

**Table 3.1** Occurrence and proposed function of CRAC-like motifs in viral and cellular proteins

Protein	Protein domain function	Motif	Localization protein domain/membrane face	References/comments
TSPO mouse	Cholesterol transport	147V <u>L</u> N <u>Y</u> Y <u>V</u> W <u>R</u> <sub>154</sub>	C-terminal TM $\alpha$ -helix/mitochondrial outer membrane	Li and Papadopoulos (1998); Li et al. (2001); Jamin et al. (2005)
TSPO human gp41, HIV-1	Membrane fusion	147T <u>L</u> N <u>Y</u> CV <u>W</u> R <sub>154</sub> 679L <u>W</u> Y <u>I</u> K <sub>683</sub>	Pre-TM ecto-domain/outer leaflet of PM and viral envelope	Binds to CHS agarose (cp. Table 3.2) Vincent et al. (2002)
M2 Influenza A virus	Incorporation of Env into virus particles	763L <u>C</u> L <u>F</u> S <u>Y</u> H <u>R</u> L <u>R</u> <sub>772</sub> 833V <u>Q</u> A <u>A</u> Y <u>R</u> A <u>I</u> R <sub>841</sub>	Endodomain: pre-LLP-2 and inside LLP-1/inner leaflet of PM and viral envelope	No binding to CHS agarose (cp. Table 3.2) Vincent et al. (2002)
M2 Influenza A virus	Assembly budding/pinching off	44D <u>R</u> L <u>F</u> F <u>K</u> C <u>I</u> Y <u>R</u> R <u>L</u> K <u>Y</u> G <u>L</u> K <sub>60</sub> <sup>1</sup> 44D <u>R</u> L <u>F</u> F <u>K</u> C <u>I</u> Y <u>R</u> R <u>F</u> K <u>Y</u> G <u>L</u> K <sub>60</sub> <sup>2</sup> 44D <u>R</u> L <u>F</u> F <u>K</u> C <u>I</u> Y <u>R</u> F <u>F</u> K <u>H</u> G <u>L</u> K <sub>60</sub> <sup>3</sup> 44D <u>R</u> L <u>F</u> F <u>K</u> C <u>I</u> Y <u>R</u> F <u>F</u> E <u>H</u> G <u>L</u> K <sub>60</sub> <sup>4</sup> 55L <u>K</u> Y <u>G</u> L <u>K</u> <sub>60</sub> <sup>1</sup> 97V <u>K</u> L <u>Y</u> R <u>K</u> L <u>K</u> <sub>104</sub>	PM and viral envelope; endodomain/cytoplasmic	Schroeder et al. (2005)
M1 Influenza A virus	Assembly budding		Matrix protein 'helix six domain' interacts with membrane and RNP	(One of three incidental CRAC motifs)
Human cardiac Na <sup>+</sup> /Ca <sup>2+</sup> exchanger <sup>5</sup>	Specific binding to PIP2 regulates activity	233D <u>R</u> R <u>L</u> F <u>Y</u> K <u>Y</u> V <u>V</u> K <u>R</u> Y <u>R</u> A <u>G</u> K <sub>269</sub> 261V <u>Y</u> K <u>R</u> Y <u>R</u> A <u>G</u> K <sub>269</sub>	Endogenous XIP region	(Incidental CRAC motifs)
NAP-22 chicken, rodent CNS	Cholesterol-dependent sequestering of PtdIns(4,5)P2	4L <u>S</u> K <u>K</u> K <u>K</u> G <u>Y</u> N <u>V</u> N <u>D</u> E <u>K</u> <sub>17</sub>	N-terminal/PM rafts, nucleus	Terashita et al. (2002); Epand et al. (2004, 2005a)

Sequences of influenza A virus strains: <sup>1</sup> Germany/27 (H7N7); <sup>2</sup> WSN/33 (H3N2); <sup>3</sup> Singapore/1/57 (H2N2); <sup>4</sup> Udom/307/72 (H3N2); cp. legend to Fig. 3.2; <sup>5</sup> accession number AAD26362

**Table 3.2** Methods and parameters in the analysis of cholesterol binding

Method/parameter		Protein					
		HIV gp41	Influenza M2 <sup>5</sup>	SFV E1	TSPO	GPCR	A $\beta$ <sup>a</sup>
Binding properties	Cholesterol incorporation	–	+	–	+ <sup>7</sup>	–	+ <sup>14</sup>
	– stoichiometry cholesterol per subunit	–	0.5–1	–	–	2 <sup>11,12</sup>	–
	– binding constant	–	–	–	6.1 nM <sup>8</sup>	–	–
	– Filipin staining	–	+	–	–	–	–
	Affinity chromatography	+ <sup>1</sup>	+	–	–	–	–
	Chemical shift of cholesterol carbon atoms ( <sup>13</sup> C MAS NMR)	+ <sup>2</sup>	–	–	–	–	–
	Complexation with cholesterol crystallites	(+) <sup>3</sup>	–	–	–	–	+ <sup>15</sup>
Binding site	CBPPA <sup>b</sup> mapping	–	–	–	–	–	+ <sup>14</sup>
	Binding site transplantation	+ <sup>1</sup>	–	–	+ <sup>9</sup>	–	–
	Photo-affinity labelling with <sup>3</sup> H steroid	–	–	+ <sup>6</sup>	+ <sup>9</sup>	+ <sup>13</sup>	–
	X-ray crystallography	–	–	–	–	+	–
	Mutant studies	(+) <sup>4</sup>	–	–	+ <sup>7,9,10</sup>	–	–

<sup>a</sup>A $\beta$  is the cleavage product of amyloid precursor protein

<sup>b</sup>CPBBA – Cholesterol–protein binding blot assay

<sup>1</sup>Affinity chromatography on cholesterol-hemisuccinate (CHS) agarose (Vincent et al., 2002)

<sup>2</sup>Epand et al. (2003)

<sup>3</sup>Experiments on short peptides, not full-length gp41

<sup>4</sup>Mutant studies on full-length protein biological function, not cholesterol binding (cf. Table 3.3)

<sup>5</sup>Schroeder and Lin (2005)

<sup>6</sup>Umashankar et al. (2008)

<sup>7</sup>Jamin et al. (2005)

<sup>8</sup>Lacapère et al. (2001)

<sup>9</sup>Li et al. (2001)

<sup>10</sup>Li and Papadopoulos (1998)

<sup>11</sup>Cherezov et al. (2007)

<sup>12</sup>Hanson et al. (2008)

<sup>13</sup>Eroglu et al. (2003)

<sup>14</sup>Yao and Papadopoulos (2002)

<sup>15</sup>Harris and Milton (2009)

The TSPO CRAC motif is currently the best-studied cholesterol-binding site, which perhaps explains why such motifs are being investigated in other proteins. Single mutants, where the signature residues Y and R of the CRAC motif were replaced by S and L abolished cholesterol uptake by bacteria expressing TSPO (Li and Papadopoulos, 1998). Transplantation of the CRAC motif into another protein,



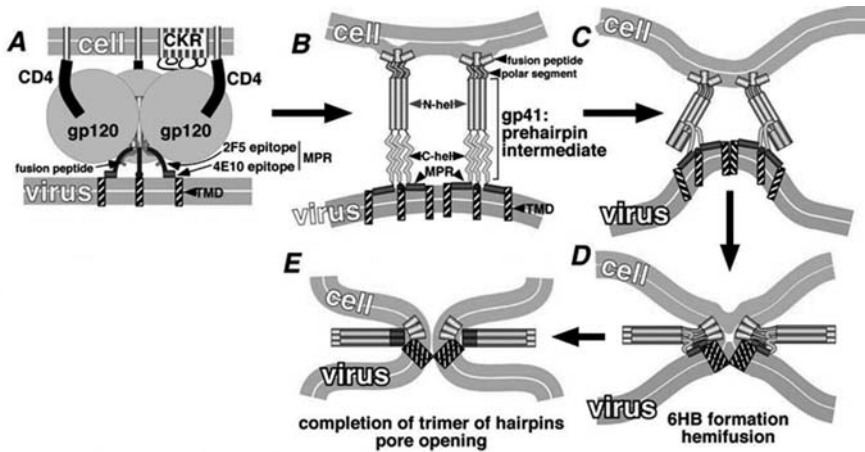
as done for TPSO and for HIV gp41, substantiated its assignment as a cholesterol-binding site. Photoaffinity labelling with  $^3\text{H}$ -promegestone of the TPSO CRAC motif transplanted onto Tat, a cell-permeating HIV protein, was competed 1000-times more efficiently by cold cholesterol than by promegestone. The triple mutant V149G, Y152S, R156L of this construct could not be photoaffinity labelled (Li et al., 2001), and the TSPO single mutant Y152S no longer bound  $^3\text{H}$ -cholesterol (Jamin et al., 2005). A cholesterol-protein binding blot assay enabled the delineation of a cholesterol-binding site in A $\beta$  (Yao and Papadopoulos, 2002). Independent studies by electron microscopy also point to specific cholesterol binding of  $\beta$ -amyloid (Harris and Milton, 2009; see also Chapter 2). The analysis of the chemical shift of cholesterol carbon atoms in complexes with short peptides containing cholesterol-binding motifs was pioneered by Epanand et al. (2003) and applied to gp41 CRAC. These authors also studied a number of sequence variations in short CRAC-containing peptides, discussed in Section 3.2.3.

## 3.2 Human Immunodeficiency Virus Fusion Protein gp41

In 2009 the notion that HIV is the paradigm of a virus that enters cells by fusion with the plasma membrane (reviewed by Gallo et al., 2003) was overturned. HIV enters the cell by receptor-mediated endocytosis, albeit at neutral pH (Miyachi et al., 2009). HIV is transmitted either by free virus particles or via fusion of infected with non-infected cells, forming multi-nucleate syncytia. Both the Env protein clusters on the donor side, and primary and secondary receptors on the acceptor side, reside in raft membrane domains (reviewed by Waheed and Freed, 2009).

Interactions of HIV gp41 with cholesterol have been investigated more extensively than those of any other viral protein. HIV gp41 is derived by proteolytic cleavage from its precursor, the envelope glycoprotein 160. In complex with the other cleavage product gp120, gp41 forms the trimeric spikes of the virus particle. The gp120 subunit presents the receptor binding sites for the primary receptor CD4 and for secondary receptors and, with the gp41 subunit, functions as a class I fusion protein. Fusion is prepared by a sequence of events triggered by adsorption to the primary receptor (Fig. 3.1). Receptor binding sets off extensive restructuring of gp41 to expose and propel the N-terminal fusion peptide into the target membrane (reviewed by Gallo et al., 2003).

The ectodomain comprises defined sub-domains, which refold into different secondary structures during fusion. From the point of view of cholesterol binding, the pre-transmembrane (pre-TM) or membrane proximal region (MPR) (Fig. 3.1) of 20 amino acids (664–683) immediately preceding the TM segment has attracted special interest: DKWASLWNWFNITNWLWYIK. It forms an  $\alpha$ -helix in lipid micelles, wherein four of the five tryptophan and the tyrosine residues align as a ‘collar of aromatic residues’ (Schibli et al., 2001). Analogous to tryptophan-rich antimicrobial peptides, the aromatic collar was predicted to engage with the aqueous interface of the membrane bilayer. The pre-TM terminates on LWIYK (679–683), the CRAC



**Fig. 3.1** Conformational transitions of HIV protein gp41 during the fusion cascade. **A** Release of the metastable state of the gp120-gp41 complex by binding to the primary and secondary receptors, CD4 and CKR. The membrane-proximal region MPR (pre-TM) is exposed adjacent to the virus envelope. The MPR-distal sequence occludes the fusion peptide. **B** gp120 trimers refold into extended  $\alpha$ -helical structure and harpoon the fusion peptide into the target membrane. Coil-to-amphipathic helix transition of the MPR-distal sequence enables immersion of the pre-TM in the membrane interfacial zone. **C** Extended  $\alpha$ -helices zip into a six-helix bundle (6HB) and clamp virus and cell membrane, causing **(D)** hemifusion and, by pulling the pre-TM into the trimer of hairpins, **(E)** fusion pore opening. Model of Bellamy-McIntyre et al. (2007), Figure 8 (modified), with permission from the American Society for Biochemistry and Molecular Biology

motif immediately proximal to the transmembrane domain. The role of the pre-TM has been studied at all levels of complexity, from mutational study of virus reproduction in the cell and effects on the various functions of gp41, to isolated proteins and peptides in artificial membrane systems.

### 3.2.1 Mutational Studies on the Pre-TM CRAC Motif in Virus-Cell Systems

Helseth et al. (1990) found that substituting the lysine of the LWYIK motif by isoleucine reduced syncytium formation by 95%. The mutation did not interfere with translation, processing and cell surface expression of Env, or with its binding to CD4, but this mutant Env expressed from a plasmid was completely unable to trans-complement the single-cycle replication of an Env-deleted virus. Salzwedel et al. (1999) explored the function of the pre-TM through substitution, deletion and insertion, and constructed a number of CRAC mutants – prior to the recognition of its cholesterol-binding significance. They found that the pre-TM is dispensable for maturation, trafficking, cell surface expression and CD4 binding, but is required for cell–cell fusion. The substitution WA within LWYIK was tolerated. In contrast,

replacing K abrogated fusion. Deletion of LWYI inhibited incorporation of gp120 into virus particles, viral entry and syncytium formation (Salzwedel et al., 1999) while lipid mixing and small molecule transfer were only reduced by 50% (Muñoz-Barroso et al., 1999). Analogously, inserting nine amino acids between Y and K inhibited entry (Salzwedel et al., 1999) and fusion, but reduced lipid and small content mixing only by about 50%. Thus, a dysfunctional LWYIK motif appeared to allow fusion pore formation, but arrest fusion pore expansion.

Ten years later, a new mutational study has now focused on the CRAC motif (Chen et al., 2009). Three deletion mutants and three point mutations were studied:  $\Delta$ LWYIK,  $\Delta$ YI,  $\Delta$ IK, KE, WA, YA. All mutant proteins underwent normal synthesis, oligomerization, cell surface expression, and incorporation with normally assembled Gag into mutant virus, i.e. budding was not impaired. However, multi-cycle replication of deletion mutants was significantly slowed, and virus infectivity and cell-cell fusion were strongly impaired (Table 3.3). The deletion mutants also interfered in *trans* with virus infectivity and to a lesser degree with fusion elicited by wild-type Env. The mutation KE suppressed infectivity in *trans* and eliminated fusion, and WA substitution was less disruptive than the other point mutations.

Overall, the recent study by Chen et al. (2009) shows that function of the LWYIK motif in virus infection is most sensitive to alteration of the CRAC consensus residues L, Y and K, confirming the earlier results of Salzwedel et al. (1999).  $\Delta$ LWYIK gp41 remains susceptible to peptides blocking the formation of the six-helix bundle fusion intermediate (Fig. 3.1), which proves a degree of functional independence of these subdomains. Thus, the effects of the mutations may be attributed to local interactions of the LWYIK motif with lipid membranes. Of interest, none of the mutations of the CRAC motif influenced raft association of gp41. In a dye transfer assay between Env-expressing effector and CD4-expressing target cells pre-TM  $\Delta$ LWYIK supported lipid mixing, tantamount to hemifusion, but inhibited small molecule content mixing (Chen et al. 2009). The Chen study

**Table 3.3** Phenotypes of CRAC motif\* mutations of HIV gp41

Mutant	Infectivity		Cell-cell fusion	Transdominant interference with		Lipid vs. content mixing
	Direct	Transcomplementation		Cell-cell fusion	Virus	
$\Delta$ <u>L</u> WYIK <sup>1</sup>	4	4	1	70	25	140 vs. 32
$\Delta$ <u>Y</u> I <sup>1</sup>	8	8	2	66	30	–
$\Delta$ <u>I</u> K <sup>1</sup>	8	8	<1	60	35	–
<u>L</u> I <sup>2</sup>	–	–	44–70	–	–	–
W <u>A</u> <sup>1</sup>	37	43	90	100	90	–
<u>Y</u> A <sup>1</sup>	46	48	35	–	–	–
<u>K</u> E <sup>1</sup>	40	17	< 10	–	–	–

% control; \*consensus residues are underlined; – not done after <sup>1</sup>Chen et al. (2009); <sup>2</sup>Epand et al. (2006)

confirms the conclusions of earlier studies (Muñoz-Barroso et al., 1999; Salzwedel et al., 1999), showing that LWYIK is required for the formation and dilation of fusion pores.

### ***3.2.2 Studies on Full-Length gp160, gp41 and Polypeptide Constructs***

One method for assaying the cholesterol affinity of a protein is by binding to cholesterol hemisuccinate (CHS) linked to agarose. Vincent et al. (2002) found that a soluble Env construct binds to CHS, but hardly at all to cholic acid agarose. As further controls, calmodulin agarose bound the construct via the Env calmodulin binding site and ConA-sepharose B via gp120-linked mannose. Maltose binding protein (MBP) fusions of gp41 sequences overlapping the pentapeptide LWYIK, even the minimal construct MBP-LWYIK, also attached to cholesterol hemisuccinate agarose, whereas others like the gp41 N-terminal fusion peptide, an immunodominant epitope or an endodomain fragment (aa752–856) did not. This sequence spans two CRAC motifs (Table 3.2), which are thus discounted. MBP fusions with complete or incomplete gp41, with or without TM, bound equally, as did a construct containing LWYIR. The study of Vincent et al. (2002) presented compelling evidence of cholesterol binding by the gp41 pre-TM CRAC motif, however, Chen et al. (2009) made the point that none of this work was replicated.

### ***3.2.3 Peptide Studies and Modelling***

The pre-TM has been analysed in detail by modelling and by experiments on isolated peptides and their membrane interaction. According to epitope mapping and hydrophathy analyses pre-TM was subdivided into short defined sequence elements. N-terminally, pre-TM overlaps the epitope of the monoclonal antibody (Mab) 2F5. Residues 666–673 constitute interfacial subdomain I, 670–676 the epitope of Mab 4E10, and 677–683 interfacial subdomain II (reviewed by Lorizate et al., 2008). Different from the TM segment, which is hydrophobic according to the classical Kyte-Doolittle (K-D) scale, the pre-TM exhibits interfacial hydrophobicity as defined by White and Wimley (1999) (W-W). The K-D scale is based on phase partitioning of hydrophobic side chains, while the W-W scale reflects whole residue partitioning of oligopeptides into the bilayer interface of POPC. For the N-terminal fusion peptide W-W and K-D hydrophathy overlap. In contrast, for pre-TM of HIV-1, 2 and SIV the distance between the W-W peak and the transmembrane K-D peak is 15–20 residues. Pre-TM interfacial hydrophobicity analysed in a narrow window of 5 aa exhibits two peaks, and mutations eliminating this bifurcation also interfere most with fusion (Sáez-Ciri3n et al., 2003), e.g.  $\Delta$ LWYI, the CRAC deletion of Salzwedel et al. (1999).

Suárez et al. (2000a) tested the ability of the gp41 fusion (FP) and pre-TM peptides (pre-TMp) to permeabilize and fuse artificial membranes. Surprisingly, induction of membrane leakage by, and fusogenic activity of pre-TMp on large unilamellar vesicles (LUVs), is greater than the activity of the fusion peptide (FP). The validity of these peptide assays with respect to the earlier virus-cell studies (Salzwedel et al., 1999) was underscored by the inactivity of pre-TMp mutant W(1–3)A and its inability to cooperate with FP (Suárez et al., 2000a, b). Equimolar mixtures of wild-type pre-TMp and FP exhibited cooperativity and an increase in tryptophan fluorescence, indicative of physical interaction. Binary peptide mixtures exhibited higher reactivity to the Mab2F5 epitope of pre-TM (Fig. 3.1), and peptide hybrids exhibited even greater antibody affinity but less membrane destabilizing power. Their binary interaction was interpreted as a ‘kinetic trap’ stalling fusion, and it was inferred that in the metastable structure of gp120/gp41 the two membrane-active segments mutually mask their hydrophobic surfaces (Fig. 3.1A; Lorizate et al., 2006a, b).

In planar supported membrane bilayers with SM and cholesterol where liquid-ordered (lo) and –disordered (ld) lipid domains co-exist, pre-TMp clusters formed exclusively at the domain boundary (Sáez-Ciri3n et al., 2002). In a strictly cholesterol-dependent manner Mab4E10 bound and blocked liposome permeabilization by these pre-TMp clusters (Lorizate et al., 2006c). These findings are consistent with the pre-TM structure being embedded in the HIV envelope (Fig. 3.1B), as seen in the low resolution pre-fusion SIV spike 3D-structure (Zhu et al., 2006).

The Epanand group investigated the potential of peptides derived from the pre-TM sequence to bind cholesterol and induce phase separation in membranes (see also Chapter 9). Differential scanning calorimetry (DSC) was used to monitor the enthalpy of acyl chain melting transition, which increases upon demixing of cholesterol (Epanand et al., 2003). As a consequence of demixing, cholesterol crystallites form. Liposomes were prepared in the presence of peptide at high peptide-to-lipid ratio, 5 to 15 mol%. Introduction of LWYIK into multilamellar vesicles (MLV) of cholesterol admixed to SOPC or POPC increased the enthalpy of acyl chain melting transition and concomitantly induced cholesterol segregation into crystallites. Nuclear Overhauser effect spectroscopy indicated deeper penetration of the aromatic amino acids into the bilayer in the presence of cholesterol. This was corroborated by the increased quench of tryptophan fluorescence in the presence of cholesterol, of LWYIK (Epanand et al., 2003) and LASWIK (Epanand, 2004; Epanand et al., 2005b), the analogous gp41 sequence of most HIV-2 strains. <sup>13</sup>C Magic angle spinning NMR suggested stronger interactions with the cholesterol A ring than with the interior of the leaflet. The complete pre-TMp was actually less prone to sequester cholesterol into domains than LWYIK itself, with LASWIK intermediate (Epanand et al., 2005b). Cholesterol sequestration in SOPC/Cholesterol and enhancement of Trp fluorescence in the presence of cholesterol generally were strongest for wild-type CRAC and were diminished most by consensus-violating substitutions (Epanand et al., 2006).

Altering the first CRAC residue L to V (within consensus) or A had a less profound effect than to I. IWYIK, unlike all other CRAC peptide variants, lowered

the melting enthalpy of pure SOPC (Epanand et al., 2006). The formation of cholesterol crystallites in SOPC/cholesterol was interpreted as displacement of cholesterol from positions adjacent to SOPC molecules rather than domain formation as with LWYIK (Greenwood et al., 2008). These three variants were also probed in the context of gp41 co-expressed with Tat from a plasmid. The mutation LI had similar effects as other single mutations of the CRAC motif (Table 3.3). A series of double mutants was made to alter the consensus or the intervening residues. Whereas GWGIK and LWGIG inhibited cell-cell fusion by > 60%, LGYGK inhibited only 25–30% (Vishwanathan et al., 2008a, b). Epanand et al. (2006) also modelled a structure maximizing cholesterol-peptide interactions. All variants partitioned in the acyl chain-polar interface, but LWYIK was unique in that cholesterol OH was both H-bond acceptor to tyrosine OH, and H-bond donor to the lysine terminal CO. In the optimized model LWYIK enwraps the cholesterol A-ring and does not contact the hydrophobic bulk of the molecule.

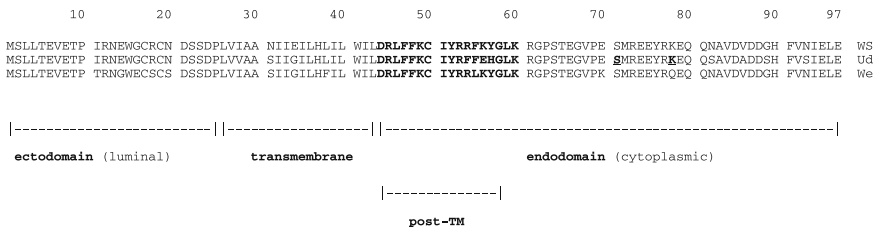
### **3.3 Influenza Virus M2 Protein**

#### ***3.3.1 Influenza Virus Entry and Egress***

Influenza virus has a segmented RNA genome packed as ribonucleoprotein (RNP) into a protein matrix, surrounded by an envelope carrying three transmembrane proteins, HA, NA and M2. The eight RNA segments are transcribed and replicated in the nucleus (reviewed by Whittaker et al., 2000). IFV invades the cell by adsorptive endocytosis (reviewed by Smith and Helenius, 2004), which delivers the virus to a perinuclear site (Lakadamyali et al., 2003). Here, the endosomal pH decreases to a threshold (pH<sub>trans</sub>) triggering conformational transition of viral hemagglutinin and activation of the proton channel M2. The fusion peptide of HA is unburied and propelled into the endosomal membrane, launching fusion of the viral with the endosomal membrane (reviewed by Cross et al., 2001). Concomitant proton influx through the M2 ion channel dissociates the dense matrix ('uncoating') making RNP susceptible to primary transcription (cf. Whittaker et al., 2000). It is critical that all eight genome segments arrive in the nucleus. Likewise, at completion of the infectious cycle during virus assembly, the eight RNA segments must be sorted into the virus particle. The M2 protein plays a role both in packing and unpacking the genome.

#### ***3.3.2 M2 Protein Structural and Functional Domains***

Influenza A M2 protein is a unique, multifunctional protein critical for initiation and completion of the infectious cycle. M2 is a tetrameric class III, single-pass TM protein (Holsinger and Lamb, 1991; Sugrue and Hay, 1991). Figure 3.2 depicts the structural and functional domains of influenza A M2. The 25 amino-acid



**Fig. 3.2** Structural and functional domains of influenza A M2 protein. Influenza A strains: WS – WSN/33 (H1N1) – NCBI accession L25818.1; Ud – Udorn/307/72 – NCBI accession J02167.1 (H3N2); We – Weybridge/27 (H7N7) – EMBL accession AX006731.1. Bold print: post-TM. Bold printed, underlined residues interact with M1 protein

ectodomain is required for the incorporation of M2 into virus particles (Park et al., 1998) and forms the channel mouth. The  $\alpha$ -helical 19 amino-acid TM segment (Lamb et al., 1985; Schnell and Chou, 2008; Stouffer et al., 2008) defines a minimal proton channel (Duff and Ashley, 1992). However, the following sequence up to at least residue 62 is also necessary for ion channel activity in vivo (Tobler et al., 1999). This amphipathic post-TM domain (D44–K60) comprises a sharp turn and a second alpha-helix according to solid-state NMR of the monomer (Tian et al., 2003). The 3D structure of the tetramer M2<sub>18-60</sub> elucidated by NMR in solution (Schnell and Chou, 2008) shows the TM and the post-TM  $\alpha$ -helices connected by a loop of residues 47–50 (Fig. 3.3). The post-TM includes the palmitoylation site and CRAC motifs overlapping a sequence with predicted affinity to PIP4,5P2 (Table 3.1). Most of the M2 endodomain is involved in virus assembly, required

**Fig. 3.3** 3D-model of the tetrameric M2 transmembrane and post-TM structure (Schnell and Chou, 2008). The four helices forming the ion channel are in the upper left, the four post-TM helices in the lower right. Key residues are indicated: S50 marks the position of the palmitoylated C50 of the wild-type sequence. L46 is the first and Y52 the central residue of a common M2 CRAC motif. The position normally occupied by a basic residue is mutated in the Udorn strain (E56) (cp. Table 3.1, Fig. 3.2) (Redrawn after MMDB ID: 62125; PDB ID: 2RLF.)



both for the incorporation of M1 and genome packing (McCown and Pekosz, 2005, 2006; Iwatsuki-Horimoto et al., 2006; Chen et al., 2008).

### ***3.3.3 Influenza Virus Membrane Rafts***

HA and NA are integral raft proteins (Skibbens et al., 1989, Kurzychalia et al., 1992; Scheiffele et al., 1997; Zhang et al. 2000; Nayak and Barman, 2002) and the budding of influenza virus particles is raft-dependent (Scheiffele et al., 1999, Zhang et al., 2000). Raft trans-membrane proteins typically possess a long TM segment, since raft membranes are thicker than bulk membrane (Coxey et al., 1993; Ren et al., 1997). M2 differs from the large spike proteins HA and NA by its short 19 residue TM segment vs. 25–30 for the latter. Compared to HA and NA only a small amount of M2 is extracted into detergent-resistant membrane (DRM) by cold TX-100 (Zhang et al., 2000, Schroeder et al., 2005). Clusters of HA can be visualized in contours of the cell surface by immuno-gold labelling, whereas non-raft mutant HA is distributed more uniformly; these clusters were identified as raft micro-domains and platforms of virus budding (Takeda et al., 2003), which also contain NA and the raft marker GM1 (Leser and Lamb, 2005). Low density of M2 staining did not allow for assessment of its surface distribution in PM contours. In planar plasma membrane sheets HA appears in large 2-dimensional clusters (Hess et al., 2005) and these enable analysis of HA, M1 and M2 co-clustering (Chen et al., 2008). M2 protein co-clustered with M1, however, an alanine-scanning mutant M2<sub>71</sub> SMR → AAA (cp. Fig. 3.2) did not. Statistical analysis of co-clustering with HA revealed that this mutant still displayed significant long-range (> 200 nm) association with HA, albeit less than wild-type M2. Moreover, M1 in the background of this M2 mutation also remains associated with HA. This analysis suggested that different sequence elements of M2 are responsible for the association with M1 on the one side and HA on the other, and that association of M2 with HA is not mediated by M1 (Chen et al., 2008). Indeed, M1-binding sites in the M2 sequence are spatially separated from the post-TM (Fig. 3.2). We hypothesized that M2 attaches peripherally to the HA-studded membrane raft (Schroeder et al., 2005). Its short TM domain should lock M2 into non-raft membrane while post-TM lipid-binding determinants form a bridge into raft domains (see Section 3.6).

### ***3.3.4 Cholesterol in the Apical Transport and Maturation of M2 Protein***

In contrast to HA and NA, M2 is recycled between the PM and the TGN (Henkel and Weisz, 1998) where it equilibrates pH and protects acid-labile HA species from premature low-pH conformational transition (Sugrue et al., 1990; Grambas and Hay, 1992; Ciampor et al., 1992a, b; Ohuchi et al., 1994; Takeuchi and Lamb, 1994). HA and M2 are apparently co-transported to the PM, sharing the



same transport pathways and vesicles. By peripherally inserting at the raft–non-raft interface, M2 may already associate to HA-bearing rafts during vesicular transport.

Cholesterol depletion slows down apical transport via sphingolipid-cholesterol rafts and causes mis-sorting of HA to the basolateral membrane (Keller and Simons, 1998). Cholesterol is also required for the maturation and stability of M2. This was first observed in a heterologous expression system. Extreme cholesterol depletion of insect cells altered the ultrastructure of the Golgi and interfered with cytotoxicity of expressed M2 (Cleverley et al., 1997). We studied cholesterol requirements of the ion channel function on M2 expressed in insect cells in the presence of cholesterol. The proton channel activity of liposome-reconstituted M2 was found to be independent of cholesterol (Lin and Schroeder, 2001). We also expressed M2 protein in *E. coli* which is intrinsically cholesterol-free. Irrespective of cholesterol content, different M2 preparations exhibited nearly the same activity and susceptibility to the antiviral drug rimantadine (Schroeder et al., 2005). While cholesterol is not directly required for ion channel activity it promotes tetramerization, a prerequisite of ion channel activity (Sakaguchi et al., 1997). Synthetic lipid bilayers of Golgi thickness (C16-C18 phospholipids) support tetramerization of the TM peptide M2<sub>19-46</sub> better than shorter phospholipids. Inclusion of cholesterol into the bilayers enhanced membrane thickness as well as M2 tetramerization. Judged by Scatchard analysis cholesterol did not directly bind to the transmembrane peptide (Cristian et al., 2003). Full-length M2 expressed in the absence of cholesterol in *E. coli* exhibited a higher dimer content and lower stability than M2 expressed in insect cells in the presence of serum (Schroeder et al., 2005). The lack of M2 activity in cholesterol-free insect cells (Cleverley et al., 1997) may therefore be attributed to a thinning of the Golgi membranes resulting in the failure of M2 to tetramerize. This is all the more likely as insect cells are cholesterol auxotroph and grow at 27°C, and their membranes are composed of shorter phospholipids than membranes of cells grown at 37°C (Rietveld et al., 1999). Introduction of cholesterol also causes the incorporation of longer chain phospholipids into insect cell membranes (Gimpl et al., 1995; Marheineke et al., 1998).

### ***3.3.5 M2 Protein-Cholesterol Binding Experiments***

M2 protein co-purifies with cholesterol which survives extensive detergent washes (Schroeder et al., 2005). Following expression and immunoaffinity purification from virus-infected chick embryo cells labelled with tritiated cholesterol the co-purified, extractable neutral lipid was subjected to thin-layer chromatography. About 69% of the extracted material coincided with the cholesterol spot. The cholesterol content of purified Weybridge M2 was 0.9 mol per M2 subunit. Sequence-identical M2 protein expressed and purified from insect cells by immunoaffinity FPLC contained 0.5 mol cholesterol per subunit. By prolonged treatment of solid-phase bound M2 with 40 mM 1-octyl- $\beta$ -D-glucoside, most but not all cholesterol could be removed

(C.S., unpublished). This M2 preparation was captured by cholesteryl hemisuccinate agarose but not by unmodified agarose (Schroeder et al., 2005). Cholesterol co-purification with M2 isolated from homologous and heterologous expression systems and adsorption to cholesterol hemisuccinate indicated cholesterol binding by M2 (Table 3.2).

### ***3.3.6 Membrane Raft Binding Determinants and CRAC Motifs in the Post-TM***

The post-TM sequence of the M2 protein (D44–K60; see Figs. 3.2 and 3.3) exhibits interfacial hydrophobicity (White and Wimley, 1999) up to residue 57 (cf. Schroeder et al., 2005) and covers three overlapping determinants of lipid raft binding, palmitoylation at C50, one or two CRAC motifs, and an XIP-like motif (Table 3.1). The M2 protein of influenza A/Udorn 307/1972 (H3N2) (Udorn) lacks R54, K56 and L55 and therefore does not possess a bona-fide CRAC motif, but basic residues are present further downstream of Y52 (Table 3.1). The M2 Weybridge post-TM sequence is most closely homologous to the XIP region of Na/Ca exchangers (Table 3.1) that has specific affinity to PIP<sub>4</sub>,5P<sub>2</sub> (He et al., 2000), a lipid species enriched in the cytoplasmic leaflet of raft membranes (Liu et al., 1998). NAP-22 is another example of a protein predicted to interact with cholesterol via an N-terminal motif similar to CRAC with longer spacers between the L, Y, and K residues (Terashita et al., 2002; Epand et al., 2004, 2005a; Table 3.1). Similar to M2 post-TM, this motif also exhibits a close overlap of elements determining raft binding, myristoylation, affinity to PIP<sub>4</sub>,5P<sub>2</sub>, and predicted cholesterol affinity. Epand et al. (2005a) determined that replacing the Y residue of this motif in a 19 residue NAP-22 peptide abolishes its ability to induce a cholesterol-depleted domain in LUVs.

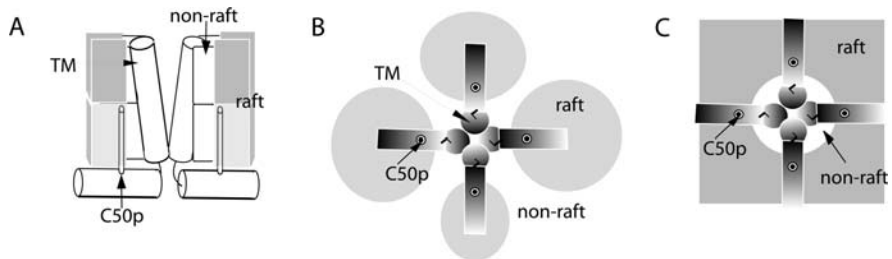
Influenza B and C viruses also encode M2-like ion channel proteins, albeit less extensively studied, with analogous roles to influenza A M2, despite lack of sequence homology, involved in virus entry and capable of equilibrating pH gradients; BM2 also has a function in viral assembly and egress similar to AM2 (Hongo et al., 2004; Imai et al., 2004, 2008; Betakova and Hay, 2007; Pinto and Lamb, 2006). Both influenza B and C M2 sequences include a CRAC motif of unknown significance (not shown).

Stewart et al. (submitted) introduced mutations at CRAC motifs of M2 of the Udorn and WSN strains. The substitution R54F that restores a standard CRAC motif into Udorn M2 (see Table 3.1) neither influenced virus replication kinetics in vitro nor affected the formation of filamentous virus or the incorporation of matrix and envelope proteins into progeny virus. Likewise, alanine substitution of the key residues L46, Y52 and R54 eliminating the CRAC motif (WSN M2delCRAC) did not affect WSN replication in vitro, but caused attenuation of virulence. At a dose of 10<sup>5</sup> TCID<sub>50</sub> infection by WSN was lethal, whereas 80% of mice infected with WSN delCRAC survived; mutant R54F had an intermediate phenotype.

M2 palmitoylation at C50 is not conserved in all influenza virus strains, e.g. C50 is present in most H3N2 but only in a third of the H1N1 strains (Grantham et al., 2009). This is in contrast to HA where palmitoylation is conserved throughout the subtypes and improves virus budding (Jin et al., 1996), raft targeting (Melkonian et al., 1999) and assembly with the M1 protein in a strain-dependent manner (Chen et al., 2005). Judged by the behaviour of M2 C50S mutants, C50 palmitoylation was not required for the replication or filamentous budding of the Udorn and WSN strains *in vitro*, however, the WSN C50S virus mutant was attenuated in mice (Grantham et al., 2009). The robustness of IFV to mutations abolishing palmitoylation and the CRAC motif of the M2 post-TM implies functional redundancy and the need to disrupt more than one membrane-targeting determinant in the post-TM to see effects *in vitro*.

The properties of the short TM and amphiphilic post-TM sequence may encode dual affinity to raft and non-raft membrane, targeting M2 to the membrane domain border as a ‘peripheral raft protein’. We proposed a simple model (Fig. 3.4) of the post-TM anchored in membrane rafts via palmitate. Palmitoylation, CRAC motifs and the PIP4,5P2-binding motif may support affinity to the periphery of rafts, while the short TM segment remains surrounded by non-raft membrane.

M2 post-TM features a conserved endocytic internalization motif at residues 52–55, YxxΦ, that often marks tight turns in the three-dimensional structures of internalized proteins (Collawn et al., 1990); also a kink at K60 was predicted by Saldanha et al. (2002). These elements may confer the structural flexibility required for a role of the post-TM region in membrane fission (see below). Figure 3.3 shows a cartoon of the 3D-model of M2<sub>18-60</sub> C50S (Schnell and Chou, 2008) indicating the position of the CRAC motif. Inclusion of C50 palmitoylation providing a second anchor perpendicular to the membrane plane should cause significant alterations to this model. Schnell and Chou (2008) address this issue: ‘Modelling shows that extending the transmembrane helix to Phe 48 would place residue 50 facing the membrane, allowing for insertion of the palmitoyl acyl chain into the lipid bilayer.



**Fig. 3.4** Peripheral raft association of the M2 tetramer. (a) Cross-section of the membrane showing the TM and post-TM of two of the four subunits of the tetramer. TM is surrounded by non-raft membrane while post-TM connects to raft membrane via the palmitate bound to C50 (C50p) and other raft-targeting sequence elements. (b) Tetramer viewed from the endodomain. Subunits bridge separate rafts. (c) Merger of rafts, trapping the tetramer in small patch of non-raft membrane within raft domain. From Schroeder et al. (2005), with permission from Springer Publishers

This minor rearrangement would also move the amphipathic helices closer to the transmembrane domain.' Only structural studies on palmitoylated M2 will reveal whether and in which membrane environments such elongated TM segments may exist.

### ***3.3.7 Morphogenesis and Budding***

Influenza virus buds at the plasma membrane as spherical (80–120 nm in diameter) or filamentous particles up to > 10  $\mu\text{m}$  long (for a recent review see Schmitt and Lamb, 2005). The latter may have a role in virus transmission in infected lung tissue by bridging infected and non-infected cells (Roberts and Compans, 1998), while spherical particles are expected to be more stable and suitable for aerosol transmission between hosts (Bourmakina and García-Sastre, 2003). Transmission electron microscopy of serially sectioned budding virus particles revealed seven RNP segments of different lengths surrounding the central eighth segment, projecting downwards from the top of the bud (Noda et al., 2006). Once the virus particle is pinched off the RNP segments become indistinguishable. An unknown packaging mechanism sorts the eight different RNP segments into each assembling virus (Fujii et al., 2003; Noda et al., 2006). The M2 protein is involved in this process (McCown and Pekosz, 2005, 2006).

Filamentous particles usually predominate irrespective of virus subtype, passage history or host species (cf. Elleman and Barclay, 2004). The filamentous phenotype is associated with gene segment 7 (Smirnov et al., 1991) encoding M2 and M1 protein and also requires a functional cortical actin microfilament array (Roberts and Compans, 1998; Simpson-Holley et al., 2002). Like spherical morphogenesis, the formation of virus filaments is raft-associated (Simpson-Holley et al., 2002). The outgrowth of virus filaments appears to bypass a decision point to complete spherical morphogenesis, as suggested by the following observations:

M2 is implicated in influenza virus morphogenesis, since a specific monoclonal antibody to the M2 ectodomain (Mab 14C2) suppressed the production of filamentous virus; strains unable to generate filamentous particles were not susceptible (Zebedee and Lamb, 1989; Elleman and Barclay, 2004). The antibody clusters the M2 protein on the PM and reduces its surface expression. Moreover, this antibody labelled M2 in spherical but not in filamentous virus particles (Hughes et al., 1995). Resistance to 14C2 mapped to the M2 endodomain, or to the M1 (matrix) protein of A/Udorn (Fig. 3.2; Zebedee and Lamb, 1989), resulting in distinct morphological phenotypes. M1 A41V generates exclusively spherical particles (Roberts et al., 1998; Elleman and Barclay, 2004). This substitution also occurs in high producer H1N1 laboratory strains PR8/34 and WSN/33 that have lost the morphotype switch, which confers no selective advantage for propagation *in vitro*. Mutations in Udorn M2 (S71Y or K78Q, Fig. 3.2) render filamentous particles less susceptible to antibody restriction but these have a much lower yield of infectious virus and may be defective in pinching-off (Hughes et al., 1995; Roberts et al., 1998). This data implicated interactions between the M2 endodomain region 71–78 with M1

31-41 (Zebedee and Lamb, 1989). Physical interaction of M1 with the M2 cytoplasmic tail has since been proven by immunoprecipitation and pull-down experiments (McCown and Pekosz, 2006; Chen et al., 2008). In the ensuing mutational studies of the M2 and M1 genes of filamentous Udorn and spherical WSN it turned out that mutations at these and other sites where the M1 s differ, or truncation of the M2 cytoplasmic tail, switch the morphotype (Bourmakina and García-Sastre, 2003; Elleman and Barclay, 2004; Burleigh et al., 2005; McCown and Pekosz, 2006; Iwatsuki-Horimoto et al., 2006; Chen et al., 2008).

Truncation of the M2 cytoplasmic tail (McCown and Pekosz, 2006) but also mutation at the extreme N-terminus of NA (Barman et al., 2004) led to 'daisy chains' of spherical, budding virus particles, defective in pinching-off (fission). A recent observation directly implicates membrane rafts in this process: Viperin is an interferon-induced protein that disperses lipid rafts (Wang et al., 2007) as evidenced by reduced copatching of HA with GM1, enhanced membrane fluidity, TX-100 extractability and lateral mobility of HA. Viperin binds and inhibits farnesyl diphosphate synthase upstream the cholesterol biosynthetic pathway. Remarkably, viperin expression also elicits 'daisy chain'-like IFV budding.

### ***3.3.8 Incorporation of M2 into Virus Particles and the Process of Membrane Fission***

Although M2 is expressed as abundantly as HA, the ratio of HA (trimer) to M2 (tetramer) in the virus envelope was estimated to be 500:15 (Zebedee and Lamb, 1988) and this sub-stoichiometric incorporation attributed to the exclusion of M2 from rafts (Zhang et al., 2000). The essential functions of M2 in virus uncoating (Kato and Eggers, 1969; Takeda et al., 2002) and genome packaging imply mechanisms specifically incorporating a few molecules of M2 into the envelope. As detailed in the previous sections: (1) The M2 endodomain at and beyond residue 71 physically interacts with matrix protein M1. (2) Although not an integral raft protein, M2 is associated specifically with raft-embedded HA, independent of M1 binding, apparently mediated by determinants for peripheral raft association in the pre-TM (Fig. 3.4). (3) M2 transits with HA through the trans-Golgi to the PM within the same transport vesicles. (4) M2 truncated at residue 70 still packages into virus particles (McCown and Pekosz, 2006), thus, the sequences responsible for M2 packaging include the post-TM. (5) The M2 post-TM is implicated as a factor in membrane fission (pinching-off) as are membrane rafts. We suggested that pinching-off occurs at the fault-line between raft and non-raft membrane at the budding pore (Schroeder et al., 2005; Schroeder and Lin, 2005), where M2 protein concentrates due to its affinity to the raft periphery. This gained further support from an experiment where transient cholesterol depletion, restricted to the process of pinching-off, actually increased the yield of spherical (WSN strain) particles (Barman and Nayak, 2007).

The proposed role of M2 in pinching-off is in agreement with electron micrographs showing immunogold-labelled M2 clustered at the neck of virus buds (Hughey et al., 1995; Lamb and Krug, 1996). The antibody 14C2 that stalls filamentous growth is not effective as Fab chains (Hughey et al., 1995), it must cross-link M2 to cluster it. We suggested that M2 cross-linking would accelerate pinching-off like a draw-string (Schroeder et al., 2005). The role of M2 in genome packaging (McCown and Pekosz, 2005, 2006) and the fact that the genome segments are coordinated to the top of the bud (Noda et al., 2006) suggests that M2 may also prime budding as an initial focus for RNP and M1 assembly. This would produce virus particles with distinct poles of M2.

In summary, the data indicates that the cholesterol affinity of the M2 protein is one of the functionally redundant elements targeting it to the periphery of membrane rafts. Association to the raft periphery appears to underlie its role during the budding and fission of virus particles.

### 3.4 Fusion Proteins of Alphavirus Species

The class II fusion proteins, E1 and E, of alpha- and flaviviruses power low pH-dependent fusion of the viral and the endosomal membrane during virus infection (reviewed by Heinz and Allison, 2000; Kielian, 1995, 2006). Alphavirus is a genus of togaviridae, flavivirus a genus of flaviviridae. In cholesterol-depleted insect cells alphavirus Semliki Forest virus (SFV) growth is 1000-fold restricted (Phalen and Kielian, 1991), whereas a less cholesterol-dependent point mutant, P226S, is only restricted 40-fold (Vashishtha et al., 1998). This mutation has arisen independently and repeatedly during appropriate selection conditions (Chatterjee et al., 2002). Likewise, the Sindbis alphavirus (SIN) is cholesterol-dependent for entry and egress (Lu et al., 1999). Fusion and infection of insect cells by SFV and Sindbis virus are stimulated by cholesterol. Despite similarity in fusion mechanism flaviviruses like yellow fever and several dengue virus (DV) strains do not require cholesterol.

Umashankar et al. (2008) demonstrated that an SFV E1 ectodomain protein (E1\*) incubated with liposomes was labelled by photocholesterol (Table 3.2) at pH 5, the pH of fusion, whereas DV2 E\* protein was not. A cholesterol-dependent cytolysin served as the positive control, which was also labelled by photocholesterol. In contrast, full length E1 membrane-inserted by its TM domain was not photoaffinity labelled, even at low pH. Labelling by photocholesterol indicates a specific interaction of the fusion peptide with cholesterol in the target membrane.

SFV E1\* inserted into liposomes encompassing a liquid ordered SM and cholesterol-enriched phase could be extracted with m $\beta$ CD, along with the cholesterol, while DV E\* was not extracted (Umashankar et al., 2008), similar to E\* of tick-borne encephalitis virus (TBE), another flavivirus (Stiasny et al., 2003). Both SFV and SIN, although requiring SM and cholesterol for infection, do not depend on membrane rafts (Waarts et al., 2002), and it is just the ectodomain during fusion but not the full length E1 protein that associates with rafts (Ahn et al., 2002).

The P226S mutation of SFV and SIN (Lu et al., 1999) is located to the ij loop which is apposed to the fusion peptide loop in the E protein 3D structure (Roussel et al., 2006) and apparently modulates cholesterol dependence of fusion. Recently the mutation A226V arose during a Chikungunya (alphavirus) epidemic. This mutation was associated with transmission by a new vector, *Aedes albopictus* and, at the same time, increased cholesterol dependence of the virus (Tsetsarkin et al., 2007).

SIN and SFV virus budding also exhibits a cholesterol requirement, attenuated by the same P226S mutation in E1 protein. In the absence of cholesterol, E1 protein is preferentially degraded rather than incorporated into progeny virus.

### 3.5 Other Cholesterol-Binding Virus Proteins

The first instance of a virus protein reported to bind cholesterol was Sendai virus class I fusion (F) protein (Asano and Asano, 1988). <sup>3</sup>H-cholesterol was added to a purified F protein preparation and a complex isolated by immuno-precipitation. The 3-OH group of cholesterol was not required for binding. Cholesterol labelled about 10% of the monomer. Cholesterol binding was blocked by a fusion inhibitory peptide. This work was apparently not followed up. Sendai virus F protein is extracted into DRM and the virus is proposed to bud from membrane rafts (Sanderson et al., 1995; Ali and Nayak, 2000).

The coronavirus spike (S) protein is a class I fusion protein, structurally and functionally similar to HIV Env. Analogous to HIV gp41, peptides representing the S protein pre-TM amphiphilic sequence are able to permeabilize and fuse membranes (Sainz et al., 2005). The pre-TM sequences of SARS and other coronaviruses, e.g. mouse hepatitis virus, harbor a CRAC motif (not shown). Cholesterol-binding studies have not been reported.

### 3.6 Conclusions

HIV gp41, influenza A M2 and SFV E1 protein are vastly different in structure and function. They have in common flexible domains which undergo conformational transition during the membrane restructuring processes and contain cholesterol binding sites. The type of studies performed and the amount of data available documenting cholesterol binding and its biological function vary greatly. For HIV gp41 cholesterol dependence of fusion is proven, a specific CRAC motif is shown to mediate cholesterol binding of gp41-derived peptides, and mutations to this motif arrest HIV infection at the hemifusion stage. The missing link in the chain of evidence is the demonstration at the virus-cell level that these gp41 mutations abrogate fusion due to impaired cholesterol binding. The influenza A virus M2 protein binds cholesterol which is, however, not required for its proton channel function. Mutation of a potential cholesterol binding CRAC motif attenuates virus virulence. This motif may be one of the lipid-binding determinants linking M2 peripherally to

raft microdomains and predicted to play a role in virus budding and fission. Since this hypothesis was first published data has accrued consistent with peripheral raft targeting and supporting the role of the M2 cytoplasmic tail in morphogenesis. The specific role of cholesterol-binding in these processes requires further study. SFV is the first virus for which direct cholesterol binding by the fusion peptide was proven. A second-site locus modulating cholesterol affinity is related to host range and virulence in a number of alphavirus species.

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