# Chapter 14

# The Role of Lipid Microdomains in Virus Biology

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**Abstract**
Many of the highly pathogenic viruses including influenza virus, HIV and others of world wide epidemiological importance are enveloped and possess a membrane around the nucleocapsid containing the viral genome. Viral membrane is required to protect the viral genome and provide important functions for attachment, morphogenesis and transmission. Viral membrane is essentially composed of lipids and proteins. While the proteins on the viral envelope are almost exclusively virally encoded, lipids, on the other hand, are all of host origin and recruited from host membrane. However, lipids on the

Abbreviations: AFM, atomic force microscopy; AIDS, acquired immune deficiency syndrome; ALV, nontransforming avian leukosis virus; ASV, avian sarcoma virus; BCR, B cell antigen receptor; BHK cell, baby hamster kidney cell; CD, cyclodextrin; CEC, chick embryo cells; CIV, Chilo Iridescent virus; CLSM, confocal laser scanning microscopy; CMV, cytomegalovirus; CoV, coronavirus; CT, cytoplasmic tail; DIG, detergent insoluble GSL-enriched domain; DRM, detergent resistant membranes; EAV, equine arteritis virus; EBOV, Ebola virus; EBV, Epstein-Barr virus; ER, endoplasmic reticulum; FRT, Fisher rat thyroid cells; FPV, fowl plague virus; FRET, fluorescence energy transfer; Gal Cer, galactosyl ceramide; GEM, GSL-enriched membrane; GlcT-1, glycosyltransferase; GSL, glycosphingolipid; HA, hemagglutinin; HaK, hamster kidney cells; $Hat^-$, cytoplasmic tail minus HA; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HSV, herpes simplex virus; IC, intermediate pre-Golgi compartment; I domain, interacting domain; $l_c$, liquid crystalline phase; $l_d$, liquid disordered phase; $l_o$, liquid

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viral membrane are not incorporated randomly and do not represent average lipid composition of the host membrane. Recent studies support that specific lipid microdomains such as lipid rafts play critical roles in many aspects of the virus infectious cycle including attachment, entry, uncoating, protein transport and sorting as well as viral morphogenesis and budding. Lipid microdomains aid in bringing and concentrating viral components to the budding site. Similarly, specific viral protein plays an important role in organizing lipid microdomains in and around the assembly and budding site of the virus. This review deals with the specific role of lipid microdomains in different aspects of the virus life cycle and the role of specific viral proteins in organizing the lipid microdomains.

## 1. INTRODUCTION

In a natural setting of viral infection, the human or animal host is infected at a very low moi (multiplicity of infection) with relatively few virus particles. Therefore, cycles of replication leading to release of new progeny viruses and infection of new host cells by the progeny viruses must be repeated many times and are critically required not only for the survival of the virus and spread from one host cell to another but also is fundamentally essential for producing the disease syndrome in the infected host. In most cases viruses must kill, destroy or alter the function of a large number of cells of a specific organ or tissue before the specific functional abnormality in the form of a disease syndrome such as pneumonia, hepatitis, or acquired immune deficiency syndrome (AIDS), etc is manifested. Furthermore, since viruses usually do not release toxins, each virus particle can only infect and kill one cell and in the process of infection of permissive cells, produces many more virus particles. Therefore, virus replication is an obligatory requirement for disease production caused by lytic viruses.

Abbreviations: Continued
ordered phase; LBPA, lysobisphosphatidic acid; L domain, late domain; M1, matrix protein of influenza virus; MAD, membrane attachment domain; MBGV, Marburg virus; MDCK cell, Madin-Darby canine kidney cell; MHC, major histocompatibility complex; MHV, mouse hepatitis virus; M-PMV, Mason-Pfizer monkey virus; MV, Measles virus; NA, neuraminidase; NDV, Newcastle disease virus; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PLD2, phospholipase D2; RSV, respiratory syncytial virus; SFV, Semliki Forest virus; SIN, Sindbis virus; SNOM, scanning near field optical microscopy; SV, Sendai virus; SV5, Sendai virus; SV40, Simian virus 40; TCR, T cell antigen receptor; TGN, *trans* Golgi network; TIM, Triton-insoluble membrane; TM, transmembrane; TMD, transmembrane domain; TR, transfection receptor; TX-100, Triton X-100; VEE, Venezuelan equine encephalitis virus; VLP, virus like particle; VSV, vesicular stomatitis virus; WT, wild type.

Among the living organisms, virus multiplication occurs by a unique process compared to that of either prokaryotes or eukaryotes which multiply as a unit. Unlike any other living forms, viruses do not multiply as a unit and use a unique strategy called the "assembly" process. In this process, viruses after infection of the host cells produce different viral components independently although temporally regulated and often in different amounts and in different location and compartments within the host cell. Finally, the components must be assembled in a specific subcellular location to produce the whole infectious virus. Therefore, the complexity in the assembly process will vary significantly among the viruses depending on the number of components involved and the location and process of assembly. Simple viruses such as polioviruses which have only a few components to assemble, need fewer steps in the assembly process and can be reproduced even in a test tube outside living cells. On the other hand, viruses like herpes- and poxviruses which are highly complex, have many components to assemble and require multiple steps in assembly involving different cellular organelles. The final step, "budding" and release of buds to generate infectious virus, cannot be reproduced outside the living cell.

With respect to the assembly process, the viruses can be classified under two major groups i.e. non-enveloped (naked) and enveloped viruses. In the non-enveloped viruses which contain the nucleocapsid without any envelope, the assembly process consists of two basic events: (i) the assembly of capsid from one or multiple protein components, (ii) incorporation of viral nucleic acid into the capsid. These two basic events which may involve further maturation and processing of proteins during and after assembly will generate infectious virus particles and can often be reproduced *in vitro* outside the living cell. However, with enveloped viruses, the assembly of nucleic acid (DNA or RNA) and capsid is not sufficient for producing infectious virus. The nucleocapsid must be enveloped in a lipid membrane by a process called "budding" for producing infectious virus particles. The envelopment of viral nucleocapsid is a critical event and obligatory requirement for assembly and release of the infectious enveloped viruses. The viral envelope not only protects the nucleocapsid from degradation by nucleases and proteases but also provides the critical functions in the infectious process by providing the exposed transmembrane receptor-binding proteins on the viral envelope for virus attachment to the new host cells and permitting efficient delivery of the viral nucleocapsid into the cytoplasm of the infected cell by a complex process called fusion for initiating the virus replicative cycle. The fusion between the viral membrane and the host membrane can occur either at the plasma membrane at neutral pH or inside the cell with the endosomal membrane at acidic pH. In addition, for some viruses like influenza virus, the envelope possesses a protein component (neuraminidase [NA]) which facilitates the release of virus after budding has occurred and aids in the transmission of virus from cell to cell and host to host.

Since viruses usually do not carry genes for lipid biosynthesis and lipid modification, viral membranes are formed by extension of the host cell membrane while virus budding occurs. During the budding process, all host proteins are essentially excluded and host lipids are selectively included within the viral membrane. Occasionally, a few host proteins which are functionally important for viral infectious cycle are selectively included in some enveloped viruses. How viruses exclude the host proteins is not fully understood. Many viruses suppress or shut off the synthesis and transport of host proteins and thereby prevent the incorporation of newly synthesized host proteins into viral envelope. However, this proposition would not explain exclusion of the preexisting membrane proteins from the viral envelope. It is generally believed that the interaction of the viral membrane proteins with the internal viral components as well as interaction among the internal viral components such as matrix proteins and nucleocapsids and also interaction among viral envelope proteins effectively crowd out the preexisting host proteins from the budding site. In addition, specific lipid composition and state of viral lipids may facilitate exclusion of host proteins from the viral envelope. Finally, although the lipid composition of the viral envelope depends on the host cell, tissues and organelles from which the viruses bud as well as the external environment and factors including the growth medium in which the host cells are grown and viruses are propagated, the viral envelope does not quantitatively represent the lipid composition of organelle or plasma membrane of infected cells. This would suggest that viruses can selectively modify host membranes and acquire different lipids at the budding site. This process in turn would imply that the host cell membrane is not homogenous or uniform with respect to lipid composition but rather is heterogeneous and mosaic in nature containing heterogeneous microdomains with varying lipid composition and that viruses selectively bud from specific microdomains on the cell membrane. In this review we will discuss the nature and heterogeneity of these microdomains and the role these microdomains play in affecting the lipid composition of viral membranes and in budding and envelopment of virus particles.

## 2. LIPID COMPOSITION OF VIRAL AND HOST MEMBRANES

Although viruses bud from host membranes and all viral lipids are acquired from the host membranes, viral lipid composition does not match quantitatively with that of the host membranes. There are a number of reasons why the lipid composition of the viral membrane is different from that of the host cell membrane and why the lipid composition varies among different viruses.

(i) *Budding site.* Different viruses bud from different subcellular organelles (Figure 1, Table 1). For example, herpes virus, a complex DNA virus, buds from the inner nuclear membrane. However, fully mature infectious herpes virus exits from the basal layer of infected epithelial cells. Hepadna, rota and

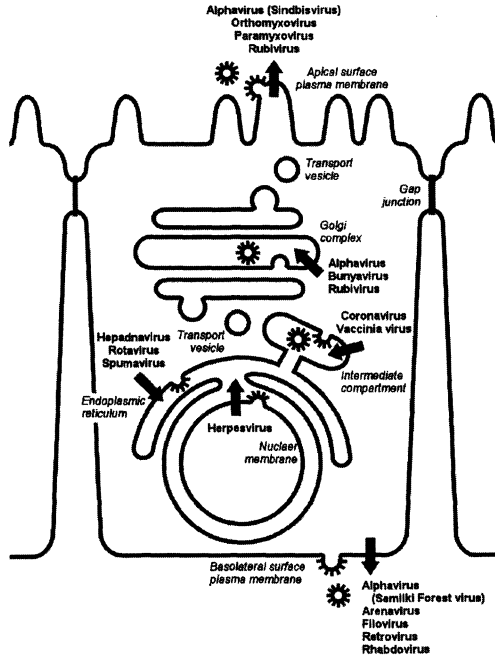


Figure 1. Budding sites of enveloped viruses. The figure depicts a polarized epithelial cell and viruses are budding from the different subcellular compartments and plasma membrane domains are shown.

Table 1. Budding site of enveloped viruses

| Host membrane | Budding site of enveloped viruses |
|---|---|
| Nuclear membrane | Herpesvirus |
| Endoplasmic reticulum (ER) | Hepadanavirus, Rotavirus, Spumavirus |
| Intermediate compartment | Coronavirus, Vaccina virus |
| Golgi complex | Alphavirus, Bunyavirus, Rubivirus |
| Plasma membrane: | |
| Apical | Alphavirus (Sindbis virus), Orthomyxovirus, Paramyxovirus, Rubivirus |
| Basolateral | Alphavirus (Semliki Forest virus), Arenavirus, Filovirus, Retrovirus, Rhabdovirus |

spuma viruses bud from the endoplasmic reticulum. It is not clear if they use the specific domain of plasma membrane (apical or basolateral) for their exit and most likely, cell lysis causes release of these viruses from infected cells. Corona and vaccinia viruses acquire their envelope from the intermediate pre-Golgi compartment (IC). Vaccinia virus further undergoes a complex maturation process before being released from the plasma membrane. Both vaccinia and corona viruses bud from the basal plasma membrane. Bunya and toga (alpha- and rubivirus) viruses acquire their envelope from the Golgi complex. However, their budding site at the plasma membrane varies. For example, Sindbis virus (SIN) appears to exit from the apical membrane whereas Semliki Forest virus (SFV) buds from the basolateral membrane although both of them are members of the alpha virus groups. Finally, the assembly and budding of some viruses such as orthomyxo-, paramyxo-, filo-, retro- and rhabdoviruses occur only at the plasma membrane. Therefore complete infectious virions of these virus groups are not found inside the infected cells. Among these viruses, orthomyxo- and paramyxoviruses bud from the apical plasma membrane whereas the filo-, retro- and rhabdoviruses bud from the basolateral plasma membrane. It should be noted that even some non-enveloped viruses such as papilloma, Simian virus 40 (SV40), polio virus, also exit from the apical plasma membrane. Furthermore, the budding or exit site from the apical or basolateral membrane also affects viral pathogensis and the disease syndrome. Usually, viruses budding from the apical plasma membrane such as influenza (orthomyxovirus) viruses do not cause viremia or infect internal organs. On the other hand, viruses budding from basolateral surface cause cell to cell fusion leading to heterokaryon or syncytium formation, vesicle formation as well as viremia and systemic infection of internal organs. However, there are exceptions to this general rule such as measles viruses which although budding predominantly from apical membrane, cause cell to cell fusion, viremia and systemic infection. In addition, other factors such as cleavage of and fusion by F protein at neutral pH play an important role in the phenotype of these viruses.

Lipid composition of viral membrane primarily depends on the budding site. Since different subcellular organelles possess varying lipid composition, viruses budding from membranes of different organelles (Figure 1, Table 1) will have different lipid composition (Table 2). Viruses budding from the endoplasmic reticulum (ER), Golgi, nucleus or plasma membrane will have different lipid composition since the lipid composition of different membranes vary both quantitatively and qualitatively. In addition, viruses budding from the different domains of the same membrane will have different lipid composition. For example, viruses budding from the plasma membrane will have different lipid composition depending on whether the virus buds from the apical or basolateral domain of the plasma membrane in polarized epithelial cells.

Table 2. Phospholipid composition of viral envelope and cellular membrane

| Cell/virus | Phospholipid composition (%) | | | | | | | | | | References |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | PA | PC | PE | PI | PS | SPH | LPE | LPC | Lec | Other | |
| Uninfected BHK cells (PM) | - | 71.7 | 13.9 | 4.3 | 2.5 | 7.6 | - | - | - | - | van Genderen *et al.*, 1995 |
| EAV from BHK | - | 54.9 | 13.9 | 7.1 | 5.1 | 19.5 | - | - | - | - | van Genderen *et al.*, 1995 |
| FPV from BHK | - | 38.5 | 28.6 | 8.2 | 6.7 | 18.1 | - | - | - | - | van Meer & Simons, 1986 |
| HSV from BHK | - | 55.8 | 13.0 | 8.2 | 7.1 | 15.9 | - | - | - | - | van Genderen *et al.*, 1995 |
| SFV from BHK | 5.6 | 33.0 | 23.0 | 1.5 | 13.0 | 21.0 | - | 1.0 | - | - | Renkonen *et al.*, 1971 |
| Sindbis virus from BHK | - | 26.2 | 35.4 | - | 20.3 | 18.2 | - | - | - | - | David, 1971 |
| VSV from BHK | - | 41.4 | 22.2 | 4.8 | 10.8 | 20.8 | - | - | - | - | van Genderen *et al.*, 1995 |
| Uninfected calf kidney cells | 2.0 | - | 16.0 | 5.0 | 4.0 | SPH + LPE: 17.0 | | - | 44.0 | 4.0 | Kates *et al.*, 1961 |
| Influenza virus from calf kidney | 12.0 | - | 8.0 | 5.0 | 5.0 | SPH + LPE: 14.0 | | - | 36.0 | 14.0 | Kates *et al.*, 1961 |
| Uninfected CE | - | - | 18.0 | 6.0 | 6.0 | SPH + LPE: 20.0 | | - | 48.0 | 2.0 | Kates *et al.*, 1961 |
| Influenza virus from CE | - | - | 22.0 | 6.0 | 7.0 | SPH + LPE: 35.0 | | - | 28.0 | 2.0 | Kates *et al.*, 1961 |
| Uninfected CEC (PM) | - | 43.5 | 23.8 | 18.8 | 11.1 | 3.3 | - | - | - | - | David, 1971 |
| Sindbis virus from CEC | - | 26.4 | 32.1 | - | 20.8 | 20.5 | - | - | - | - | David, 1971 |
| Uninfected CEF (PM) | - | 36.0 | 26.0 | - | 13.0 | 22.0 | - | - | - | 2.0 | Quigley *et al.*, 1971 |
| ALV (RAV-2) from CEF | - | 20.0 | 30.0 | - | 16.0 | 33.0 | - | - | - | 1.0 | Quigley *et al.*, 1971 |
| ASV (B77) from CEF | - | 21.0 | 29.0 | - | 17.0 | 30.0 | - | - | - | 3.0 | Quigley *et al.*, 1971 |
| NDV from CEF | - | 26.0 | 33.0 | - | 10.0 | 26.0 | - | - | - | 4.0 | Quigley *et al.*, 1971 |
| RSV from CEF | - | 20.0 | 33.0 | - | 12.0 | 29.0 | - | - | - | 4.0 | Quigley *et al.*, 1971 |
| Sendai virus from CEF | - | 27.0 | 31.0 | - | 14.0 | 24.0 | - | - | - | 5.0 | Quigley *et al.*, 1971 |
| Sindbis virus from CEF | - | 21.0 | 33.0 | - | 15.0 | 29.0 | - | - | - | 2.0 | Quigley *et al.*, 1971 |
| Uninfected CF cells | - | 51.2 | 20.1 | 7.5 | 6.6 | 6.3 | - | - | - | 8.3 | Heydrick *et al.*, 1971 |
| VEE virus from CF | - | 35.4 | 28.1 | 1.8 | 16.9 | 13.1 | - | - | - | 3.7 | Heydrick *et al.*, 1971 |
| Uninfected L cells | - | 47.8 | 16.1 | 3.9 | 2.9 | 9.3 | - | - | - | 20.0 | Heydrick *et al.*, 1971 |
| VEE virus from L cells | - | 23.3 | 29.1 | 0.5 | 14.7 | 26.2 | - | - | - | 5.1 | Heydrick *et al.*, 1971 |
| Cf 124 Ti cells (PM) | - | 48.0 | 25.0 | 8.5 | 7.5 | 0.8 | 1.4 | 3.0 | - | 4 | Balange-Orange & Devauche, 1982 |
| CIV from Cf 124 Ti | - | 35.0 | 26.0 | 27.0 | 6.0 | 2.0 | 4.0 | t | - | t | Balange-Orange & Devauche, 1982 |

Table 2. Continued.

| Cell/virus | Phospholipid composition (%) | | | | | | | | | | References |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | PA | PC | PE | PI | PS | SPH | LPE | LPC | Lec | Other | |
| Uninfected H9 cells (PM) | 0.6 | 47.6 | 23.3 | 4.9 | 10.3 | 9.6 | - | - | - | 3.4 | Aloia *et al.*, 1993 |
| HIV-$1_{RF}$ from H9 | 1.2 | 29.9 | 24.6 | 0.4 | 9.0 | 24.1 | - | - | - | 8.3 | Aloia *et al.*, 1993 |
| HIV-2-L from H9 | 1.2 | 27.6 | 27.3 | 1.1 | 15.5 | 23.0 | - | - | - | 4.8 | Aloia *et al.*, 1993 |
| Uninfected HaK cells (PM) | - | 46.8 | 13.0 | 11.7 | 5.0 | 24.4 | - | - | - | - | Klenk & Choppin, 1970 |
| SV5 from HaK | - | 43.8 | 17.1 | 8.5 | 5.0 | 25.8 | - | - | - | - | Klenk & Choppin, 1970 |
| Uninfected MDBK cells (PM) | - | 44.5 | 27.2 | 2.9 | 2.2 | 22.8 | - | - | - | - | Klenk & Choppin, 1970 |
| SV5 from MDBK | - | 23.8 | 40.9 | 5.2 | 2.0 | 27.3 | - | - | - | - | Klenk & Choppin, 1970 |
| Uninfected MDCK cells | - | 62.5 | 20.1 | 5.2 | 4.2 | 7.7 | - | - | - | - | van Meer & Simons, 1986 |
| FPV from MDCK (apical) | - | 9.6 | 44.8 | 2.8 | 22.4 | 20.5 | - | - | - | - | van Meer & Simons, 1986 |
| VSV from MDCK (basolateral) | - | 28.9 | 33.5 | 3.2 | 18.9 | 15.6 | - | - | - | - | van Meer & Simons, 1986 |
| Uninfected Sac(-) cells | - | 71.8 | 16.8 | 5.9 | 3.8 | 1.8 | - | - | - | - | van Genderen *et al.*, 1995 |
| MHV from Sac(-) | - | 71.1 | 10.8 | 10.1 | 5.7 | 4.2 | - | - | - | - | van Genderen *et al.*, 1995 |

-, Not determined; t, trace amount.

Compared to the basolateral domain, the apical domain of the plasma membrane of intestinal epithelial cells and Madin-Darby canine kidney (MDCK) cells possesses 2 to 4 fold enrichment of phosphatidylcholine (PC) but similar level of phosphatidylethanolamine (PE). Therefore in polarized epithelial cells like MDCK cells, both influenza virus (fowl plague virus [FPV]) budding from apical surface and vesicular stomatitis virus (VSV) budding from the basolateral surface will have some common lipids representing the plasma membrane but some different lipids representing the domain of their origin. Both FPV and VSV possessed less PC and more SPH and compared to the total cellular lipid representing their origin from the plasma membrane. On the other hand, FPV, an apical virus, has 4 fold less PC compared to VSV, a basolateral virus, when grown in polarized MDCK cells. In polarized cells, tight junctions restrict the lateral diffusion of lipids on the exoplasmic leaflet but permit free diffusion of lipids in the cytoplasmic leaflet between the apical and basolateral domains. This will affect the lipid composition of the apical and basolateral domains and consequently, will affect the lipid composition of viruses budding from these respective plasma membranes. However, when both viruses were grown in baby hamster kidney (BHK) cell, a nonpolarized cell, the PC content of both FPV and VSV were similar (van Meer and Simons, 1986).

(ii) *Other factors*. The lipid composition of the same virus will also depend on the cell type in which the viruses were grown, composition of the growth medium used for cell culture and virus propagation. In addition, the state of the cell monolayer layer such as stationary vs actively growing and association with the neighboring cells, i.e. polarized vs nonpolarized or transformed vs nontransformed, etc. will also affect lipid composition of virus particles.

(iii) *Mosaic nature of the membrane lipids*. Membrane lipids form an amphipathic barrier between the cell and its outside environment and exist as a lipid bilayer. Numerous studies have convincingly shown that lipid bilayers of plasma membrane are not homogenous or uniform but mosaic in nature containing heterogenous microdomains and viruses bud from these microdomains.

(iv) *Viral envelope proteins* are also critical factors and play important roles in determining the lipid composition of the viral membrane. This was evident in early studies describing the requirement and selection of specific lipids in the envelopment of bacteriophage PM2, an enveloped DNA virus (Brewer, 1979). Authors showed that only one structural protein sp6.6, an integral membrane protein, was able to mobilize specific lipids into newly assembled membrane of the desired shape, size and lipid composition. These lipid vesicles containing a single viral protein were composed of phosphatidylglycerol and phosphatidylethanolamine whose ratio was essentially inverse of that found in host membranes but same as in the wild type (WT) virus. With enveloped animal viruses, integral transmembrane viral proteins associate with different

lipids present in the liquid-ordered phase ($l_o$ phase) or liquid-disordered phase ($l_d$ phase) during their transport from *trans* Golgi membrane to the plasma membrane and during their residence on the plasma membrane prior to and during the virus budding process. The lipid-protein association is further modified and stabilized by the internal viral proteins which interact with the transmembrane viral proteins and/or with the cytoplasmic leaflet of the lipid bilayer. Accordingly, the lipid composition of virus membrane will vary depending on microdomains from which the virus will bud.

## 3. LIPID MICRODOMAINS

Membrane lipids are generally viewed to exist as lipid bilayer in two dimensional fluid mosaic state forming an amphipathic barrier between the cell and its external environment. Their main functions are to prevent free leakage of cytoplasmic content into outside environment and unregulated entry of external elements into the cell and also to provide suitable means to respond to the external environmental stimuli by means of signal transduction pathways. It is generally believed that membrane lipids exist in liquid-crystalline ($l_c$) or $l_d$ phase and function as a solvent for membrane proteins. However, recent studies over the last decade have argued against such simplified view of the physical state of membrane lipids and demonstrated the existence of heterogeneous lipid microdomains. Heterogeneity among the membrane lipids leads to lateral organization and phase separation forming $l_o$ phase and causes asymmetric distribution of different membrane lipids in exoplasmic vs cytoplasmic leaflets forming assembly of various microdomains. These lipid microdomains exhibit a common property of relative insolubility in nonionic detergents such as Triton X-100 (TX-100) at low temperature (Simmons and Ikonen, 1997). However, there appears to be heterogeneity among the microdomains. In general, three types of microdomains with varying lipid composition and function have been identified. These are: (a) caveolae, (b) lysobisphosphatidic acid (LBPA) domain, and (c) lipid rafts enriched in cholesterol and sphingolipids. There are further variations within each of these classes of microdomains.

### 3.1. Caveolae

Caveolae are flask shaped invaginations (50–100 nm in diameter) formed on the plasma membrane, predominantly on the basolateral surface containing both caveolin 1 and caveolin 2 proteins. Approximately, 14 to 16 caveolin 1 monomers assemble into discrete patch forming homooligomers by the interacting $NH_2$ termini with $NH_2$ termini and COOH termini with COOH termini

at the cytoplasmic side of the membrane. Membrane attachment of caveolin 1 is mediated by three separate membrane attachment domains (MAD) (N-terminus MAD amino acid [aa] 82–101, transmembrane MAD aa 102–134 and C-terminus MAD aa 135–150) (Galbiati *et al.*, 2001). Caveolin 1 binds to cholesterol, and exhibits TX-100 insolubility supporting the $l_0$ phase separation of membrane lipids. Caveolin containing microdomains are also TX-100 insoluble but can be separated from lipid rafts by antibodies against caveolin 1. Caveolin containing microdomains possess caveolin, glucosylceramide, Ha Ras, and a large quantity of cholesterol but only a small amount of sphingomyelin whereas lipid raft microdomains contain GM3 gangloside, sphingomyelin, cholesterol, c-Src, Rho A but no caveolin. Caveolae have been shown to play important role in endocytosis and in apical transport of proteins in some cells. Antibodies to caveolin 1 inhibit transport of influenza virus hemagglutinin (HA), an apical protein but not of VSV G, a basolateral protein, to the cell surface in MDCK cells (Scheiffele *et al.*, 1998). However, in Fisher rat thyroid (FRT) cells which do not synthesize caveolin 1, both influenza virus HA as well as GPI-anchored PLAP are efficiently transported to apical surface (Martin-Belmonte *et al.*, 2000) suggesting that caveolin may not be an absolute requirement for transport of apical proteins. The role of caveolae in virus biology is not well defined. However, caveolae have been shown to be involved in the entry process of SV40, a nonenveloped double stranded small DNA virus, which replicates in the nucleus although the function of caveolae in SV40 entry and uncoating process remains yet to be elucidated. Specific role of caveolae in virus morphogenesis of enveloped viruses has not been defined. Therefore, properties and function of caveolae will not be discussed further in this review.

## 3.2. Lysobisphosphatidic acid (LBPA) domain

Recently, the existence of another lipid membrane microdomain which is different from glycosphingolipid (GSL)-rich lipid raft, has been identified (Kobayashi and Hirabayashi, 2001). LBPA microdomain was discovered in GM-95 cells, a mutant melanoma cell line lacking all GSLs due to a deficiency of ceramide glucosyltransferase (GlcT-1), suggesting that TX-100 resistant microdomains can be formed in the absence of GSLs and that GSLs are not essential for the formation of TX-100 resistant raft-like microdomains. LBPA microdomains were shown to be present in endocytic pathway and is enriched in a unique phospholipid, LBPA which is localized primarily in the internal membrane of multivesicular endosome (late endosome), and is involved in lipid and protein sorting within the endosomal system. Like lipid rafts, LPBA microdomains exhibit common properties such as TX-100 resistance

supporting the $l_o$ state of the membrane lipids in the LPBA microdomains. However, little is known about the role of this LBPA microdomain in viral membrane biogenesis and in virus budding. Therefore, LPBA microdomains will not be discussed further in this review.

## 3.3. Lipid rafts

### 3.3.1. Structure and function of lipid rafts

Lipid rafts are operationally defined as cholesterol dependent microdomains resistant to solublization by nonionic detergents such as TX-100 at low temperature. Lipid rafts consist of sphingolipid-cholesterol clusters, usually varying in size and are present on the plasma membrane, apical transport vesicles as well as on Golgi and *trans* Golgi membranes. Lipid rafts vary in size, ~50 nm in diameter (Pralle *et al.*, 2000) and smaller than the caveolae which also exhibits TX-100 insolubility similar to the lipid rafts. However, as indicated above, caveolae contain caveolin, a cholesterol-binding protein, which is absent in lipid rafts and exhibit a different lipid composition. Furthermore, caveolae are present predominantly in the basolateral membrane whereas lipid rafts are found primarily in the apical domain of the plasma membrane of polarized cells but are also present in non-polarized cells such as fibroblasts.

Lipid raft microdomains are formed by lateral organization and phase separation of lipids between $l_o$ phase and $l_d$ or $l_c$ phases. $l_o$ phase separation also leads to asymmetric distribution of different lipids in the exoplasmic vs cytoplasmic lipid leaflets. These lipid microdomains containing $l_o$ phase have been variously called by different names such as detergent insoluble GSL-enriched domains (DIGs), GSL-enriched membrane (GEMs) or microdomain, detergent resistant membranes (DRMs), Triton-insoluble membranes (TIMs), GSL/or sphingolipid-cholesterol rafts, lipid rafts or simply rafts (Simons and Toomre, 2000). We will refer these microdomains as lipid rafts or rafts in this review. As mentioned above, although these rafts are found in the plasma membrane primarily on the apical domain, they are also present in the basolateral domain of plasma membrane as well as in the Golgi and *trans* Golgi network (TGN) membranes.

Detailed structural and functional analysis of lipid rafts will not be presented here as they have been described in a number of recent reviews (Simons and Ikonen, 1997; Brown and London, 1998a, 1998b; Nayak and Barman, 2002). Our emphasis in this review will be to discuss the functional and structural properties of the membrane lipids that affect virus membrane assembly and virus budding and in turn, aid in the incorporation of specific lipids into the

viral membrane and thereby affect the lipid composition of virus particles. The lipid composition of viral membrane and host cell domain are not only important in the final steps of the infection cycle such as virus assembly and budding but for many viruses, play critical roles in the initial steps such as virus entry, infectivity including virus attachment, fusion between viral and cell membrane and delivery of viral genome into the host cell for initiating the infectious cycle (Samsonov *et al.*, 2002; Chatterjee *et al.*, 2002). However, the major emphasis of this review will be to discuss the physical state of membrane lipids at the budding site and their role in the final steps of the infectious cycle, namely the virus assembly, envelopment and release of virus particles.

Specific classes of lipids are critical for the formation of functional microdomains including lipid rafts. It has been shown that sphingolipids and cholesterol when mixed *in vitro* can form detergent resistant raft-like artificial membranes in the absence of any protein. This would argue against the absolute requirement of proteins in the formation of lipid rafts and support that proteins may be present but are not absolutely essential for maintaining the physical state of lipids such as lateral packing of lipids leading to the formation of lipid rafts. To explain the behavior of certain lipids particularly sphingolipids (glycosphingolipids, GSL) and sphingomyelin and cholesterols as well as GPI-anchored proteins to non-ionic detergent treatments such as Triton X-100, NP40, Brij at low temperature (4°C), Simons and colleagues (1997) proposed the "lipid raft" hypothesis. They proposed that lipid rafts are formed as microdomains or specific clusters of lipids by lipid–lipid interactions depending on the biophysical characteristic of the lipid components. GPI anchored proteins which also exhibited TX-100 insolubility was also considered as part of the lipid raft microdomains. However, since demonstration of these complexes was dependent on extraction by TX-100 detergents at low temperature, questions were raised whether these microdomains were present in the living cell membrane or formed during TX-100 treatment. Later, however, lipid rafts were demonstrated in living cells and studied by a number of independent techniques such as fluorescence energy transfer (FRET), electron microscopy (EM) with immunogold beads, atomic force microscopy (AFM), scanning near field optical microscopy (SNOM), confocal laser scanning microscopy (CLSM) (Damjanovich *et al.*, 2002). Therefore it is now an accepted view that lipid rafts are present in living cells and provide critical role in a number of important cellular functions such as endocytosis (Sharma *et al.*, 2002; Nabi and Le, 2003), protein transport (Alonso and Millán, 2001), protein sorting (Ikonen, 2001; Aït Slimane and Hoekstra, 2002), signal transduction (Muller, 2002), immunological responses to specific antigens (Langlet *et al.*, 2000; Katagiri *et al.*, 2001; Miceli *et al.*, 2001; Alonso and Millán, J. 2001; Vogt *et al.*, 2002) as well as in virus assembly and budding (Barman *et al.*, 2001; Campbell *et al.*, 2001; Ono and Freed, 2001; van der Goot and Harder,

2001; Nayak and Barman, 2002; Suomalainen, 2002; Briggs *et al.*, 2003; Chazal and Gerlier, 2003; Harder, 2003).

### 3.3.2. Requirement of specific lipids in lipid rafts

Lipid rafts are dynamic in nature. They can coalesce or dissociate to form larger or smaller rafts with individual lipids moving in and out of the raft complex (Kusumi and Sako, 1996). Since cholesterol-sphingolipid complexes are particularly insoluble in TX-100 extraction at low temperature and float to the top of the gradient because of low density and high lipid content, this procedure has been used extensively to isolate and study the property of the lipid rafts. Such analysis demonstrated varying degrees of insolubility to TX-100 treatment of different membrane functions and therefore raised the possibility of existence of different classes of lipid rafts. Bulk membranes are enriched in glycophospholipids (frequently containing unsaturated fatty acids) and are highly soluble to TX-100 extraction at low temperature. Lipids in these membranes are loosely packed in $l_d$ state and display a state of high fluidity. Lipid raft microdomains, on the other hand, contain glycerophospholipids, (glyco)sphingolipids, GPI lipids bearing predominantly saturated fatty acids and cholesterol. These lipids can form tight packing and cholesterol in the region contributes to tight packing by filling the interstitial space between the long saturated acyl chains and sphingolipids resulting in the formation of $l_o$ state of lipids in lipid raft microdomains. Most of the membrane proteins including the transmembrane (TM) proteins except the GPI-anchored proteins are excluded from these microdomains. These microdomains also contain gangliosides such as GMI and GM2 (Alonso and Millán, 2001). The tight lateral packing of sphingolipids and cholesterol leads to TX-100 insolubility at low temperature. However, these microdomains are not homogenous and vary in TX-100 insolubility and lipid composition (Kobayashi and Hirabayashi, 2001). Resistance of these microdomains to cold nonionic detergents and alkaline sodium bicarbonate are sensitive to cholesterol depletion indicating the role of cholesterol in the formation and stability of lipids and lipid packing in lipid rafts (Müller, 2002). In addition to cholesterol, sphingolipids are also essential components of lipid rafts. There are two major sphingolipid complexes- GSLs and sphingomyelin in mammalian cells. Studies using inhibitors for GSL biosynthesis and mutant cells showed that the presence of GSLs is not required for raft formation. Therefore, the presence of either GSLs or sphingomyelin on the outer leaflet along with cholesterol may be sufficient for the formation of raft-like microdomains.

Organization of lipids in membranes can lead to different physical state of the membranes: (i) quasi solid gel phase, (ii) $l_c$, also called $l_d$ phase and (iii) $l_o$ phase. However, the quasi solid gel phase is usually formed in test tube *in vitro*

and unlikely to be found in living cells. Living cells contain lipids in $l_c$ and $l_o$ phases depending on the physical state of the lipids. The majority of lipids in the membrane of living cells are in an intermediate ($l_c$ or $l_d$) phase exhibiting high fluidity and low melting point. These lipids are less packed and have a high rotational and lateral mobility. On the other hand, lipid rafts are specialized lipid microdomains containing lipids in $l_o$ phase. The organization of lipids in the lipid raft depends on the physical states of lipids, which differ in packing, degree of the order and mobility of the constitutent lipids. In $l_o$ phase, hydrocarbon chains are in extended conformation and lipids are highly ordered, densely packed and have reduced mobility in the presence of cholesterol. In the test tube, they exhibit a relatively high melting point, resist solubilization by TX-100 and possess raft-like properties. Cholesterol tends to intercalate its rigid planar hydrophobic ring between the hydrocarbon chains of lipid with its hydroxyl group oriented towards the water phase. Cholesterol aligns with phospholipids and sphingolipids and supports the extended *trans*conformation and increases the order but decreases the lateral and rotational mobility. Glycoglycerophospholipids with kinked structure interferes with packing and have lower affinity for $l_o$ phase. On the other hand, ceramide backbone of sphingolipids contains saturated hydrocarbon chains and favors packing and $l_o$ phase formation. Cholesterol aligns with saturated hydrocarbon leading to the formation of sphingomyelin-cholesterol coupling and increases resistance to TX-100 and Brij 58. Glycosphingolipids as well as sphingomyelins are present in lipid rafts. Phospholipids that are present in the lipid raft are enriched with saturated and monounsaturated but depleted in polyunsaturated acyl chains. Although lipid components including ceramides as well as cholesterol are synthesized ER and may be assembled as preraft-like microdomains in ER, formation of detergent resistant raft domains does not occur until in the Golgi where ceramides are further modified by glycerol transferases. Sphingomyelin/cholesterol ratio as well as total cholesterol concentration in the raft are critical for TX-100 insolubility (Slimane and Hoekstra, 2002). Also, glycosphingolipids may distribute differently in rafts causing variation in concentration in individual rafts and result in varying detergent resistance within lipid rafts.

### 3.3.3. Requirement of proteins in lipid rafts

As indicated above, although lipid bilayer representing lipid rafts can be formed using only different lipid molecules which can assemble into microdomains *in vitro*, lipid rafts present in cellular membrane invariably contain protein molecules. However, it is not clear whether proteins assemble microdomains around them by attracting particular lipids or partition into already formed microdomains. These proteins include GPI anchored proteins

exposed outside and linked to outer plasma membrane leaflet via phosphatidylinositol moiety. The saturated acyl and alkyl chains of GPI increase the affinity for ordered lipids in the membrane phase. On the other hand, the majority of the transmembrane (TM) proteins are excluded from the lipid rafts. However, some TM receptors are selectively present in raft microdomains. For example CD44 (type I), CD26 (type II) are found in raft microdomains. Also, multispanning proteins such as MAL (tetraspan) are present lipid rafts and in apical transport system. Similarly, CD20 (tetraspan), LMP1 (hexaspan) in Epstein-Barr virus (EBV)-infected cells as well as influenza virus NA without any lipid modification are also present in lipid rafts (Hoessli *et al.*, 2001). Lipid rafts are present in Golgi and subapical compartments (SAC) along with MAL and annexin IIIB. Protein– lipid and protein–protein interactions may contribute to coalescence, growth and stability of some lipid rafts. Sphingolipid-cholesterol packing may coexist with more fluid membrane lipid and actin-based cytoskeleton may aid in creating diffusion-restricted membrane compartments. Cytoskeleton-associated TM proteins like CD44, CD43 may affect domain organization at basolateral surface. GM3, GM1 enriched rafts segregate at the leading edge of uropod. Identification of proteins as a component of raft requires the detergent insolubility of the protein and in turn, depends on the presence of cholesterol in membrane. GPI-anchored proteins are present on the outer leaflet whereas proteins with palmitoylation, prenylation, acylation, myristoylation are usually present in the inner leaflet of lipid bilayer.

Although rafts have been shown to exclude most membrane proteins, particularly TM proteins, they are responsible for concentrating GPI-linked and myristylated proteins such as doubly acylated src kinases as well as G proteins for receptor signaling and receptor trafficking in polarized epithelial and endothelial cells. Rafts function in immune cell activation upon ligand binding by (i) concentrating key receptors such as T-cell antigen receptor (TCR) (Janes *et al.*, 1999; Langlet *et al.*, 2000; Drevot *et al.*, 2002), B cell antigen receptor (BCR) (Guo *et al.*, 2000) as well as certain Fc receptors such as FcεRI (Holowka *et al.*, 2000) and FcγRIIB1 (Aman *et al.*, 2001), (ii) recruiting additional components such as Src family kinase Lyn and others for receptor signaling, (iii) providing platform for trafficking for BCR. In addition, entry of receptors into rafts may be controlled to regulate cell activation. Finally, pathogens may use a strategy to target rafts for blocking immune cell function. For example, LMP2 of EBV is present in the rafts, blocks BCR entry into rafts and BCR trafficking and thus shuts down BCR function (Cherukuri *et al.*, 2001).

On the cytoplasmic sides, Src kinases and some Gα subunit of heterotrimeric GTPases interact via their acyl chains (e.g. saturated palmitic, and also N-terminal myristic acids) with the cytoplasmic leaflet of lipid rafts. Although most of the TM proteins are excluded from the raft, some TM proteins like influenza virus HA, human immunodeficiency virus (HIV) gp160

use acyl modification of cysteine as well as specific amino acid residues of in the TM for interaction with lipid rafts. However, now it is well established that some TM protein like influenza virus NA (type II protein) which does not have any acyl modification of transmembrane domain (TMD) also associates with lipid raft (Barman and Nayak, 2000; Zhang *et al.*, 2000). Furthermore, cytoplasmic tail can also affect lipid raft-association of NA (van der Goot and Harder, 2001).

Proteins in the raft may provide stability in raft domains since rafts can vary in size (Sheets *et al.*, 1997; Pralle *et al.*, 2000; Schütz *et al.*, 2000). Raft-like patches as large as 350–700 nm can be assembled from multiple small elementary raft-ordered membrane domains through protein-based lattices or anchored to cytoskeletal elements such as actin (Harder and Simons, 1999).

Furthermore, plasma membrane is in a dynamic equilibrium between $l_o$ raft and $l_d$ non-raft phases. Proteins such as Src related tyrosine kinase Lck remain separated from CD45-phosphatase which regulates Lck's kinase activity. They remain segregated in raft and nonraft regions of the plasma membrane. Also, raft-associated proteins could be separated in different raft domains. Ligand binding causes linking outside of the membrane that brings different raft or non-raft-associated proteins together by coalescing. Their contents are mixed causing lateral cross-linking of raft-associated proteins. This type of clustering will affect functional activation and initiate chain reaction as in signal induction.

Presence of specific peptides in a specific conformation may increase the order of the lipids in the lipid raft. The influenza virus HA TM peptide is present in an α-helical conformation in detergent micelles and in phospholipid bilayers. The helicity of the peptide increased in lipid bilayers composed of acidic lipids and in turn, the presence of the peptide also increased the acyl chain order of the lipid bilayer. The mutual interactions of the TM peptide and the surrounding lipids may be reciprocal. Ordered lipids attract TM domains and TM domains in turn increase the order of the lipids surrounding them. This process may aid in targeting HA and NA TM proteins to the ordered lipid rafts and organizing ordered lipid rafts around them (Tatulian and Tamm, 2000). However, incorporation of HA alone is not sufficient to organize the ordered lipid environment since HA incorporated in VSV envelope is TX-100 soluble (Scheiffele *et al.*, 1999).

In addition, raft-dependent protein–protein interactions could bring proteins that are present in less-ordered membrane to lipid rafts by interaction with raft-associated proteins. Interaction between influenza virus matrix (M1) and HA brings M1, a nonraft-associated protein, into lipid raft by its interaction with HA (Ali *et al.*, 2000a). Also raft-ordered membrane domains may be formed *de novo* around TM proteins on the plasma membrane such as the engaged immune receptors. Affinity of the $l_o$ domains can be increased by organization, acylation, coupling to raft-associated molecules or by conformational changes (Harder *et al.*, 1998).

Despite extensive studies of the nature and the function of lipid rafts, less is known about the heterogeneity among the lipid rafts. It is not clear if there are specific classes of distinct lipid rafts. The common criteria of TX-100 insolubility used for assaying raft-association of membrane proteins show wide variation among different proteins or among the various mutants of the same protein. These results could be due to either varying affinity of different proteins for the same class of lipid rafts or similar affinity for different classes of lipid rafts exhibiting different TX-100 insolubility (Röper *et al.*, 2000).

### 3.3.4. Function of lipid rafts

Lipid rafts have been shown to be critically involved in numerous cellular functions (Simons and Ikonen, 1997). These include clathrin-independent endocytosis, protein transport, protein sorting, protein targeting, membrane-trafficking, membrane-signaling, antigen-mediated immune response, as well as in the assembly and budding of some enveloped viruses.

In polarized epithelial cells such as MDCK cells, lipid rafts have been implicated in cell surface transport as well as in sorting and targeting apical proteins. Although the majority of these apical vesicles contain lipid rafts as evidenced from their lipid composition and TX-100 insolubility, some apical proteins such as bovine enteropeptidase (Zheng *et al.*, 1999), intestinal brush border enzymes (Danielsen, 1995), transferrin family proteins (Danielsen and van Deurs, 1995) as well as influenza C virus HEF and influenza A virus M2 protein (Hughey *et al.*, 1992), both of which are acylated (Viet *et al.*, 1990; 1991), do not use lipid rafts (Zhang *et al.*, 2000) for apical delivery.

Using influenza virus HA, a model transmembrane apical protein as a marker, apical carrier vesicles which are involved both in the transport of HA to cell surface in nonpolarized cells such as fibroblasts as well as to the apical surface in polarized epithelial cells such as MDCK were shown to contain lipid raft lipids as well as proteins such as caveolin 1, annexin XIIIb, MAL/VP17, phospholipase D2 (PLD2). Therefore these proteins are presumed to function in apical targeting. Antibody to caveolin-1 inhibited the transport of influenza virus HA to the cell surface and to the apical but not of VSV G, basolateral protein, implicating the presence of caveolin-1 in apical vesicle and its function in apical transport (Scheiffele *et al.*, 1998). However, caveolin-1 is not an essential element for apical transport of HA since in FRT cells which do not express caveolin 1/VIP21 (Zurzolo *et al.*, 1994), HA as well as GPI-anchored placental alkaline phosphatase (PLAP) are efficiently transported to the apical plasma membrane (Martin-Belmote *et al.*, 2000). Annexin XIIIb, a member of the annexin XIII sub-family, in its myristoylated form is present in sphingolipid-cholesterol rafts and stimulates apical transport (Lafont *et al.*, 1998). MAL/VIP17, a tetraspanning membrane proteolipid, is a nonglycosylated

integral membrane protein, present in lipid rafts and functions as an element in apical transport pathway since depletion of MAL inhibits both the cell surface transport and apical transport of influenza virus HA (Puertollano *et al.*, 1999). PLD2 is present in TX-100 insoluble lipid rafts and is upregulated by caveolin-1 and therefore may function as a component of apical vesicles (Czarny *et al.*, 1999). Others such as Thy-1 CD59 as well as ganglioside GM1 are preferentially partitioned into lipid rafts, although their function in apical transport pathway is not well defined.

Lipid rafts are involved not only in protein sorting and protein targeting to apical membrane in polarized cells but also in post Golgi transport of apical proteins to cell surface in nonpolarized cells. Depletion of cholesterol which leads to disorganization of lipid rafts causes missorting of apical proteins like HA, also reduces the overall transport of HA to the cell surface in both polarized and nonpolarized cells (Keller and Simons, 1998). Lipid rafts are also involved in endocytosis of proteins from apical surface via endocytic pathways independent of clathrin-coated vesicles or caveolae (Danielsen and van Deurs, 1995), as well as in endocytosis and transcytosis of proteins from basolateral surface via caveolae (Schnitzer *et al.*, 1994). Lipid rafts are also involved in membrane signaling. Several signaling molecules such as trimeric G proteins, and Ras are also associated with lipid rafts (Li *et al.*, 1995; Song *et al.*, 1996). Src family kinases, Lck ($p56^{lck}$) and Fyn ($p59^{fyn}$) implicated in antigen-mediated B and T-cell activation, are also associated with lipid rafts. Raft-association may concentrate several members of proteins involved in signal transduction and thus facilitate cross-talk among the protein molecules involved.

## 4. ROLE OF LIPID RAFTS IN VIRUS BIOLOGY

Lipid rafts have been shown to be involved in many aspects of virus life cycle. They have been implicated in virus attachment, entry, uncoating, RNA replication, protein transport, and most significantly, assembly as well as budding (Nayak and Barman, 2002; Chazal and Gerlier, 2003). Although lipid rafts clearly play the most important role in budding, they may also play critical roles in other processes of virus life cycle.

### 4.1. Role of lipid rafts in virus–host interactions, virus entry and virus replication

Rafts have been shown to be the preferential site for interaction of many pathogenic organisms (bacteria, parasite, viruses) with target cells (Campbell *et al.*, 2001). With respect to viruses, lipid rafts may provide platforms for

Table 3. Role of lipid rafts in viral entry

| Virus | Mechanism of use | References |
|---|---|---|
| Semliki Forest virus (SFV) | Fusion of viral envelope requires the presence of lipid rafts. | Kielian & Helenius, 1984<br>Phalen & Kielian, 1991<br>Waarts *et al.*, 2002 |
| Sindbis virus (SIN) | Highly dependent on cholesterol for fusion. Fusion of viral envelope involves lipid rafts. | Lu *et al.*, 1999<br>Waarts *et al.*, 2002 |
| HIV-1 | HIV-1 enters epithelial cells via raft-mediated endocytosis and transcytosis. | Nguyen & Hildreth, 2000<br>Alfsen *et al.*, 2001<br>Narayan *et al.*, 2003 |
| Simian virus 40 (SV40) | Virus enters the cell by binding MHC class 1 molecules that are located in caveolae. | Stang *et al.*, 1997 |
| Ebola virus | Viral entry requires functional rafts. | Bavari *et al.*, 2002 |
| Marburg virus | Viral entry requires functional rafts. | Bavari *et al.*, 2002 |

attachment and entry by providing means to concentrate receptors, thereby increasing the binding affinity of viruses. Certain viruses may preferentially interact with raft as the site for attachment and internalization (Table 3). For example, SV40, a DNA containing non-enveloped virus was shown to interact with major histocompatibility complex (MHC) class I molecules leading to recruitment of caveolin around the attached virus and generating caveolae like invagination (caveosome) with virus attached and thereby facilitating internalization of the attached virus (Pelkmans and Helenius, 2003). However, virus in the invaginated raft somehow escapes proteolytic degradation and finds its way into the endoplasmic reticulum and then into the nucleus, the site of virus replication. During HIV infection, the HIV-host cell fusion was shown to require reorganization of membrane lipids. It has been proposed that initial interaction of gp120 with CD4 promotes clustering of raft domains and bringing coreceptors at the infection site. It has been shown that HIV receptor CD4, coreceptor CXCR4 as well as other associated molecules favoring HIV infection such as glycosphingolipids, CD44, all reside in lipid rafts. Recently, SFV was shown to require lipid raft for promoting fusion of viral membranes with the cell membrane (van der Goot and Harder, 2001). The role of lipid rafts in the fusion process between the viral and plasma membrane has been demonstrated for a number of viruses. For SFV, the presence of sphingolipids was critical for the fusion of viral membrane to the target membrane (Nieva *et al.*, 1994). Also, fusion among measles virus-infected Vero cells was affected by drugs that inhibit cholesterol biosynthesis (Malvoisin and Wild, 1990).

In the case of HIV, which causes AIDS by complex pathogenic process involving both virus and host, rafts are involved in multiple steps of the HIV replication process involving entry, signal transduction, trafficking and assembly of HIV proteins as well as budding of HIV particles. Lipid raft-association of HIV promotes viral entry. Cholesterol depletion by the methyl-β-cyclodextrin which disrupts rafts, inhibits HIV infection (Dimitrov, 2000; Hug *et al.*, 2000). Inhibitors of GSL biosynthesis affected HIV infection and syncytia formation and could be reversed by addition of specific GSL. Also, GSL-depleted cells lost their ability to induce conformational changes in Env-expressing cells although gp120 could interact with CD4 and coreceptors (CCR5 and CXCR4). Some receptor protein such as ATRC1 for ecotropic murine leukemia virus is raft-associated. SFV requires the presence of cholesterol and sphingolipids in the target membrane. E1 protein of SFV in low pH underwent conformation at changes, interacted with target membrane via insertion of its fusion peptide leading to association of E1 with lipid rafts. However, the fusion peptide of influenza virus HA did not associate with lipid raft. Cleaved HA requires its membrane domain for complete fusion since GPI-linked HA lacking TMD, although more strongly raft-associated than the transmembrane HA, leads to hemifusion but not complete fusion of membrane.

Lipid rafts not only facilitate HIV entry during fusion and uncoating of infecting virus but also play an important role in pathogenesis by aiding the virus in penetrating the intact barriers of epithelial cells present at the surface of gastrointestinal and genitounrinary tracts. It is believed that HIV uses transcytosis to gain entry into the human host but the infection process does not involve infection of surface epithelial cells. According to this model, HIV binds to glycosphingolipid galactosyl ceramide (Gal Cer) which has been shown to function as an alternative receptor of HIV glycoproteins (Alfsen *et al.*, 2001). Gal Cer is a component of the lipid raft, enriched in the apical membrane of epithelial cells and HIV becomes associated with lipid rafts and the intact virus then traverses the epithelial cell from the apical to the basal side by transcytosis and subsequently, the transcytosed virion infects the mononuclear cells containing CD4 and chemokine co-receptors which are also raft-associated leading to entry into host and productive infection process. It has also been shown that raft-association of HIV protein may play a critical role in altering signal transduction and disease progression. Nef (negative factor) protein which becomes myristoylated and raft-associated (Wang *et al.*, 2000), has been implicated with high viral load and increased pathogenesis. Nef is a raft-associated protein. Association of Nef with lipid raft may affect clustering of signaling molecules such as Lck, Hck, Lyn, Fyn, and may interfere with T cell activation (Cheng *et al.*, 1999). Nef contains a highly conserved proline rich motif (PXXP) which interacts with SH3 domains of Hck and Fyn (Collette *et al.*, 2000; Hiipakka and Saksela, 2002).

Also, host proteins such as HLA class II which are raft-associated and become incorporated in the HIV envelope, may influence host signaling to facilitate HIV entry into the target cells (Bastiani *et al.*, 1997). HIV envelope protein (gp160 or gp120/gp41) is a type I transmembrane basolateral protein and HIV also buds from the basolateral surface of polarized epithelial cells. However, unlike most basolateral proteins and basolateral viruses which are not raft-associated, HIV as well as HIV env are raft-associated and raft-association of HIV is critical for virus infectivity. Ebola virus (EBOV) and Marburg virus (MBGV) are among the two most pathogenic viruses and belong to the filoviruses. It has been shown that transient treatment of cells with filipin and nystatin, raft disrupting agents, interfered with virus infection suggesting that lipid raft may be important as a gateway of entry of filoviruses. However, it was noted that virus entry and not virus attachment involved the folate receptor which can function as a cellular cofactor for filoviruses and was also shown to reside in lipid raft (Bavari *et al.*, 2002).

In addition to attachment and entry, lipid raft may provide important function in RNA replication. For many positive strand RNA viruses, whether non-enveloped (picornavirus) or enveloped (hepatitis C virus [HCV]), RNA replication occurs in membrane-associated compartments. For HCV, the membrane-associated replication complex seems to be associated with lipid raft (Shi *et al.*, 2003). HCV nonstructural proteins including NS5A, NS4B as well as the newly synthesized HCV RNA were present in a detergent-resistant membrane complex and cofractionated with caveolin 2, a lipid raft-associated intracellular membrane protein. HCV RNA synthesis was protected from RNase in the membrane complex but became sensitive after detergent treatment. Depletion of cellular cholesterol also reduced HCV RNA synthesis. These results suggest that HCV RNA synthesis occurred in raft-associated membrane complex.

## 4.2. Role of lipid rafts in assembly and budding processes

In the virus life cycle, budding and release of the virus particles into the outside environment are last events. For budding to occur, three steps are obligatorily required. However, successful completion of these three steps does not guarantee that the budding and release of infectious virus particles will occur since additional steps may be required for the completion of the bud and release of the particles from the host cell as well as maturation of virus particles. These three obligatory steps are: (i) Following or concomitant to the synthesis of different viral components, they must be sorted, targeted and delivered to the assembly site. (ii) During this transport process or upon arrival to the assembly site, different viral components must interact with each other

in an orderly fashion for bud formation to occur. (iii) Physical process of bud formation including the curvature of the membrane and eventual closure of the bud by membrane fusion and fission must occur to separate the virus particle from the host membrane. Lipid rafts appear to play important roles in each of these three steps.

### 4.2.1. Transporting and targeting viral proteins to the assembly site

As indicated earlier, lipid rafts actively participate in transporting and targeting many of the integral membrane proteins to the apical domains of the plasma membrane. Protein components of enveloped viruses are synthesized either in membrane-associated polyribosomes in ER or in free cytoplasmic polyribosomes. The proteins that are translated on the membrane-associated polyribosomes are either the integral membrane proteins present in the virus envelope or the secretory proteins lacking the membrane anchor region, usually not encoded by the viral genome. The other viral proteins which constitute the internal core viral components including both capsid and matrix proteins, are synthesized in the free polyribosomes as soluble cytoplasmic proteins. As indicated earlier, lipid components of lipid rafts are also synthesized in the ER and Golgi compartments and use the same exocytic pathway and vesicular transport system used by the integral membrane proteins to reach the plasma membrane. Close proximity of the integral membrane proteins with the lipid components in the exocytic pathways and their affinity for each other may facilitate their interaction and sorting in the same vesicle during their exocytic transport from the *trans* Golgi compartment to the plasma membrane. In polarized epithelial cells, integral membrane proteins are destined for either the apical or basolateral domains of the plasma membranes. It has been shown that both the basolateral (eg. VSV G protein) and apical (eg. influenza virus NA and HA) are transported together by the same vesicles to the TGN. However, the transport of apical and basolateral proteins from the TGN to the plasma membrane occurs via separate transport vesicles. Therefore, it is postulated that assembly and budding of vesicles containing apical and basolateral proteins occur at the separate domains of the TGN membrane and that the presence of lipid rafts in TGN and the affinity of the specific transmembrane proteins for lipid rafts provide platforms for segregation of apical proteins from the basolateral proteins in TGN. Influenza viral proteins have been extensively used as model for studying raft-association of proteins during transport and sorting. Influenza viral envelope possesses three integral transmembrane proteins namely HA, NA and M2. Among these both HA (type I) and NA (type II) proteins use lipid raft as a platform for apical

transport (Nayak and Barman, 2002; Nayak and Hui, 2002) but M2, although an integral membrane protein, does not use lipid raft for apical transport (Zhang *et al.*, 2000). Furthermore, in the envelope of released virions, both HA and NA remain raft-associated but M2 does not associate with the lipid raft, indicating that the influenza viral envelope also exhibits a mosaic mixture of both raft and non-raft lipid microdomains even though the majority of lipids present in the lipid bilayer of the viral envelope are in $l_o$ phase. Influenza HA associates with lipid raft and becomes TX-100 insoluble after acquiring the complex sugars in the Golgi complex. However, association of HA with lipid rafts is not dependent on oligosaccharide modification or its association with other viral proteins or its assembly into virus particles. Neither, the association of HA with lipid rafts depends on the polarity of cells (Skibbens *et al.*, 1989). TMD of HA has critical determinants for interacting with lipid rafts since chimeric HGH or HCH proteins containing TMD of VSV G or HSV C proteins and the ectodomain and cytoplasmic tail of HA did not associate with the lipid rafts. Furthermore, the exoplasmic half of the HA TMD and the amino acids contained in this region were critical for lipid raft-association (Scheiffele *et al.*, 1997; Lin *et al.*, 1998). The interaction of HA TMD with lipid raft also depended on a number of factors such as palmitoylation of three cysteine residues present in the HA TMD and cytoplasmic tail as well as the structural features such as α-helical conformation of HA TMD peptide. Furthermore, as indicated earlier, the HA TMD peptide was also shown to increase the acyl chain order of the lipid bilayer and thereby may have actively participated in stabilizing lipid rafts. In addition to TMD, the HA cytoplasmic tail may have an effect on the preferential association of HA with lipid raft since cytoplasmic tail minus HA ($Hat^-$) exhibited markedly reduced TX-100 resistance both in released virus particles and in cDNA transfected cells (Zhang *et al.*, 2000). This could be partly due to the loss of two cysteine residues in the cytoplasmic tail.

NA, a type II integral influenza virus protein, associates also with lipid raft via its TMD during intracellular transport. Chimeric analysis of using human transfection receptor (TR), also a type II protein, demonstrated that the NA TMD was responsible for interacting with lipid raft and apical transport signal (Kundu *et al.*, 1996; Barman and Nayak, 2000). However, unlike HA, interaction of NA TMD with lipid raft was not dependent on acylation of cysteine residues. Also like HA, the cytoplasmic tail of NA affected the interaction of NA in the lipid raft since removal of the conserved cytoplasmic tail reduced raft-association and increased TX-100 solubility of NA. Furthermore, NA has been implicated in facilitating the raft-association of HA (Zhang *et al.*, 2000). Like influenza virus, Sendai virus (SV) F and HN protein also associate with lipid rafts. However, both F and HN appear to associate with different classes of lipid rafts since TX-100 resistance of mature F and HN varied and was also

different from that of influenza virus HA or NA (Ali *et al.*, 2000a). Similarly, SV5 as well as respiratory syncytial virus (RSV), members of paramyxovirus group, also bud from apical domain of plasma membrane are likely to contain lipid rafts in their viral envelope. However, detailed information regarding the presence of ordered lipids in the viral envelope or the raft-association of these glycoproteins is not available. Measles virus (MV), another number of paramyxovirus, also bud from the apical surface in polarized epithelial cells (Blau and Compans, 1995; Vincent *et al.*, 2000) and become raft-associated. However, both the process of raft-association as well as apical budding of MV appear to be the result of complex interactions among the viral proteins during intracellular transport, sorting and budding on the plasma membrane. In the MV-infected cells, both surface glycoproteins (F and H proteins) as well as internal M and N proteins became lipid raft-associated. However, in cDNA transfected cells, measles virus F but not H protein when expressed alone, became raft-associated. But upon coexpression of H protein with F protein, both F and H proteins became lipid raft-associated suggesting that F protein somehow facilitated raft-association of H protein (Vincent *et al.*, 2000). On the other hand, M and N proteins, the core viral proteins, did not depend on H and F proteins for lipid raft-association (Manié *et al.*, 2000). However, it is unclear how M and N proteins which are synthesized by free polyribosomes and not transported by exocytic pathways, became raft-associated. Furthermore, relative ratios of raft-associated cell surface MV glycoproteins such as H protein was lower than that of intracellular proteins suggesting that H protein become dissociated from lipid raft after it reached cell surface. Analysis of virus envelope from released viruses showed that only about 30% of H and 10% of F glycoproteins present in the viral envelope were raft-associated (Manié *et al.*, 2000). This would suggest that MV budding occurred not from the typical lipid raft as determined by TX-100 resistance or that H and F proteins became dissociated from lipid rafts during or after budding and/or release of MV particles. Since specific domains of glycoproteins were involved in raft-association, it is expected that both the TMD of F and as well as palmitoylation of the membrane proximal cysteine residue of F protein are likely to be involved in lipid raft-association.

It has been shown that palmitoylation of HIV env protein in the Golgi is critical for raft-association and for packaging in viral envelope (Rousso *et al.*, 2000). Association of HIV env protein with cholesterol sphingolipid rafts in the Golgi is also critical for intracellular trafficking of env protein to reach the plasma membrane microdomain for assembly and budding of HIV to occur. In addition, another HIV protein, the Gag protein present in the core complex of HIV, is translated as a soluble cytoplasmic protein using free polyribosome, but becomes raft-associated because of its myristoylation and interaction with the inner leaflet of lipid raft. It is likely that raft-association of both HIV env

and Gag proteins are required for targeting HIV protein complex to the proper assembly site and that Gag-env interaction is required for conformational change and proteolytic cleavage of Gag-pol for budding and release of infectious virions. As mentioned earlier, Nef another critical HIV protein in virus budding is also raft-associated (Wang *et al.*, 2000; Zheng *et al.*, 2001). It was further shown that mature HIV released in the medium contains lipid raft in its envelope suggesting that the budding process may also be facilitated by the presence of lipid rafts at the assembly site (Aloia *et al.*, 1993). It appears that lipid rafts are not only involved in targeting HIV protein complexes to the assembly site and in budding and release of the virion, but is also critically required for maintaining the structure and conformation of matured HIV virion particles. Treatment of mature HIV with 2-hydroxy-propyl-β-cyclodextrin or methyl-β-cyclodextrin which removes cholesterol or with cholestenone (cholesterol analogue), destabilizes HIV structure and reduces infectivity. Furthermore, virion particles become denser, smaller and noninfectious suggesting that cholesterol (or lipid raft) is present on HIV envelope and is required for maintaining the integrity of HIV structure (Campbell *et al.*, 2001; Graham *et al.*, 2003). SFV treated with cyclodextrin (CD) also showed morphological defect (Scheiffele *et al.*, 1999).

Budding of the Rubella virus, a member of the Togaviridae family, genus Rubivirus, occurs from the Golgi membrane and virus particles are released from the apical side of cultured polarized epithelial cells (Garbutt *et al.*, 1999). However, little is known about the interaction of E1 and E2 proteins with lipid rafts or the role of lipid raft in the transport of envelope protein and in virus release. Two other Toga virus, SIN and SFV, bud apically in FRT cells but basolaterally from CaCo-2 cells and the distribution of p62/E2 envelope proteins is also apical in FRT cells but basolateral in CaCo-2 cells (Zurzolo *et al.*, 1992). Again, the role of lipid raft in neither of the transport of p62/E2 protein nor budding of these viruses is known. SFV and SIN are members of the alphavirus of Togavirdae family and possess similar structure as the rubella virus but in addition, they have a 6K TM protein. The 6K protein undergoes palmitoylation and is likely to interact with lipid rafts and thereby may facilitate virus budding in BHK cells. However, 6K protein appears not to be required for budding in virus-infected insect cells which possess more fluid membrane and compared to BHK cells, contain less sphingomyelin (9% vs 25%), and PC (14% vs 27%) (Cadd *et al.*, 1997). Lipid rafts may not actively participate in the assembly and budding of SFV and possibly, other members of alpha viruses since SFV budding requires precise interaction of trimeric envelope proteins forming spike with hexagonal capsid. On the other hand, for majority of enveloped viruses, such precise interaction of envelope proteins with capsids is exception rather than the rule and the incorporation of other proteins in viral envelope is often permitted (Briggs *et al.*, 2003).

Filovirus assembly and budding is also raft-associated (Bavari *et al.*, 2002). Filovirions incorporated raft-associated lipid molecules such as GMI. Ebola and Marburg virus glycoprotein (GP) were found to reside in lipid and the lipid raft-association of GPs was dependent on the palmitoylation of cysteine residues. Lipid raft-associated patches on the plasma membrane were involved in the assembly and budding of filovirus particles. In summery, lipid rafts often interact with the envelope as well as core proteins of many viruses and thereby facilitate their sorting and transport to the assembly site.

### 4.2.2. Selection of assembly site

As noted earlier, some enveloped viruses assemble and bud from the intracellular organelles (Hobman, 1993), while others bud from the plasma membrane (Figure 1, Table 1) (Garoff *et al.*, 1998). Among the viruses that assemble and bud at the plasma membrane, some bud predominantly from the apical while others from the basolateral but usually not from both domains of the plasma membrane of polarized epithelial cells (Compans, 1995). However, what makes the virus to assemble and bud from a specific site in a cell or a specific domain of plasma membrane remains unclear.

It is generally accepted that viral glycoproteins determine the site of virus assembly and budding. This notion comes from the fact that viral glycoproteins accumulate at the site of the virus budding even when expressed alone. For example, glycoproteins of viruses such as coronaviruses (CoV), hepatitis B virus (HBV), bunyaviruses and others which bud from the internal sub-cellular organelles, possess intrinsic determinants for the same sub-cellular localization as the site of virus budding (Hobman, 1993). On the other hand, glycoproteins of viruses that bud from the plasma membrane are not retained in the internal organelles but are transported to the plasma membrane. Moreover, for the latter group of viruses, viral glycoproteins possess either apical or basolateral sorting signals and are directed to the apical or basolateral surface, the specific site where virus assembly and virus budding occur in polarized epithelial cells. Furthermore, in different cells and tissues where some viruses bud from the opposite membrane, their glycoproteins are distributed accordingly. For example, SFV bud apically from FRT cells but basolaterally from CaCo-2 cells; similarly, p62/E2, the envelope glycoproteins of SFV, are targeted apically in FRT cells but basolaterally in CaCo-2 cells (Zurzolo *et al.*, 1992). However, since apical and basolateral distribution of p62/E2 glycoproteins were determined in virus-infected cells, it can not be ascertained from these experiments if other viral components affected the distribution of envelope glycoproteins of these viruses.

For retroviruses, particularly HIV, which buds from basolateral surface in polarized epithelial cells, it appears that HIV env protein plays a critical role in

determining the basolateral budding site. It was observed that HIV capsid proteins when expressed alone released particles randomly from both apical and basolateral surfaces, whereas upon co-expression with the envelope protein gp160, virus particles were released predominantly from basolateral surface (Owens *et al.*, 1991). Authors concluded from these studies that the HIV envelope protein, which is targeted to the basolateral surface in polarized epithelial cells, determines the site of virus budding. Further role of the envelope protein in polarized budding of HIV was supported by a number of experiments where mutation in the cytoplasmic tail of gp41 caused non-polar distribution of the envelope protein resulting in the non-polar budding of HIV (Lodge *et al.*, 1994; 1997) and affecting cell to cell transmission (Deschambeault *et al.*, 1999). However, attempts to redirect virus budding into ER and Golgi by introducing specific retention signals into the HIV envelope protein did not succeed (Salzwedel *et al.*, 1998). Although, in this case, one can argue that the Golgi and ER environments are much different from that of the plasma membrane and that the glycoproteins may not attain the proper structural conformation for interacting with HIV capsids to cause budding. On the other hand, mutations in the matrix domain of the Gag protein caused redistribution of both Gag and env protein on the plasma membrane (Hermida-Matsumoto and Resh, 2000) suggesting that the envelope protein may not be the only determinant for selecting the site for HIV budding.

Experiments with measles virus have shown that budding site in polarized epithelial cells was not determined by glycoproteins (Maisner *et al.*, 1998). Although viral glycoproteins H and F were transported in a random fashion and to basolateral membrane respectively, virus budding occurred predominantly from the apical surface of polarized MDCK cells. Similarly, although Marburg virus buds predominantly from the basolateral surface, its glycoprotein is transported to the apical surface (Sanger *et al.*, 2001). With human cytomegalovirus (CMV), p150, a nonenvelope tegument protein, was critical for the site of virus assembly (Sanchez *et al.*, 2000). Influenza virus, which assembles and buds from the apical plasma membrane in polarized epithelial cells, has been used extensively as a model for studying protein targeting. As indicated earlier, it possesses three transmembrane proteins HA, NA and M2, all of which are apical. Both HA and NA interact with lipid raft by the sequences present in their transmembrane domains which also possess signals for apical transport (Kundu *et al.*, 1996; Lin *et al.*, 1998; Barman and Nayak, 2000; Barman *et al.*, 2001). M2, which is also an apical protein (Hughey *et al.*, 1992), does not interact with lipid raft (Zhang *et al.*, 2000) and the apical signal of M2 is unknown at present. Of these three envelope proteins, HA, the major glycoprotein, constitutes over 80% of the envelope proteins present in virus particle. In transfected cells, a single amino acid change (Cys543→Tyr543) in HA (H2) was shown to direct HAtyr to the basolateral

side and did not affect the intracellular transport and cell surface expression of the mutant protein (Brewer and Roth, 1991). However, a mutant virus containing the basolateral HA (HAtyr), budded almost exclusively from the apical side (>99%) i.e. from the same site as the wild type virus, even though majority of HAtyr was directed to the basolateral side (Mora *et al.*, 2002; Barman *et al.*, 2003). These results demonstrated that HA, the major envelope protein, was not the determinant for the site of assembly and budding of influenza virus particles. More recently, it was also shown that VSV G protein (targeted basolaterally) does not determine the basolateral budding of that virus (Zimmer *et al.*, 2002). These studies suggest that viral glycoproteins may not be the only or major determinant for selecting the budding site.

Since the major glycoprotein was not responsible for selecting the site for virus assembly and budding, other factors of both host and virus origin are likely to be involved in selecting the budding site. Among the viral components, the matrix protein (M1) may play critical role in site selection. We have recently shown that influenza virus M1 possesses a "late" (L) domain (Hui *et al.*, 2003) which may interact with host components for virus budding. Such host components may be restricted at the apical domain for influenza virus budding and at the basolateral side for VSV budding. With measles virus, which buds predominantly from the apical surface, although its F and H are expressed predominantly on the basolateral surface, it has been suggested that polarized expression of M protein or interaction of M protein with glycoproteins at the apical plasma membrane domain may favor apical budding (Naim *et al.*, 2000). In addition to M1 protein, NP or vRNP or M1/vRNP complex of influenza A virus could have polarized distribution and may facilitate apical budding. However, how such cytoplasmic proteins which are not acylated could have a targeted polarized distribution remains unknown.

It is also likely that influenza as well as other viruses require lipid rafts as budding platform (Table 4), which are predominantly present on the apical membrane to produce virus particles with ordered lipid membrane (Brown and London, 1998a; 1998b) and that the fraction of HAtyr transported to the apical surface (but not to the basolateral surface) interacted with lipid raft. Furthermore, it has been shown that lipid rafts play an important role in influenza virus budding. With $HAtail^{-}/NAtail^{-}$ viruses where HA was more TX-100 soluble and less lipid raft-associated, virus production was low and virus particles were deformed and bizarre in shape (Zhang *et al.*, 2000). Lipid rafts in addition to transporting, targeting and concentrating viral and host components to the assembly site may have some intrinsic properties for budding to occur and thereby facilitate budding from a specific site of a membrane for a given virus.

Although viruses can bud and form particles in the absence of glycoproteins and although the Gag protein of HIV and matrix proteins of many

Table 4. Role of lipid rafts in viral exit

| Virus | Mechanism of use | References |
|---|---|---|
| Sindbis virus (SIN) | Virus buds from cholesterol-rich domains. | Lu *et al.*, 1999 |
| Vesicular stomatitis virus (VSV) | VSV-M and -G protein redistribute lipids during virus formation. | Luan *et al.*, 1995 |
| HIV-1 | Virus exits by budding selectively from lipid rafts.<br>Gag specifically associates with raft like membrane. | Nguyen & Hildreth, 2000<br>Alfsen *et al.*, 2001<br>Ono & Freed, 2001 |
| Influenza virus | Virus exits by assembling viral glycoproteins in lipid rafts and trafficking to the apical cell membrane. | Scheiffele *et al.*, 1997;<br>Keller & Simons, 1998;<br>Scheiffele *et al.*, 1999;<br>Barman *et al.*, 2001 |
| Measles virus (MV) | Virus assembly occurs in lipid rafts. | Manié *et al.*, 2000 |
| Sendai virus (SV) | Structural glycoproteins assemble in lipid rafts for virus exit. | Ali & Nayak, 2000 |
| Respiratory syncytial virus (RSV) | Matures at regions of the cell surface that are enriched in the protein caveolin-1 and lipid raft ganglioside GM1. | Brown *et al.*, 2002a<br>Brown *et al.*, 2002b |
| Ebola virus (EBOV) | Viral proteins within lipid rafts during viral assembly and budding. | Bavari *et al.*, 2002 |
| Marburg virus (MBGV) | Viral proteins within lipid rafts during viral assembly and budding. | Bavari *et al.*, 2002 |
| Murine leukemia virus (MuLV) | Envelope protein is lipid raft associated through palmitoylation. | Li *et al.*, 2002 |

negative strand viruses can bud and acquire envelope, the lipid composition of such virus like particles (VLPs) are not known. Whether such particles contain lipid raft in their envelope or glycoproteins are required for acquiring lipid rafts in their envelope remains to be determined. If such VLPs do not possess lipid rafts, it is likely that virus budding can occur from plasma membrane outside the lipid raft microdomain. If, on the other hand, such VLPs do possess lipid rafts as found in infectious virus particles, it would imply that the core components including matrix proteins can interact with lipid raft in the absence of glycoproteins and that budding requires the presence of lipid raft microdomain.

### 4.2.3. Interaction among viral components

Lipid rafts play important roles in different steps of the budding process. One important step involves the interaction among the viral components

required for producing the infectious virus. Lipid rafts not only promote transporting and targeting viral components to the assembly site but aids in the interaction among the viral components in a number of ways.

(a) Firstly, it can bring the viral components in the proximity of each other by partitioning and concentrating the proteins in specific microdomains. This is evident for proteins which can independently associate with lipid rafts and therefore, can interact with each other because of their proximity. This appears to be the case in HIV-infected cells. In HIV-infected cells, coalition of lipid rafts will concentrate HIV env and MA proteins both of which are acylated, and already present in the similar lipid compartments and thereby will facilitate their interaction with each other and the budding process. Some host proteins such as CD55, CD59, inhibitors of complement pathways, as well as other GPI-linked proteins that are present in lipid rafts are also included in HIV particles although they are not known to interact with any specific viral proteins (Nguyen and Hildreth, 2000).

In MV-infected cells, F and HN, the TM proteins, become raft-associated. However, it has been reported that M and N the intracellular cytoplasmic proteins synthesized on free polysomes, also become raft-associated (Manié *et al.*, 2000), although the mechanism by which these two proteins which are not known to be acylated, become raft-associated is unclear. Raft-association of N, M, F and HN will facilitate interactions among these viral components.

(b) Secondly, lipid rafts can also bring nonraft-associated proteins in the raft microdomain because of their interaction with raft-associated components. This appears to the case with a number of viruses. For example, Ortho- and Paramyxovirus glycoproteins are lipid raft-associated whereas their matrix proteins (M and M1) are nonraft membrane-associated. Matrix proteins after their interaction with glycoproteins become raft-associated (Ali *et al.*, 2000a, 2000b). Interactions of matrix proteins with glycoproteins as well as raft-association of matrix proteins will facilitate interaction among the matrix proteins by increasing their concentration at the budding site and this interaction among the matrix proteins is critical for virus budding.

Also, raft-ordered membrane domains may be formed de novo around TM proteins on the plasma membrane such as the engaged immune receptors. Affinity of the $l_o$ domains can be increased by organization, acylation, coupling to raft-associated molecules or by conformational changes (Harder *et al.*, 1998). All of these processes may aid in the M1/M1 and M1/glycoprotein interactions.

In studies involving VSV G and M proteins, it was shown that both proteins were needed for inducing sph- and cholesterol-enriched domains and either of these proteins alone were not effective (Luan *et al.*, 1995), again implying the role of lipid domains in bringing the interacting proteins together. Therefore,

lipid rafts play an important role in promoting interaction among viral components required for assembly and budding.

(c) Thirdly, pseudotyping is a common phenomenon observed in cells mixedly infected with two or more enveloped viruses where progeny viruses containing the genome (and capsid) of one virus and the envelope proteins of a second virus are formed. This type of mixing of core (capsid) components with envelope components has been observed with many DNA and RNA viruses and provides an important tool for gene delivery and is required for either broadening or selecting host range and tissue tropism. This well-documented phenomenon of pseudotyping is at odds with the common notion that specific interaction between the core component and envelope proteins governs the assembly and budding of most viruses. It appears that lipid forms a common platform for mixing the envelope proteins of different viruses and cellular membrane proteins. Even viruses belonging to diverse groups such herpes virus and VSV can produce pseudotype viruses. A common property among all these diverse viral and cellular proteins is that they are raft-associated and often myristoylated or palmitoylated. Even basolateral proteins like VSV G are palmitoylated and raft-associated. (Harder *et al.*, 1998). It was observed that in mixed infection, envelope proteins of different viruses as well as some of the core proteins such as HIV Gag are often raft-associated and non-ionic detergent insoluble (Pickl *et al.*, 2001). Therefore, detergent-resistant lipid rafts on the plasma membrane are common meeting grounds for core and transmembrane envelope proteins of different viruses. Also, these lipid rafts function as a platform for envelopment and budding resulting in the production of pseudotype viruses. Some of the host components such as CD4, CXCR4, as well as env proteins of other retroviruses and GPI-anchored proteins can be incorporated in viral envelope. Lipid rafts therefore provide the basis for promiscuity of incorporation of foreign proteins into number of virus particles such as VSV, HIV and support the passive incorporation of integral membrane proteins into virus particles. However, envelope protein and core interactions also play a critical role in selecting the incorporation of specific viral proteins and excluding most membrane-associated host proteins from the budding site and virus particles. Therefore, lipid microdomains such as lipid rafts facilitate mixing and interaction among the viral components required for assembly and budding of infectious viruses as well as in pseudotype formation.

### 4.2.4. Budding process and release of virus buds

4.2.4.1. Budding site: Role of viral proteins and lipid rafts

As indicated earlier, viruses do not bud randomly from host membrane but selectively from specific organelles or from the specific domains (apical or

basolateral) of plasma membrane. It also appears that within the specific membrane domain, virus budding occurs from a few select "hot spots" as if the same site in being used for multiple rounds of virus budding. Observation that multiple incomplete virus buds are joined together in HIV-infected cells defective in Tsg101 function supports this hypothesis of hot spots of virus budding (Garrus *et al.*, 2001; Demirov *et al.*, 2002; Goila-Gaur *et al.*, 2003). Release of influenza virus particles in HeLa cells after cytoskeletal disrupting agents from a few spots on the membrane (Gujuluva *et al.*, 1994) as well as incomplete virus budding of virus particles joined together budding defective influenza virus mutants also support virus budding from selective spots on the membrane (Roberts and Compans, 1998; Simpson-Holley *et al.*, 2002; Barman *et al.*, 2003; Hui *et al.*, 2003). The biophysical and biochemical properties of these hotspots of budding site are yet unknown. We speculate that at least lipid rafts are an important contributing factor for the budding site since many viruses use lipid rafts for budding and incorporation into the membrane of mature virus particles. It then becomes important to determine how the membrane is modified for budding site. Viral membranes are not only enriched with lipids assembled as lipid rafts but also contain virally encoded proteins. Two types of proteins are associated with viral membrane: (i) TM proteins that traverse the entire lipid bilayer and may have acyl modifications in TMD and cytoplasmic tails but some proteins such as influenza virus NA, a type II TM protein, are purely TM proteins without any acyl modification. TM proteins with or without acyl chains primarily interact with exoplasmic leaflet but also may interact with the cytoplasmic leaflet of the lipid bilayer by the acyl chains. (ii) Proteins present in the cytoplasmic side interact with the inner leaflet of the lipid bilayer. These cytoplasmic proteins are either modified with acyl groups and thus interact with the lipid bilayer or interact with the cytoplasmic tail of TM proteins and thereby become indirectly associated with lipid raft. As indicated earlier, some TM proteins, particularly the apical proteins also use lipid raft for sorting and transporting to the virus assembly site.

Since viral envelope proteins are present in the lipid rafts and since lipid rafts essentially constitute viral membranes, it becomes an important question whether the viral proteins present in the lipid rafts are passively incorporated into virions or they play an active role in the assembly and budding process. One possibility is that the lipid rafts are assembled into microdomains due to the physical and structural properties of lipid molecules as has been shown for the assembly of the lipid rafts in artificial membranes and viral proteins or proteins in general are just passive participants and become incorporated into lipid rafts because they are present in the vicinity and have affinity for the lipid raft. These lipid rafts containing viral TM proteins become the assembly and budding site of enveloped viruses. The second possibility is that the viral TM proteins actively participate in selecting and assembling lipids and packing

the lipids into lipid rafts for functioning as viral membranes. Current evidence suggests that proteins and lipids can interact with each other at multiple levels of organization of rafts and their incorporation into viral envelope may vary with different enveloped virus groups. For example, as indicated earlier, the influenza HA TMD peptide assumes a predominantly alpha helical conformation in detergent micelles and phospholipid bilayers. Furthermore, depending on the composition of lipid bilayer such as lipid bilayer composed of pure phosphatidylcholines, the helicity of HA peptide is increased. On the other hand, the HA TMD peptide also increased the acyl chain order and packing of lipid bilayer supporting the role of protein component in assembling raft and preferential association with lipid rafts. Possible interaction of the specific amino acids of HA and NA TMD peptides with the outer bilayer of lipid rafts has been supported by mutational analysis (Scheiffele *et al.*, 1997; Lin *et al.*, 1998; Barman *et al.*, 2001). Furthermore, cytoplasmic tail of HA may also affect interaction of HA TMD with lipid raft as the tail minus HA becomes more TX-100 soluble (Zhang *et al.*, 2000). In addition to the amino acid sequence of HA TMD, the palmitoylation of cysteine residues and their interaction with the inner leaflet of lipid bilayer also affect HA interaction with lipid rafts. Mutational analysis also showed that for NA, a type II protein, which does not undergo any acyl modification, the amino acid sequences at COOH terminus of TMD interact with the exoplasmic lipid leaflet of the raft containing glycosphingolipids and cholesterol. Also like HA, removal of the NA cytoplasmic tail affected its interaction with lipid rafts. It is not clear how the cytoplasmic tail outside the lipid bilayer would affect its association with the lipid raft. It is possible that the removal of cytoplasmic tail may affect structure of the TMD such as alpha helicity which is known to have affinity of lipid raft. In addition, oligomerization among the NA TMD as well as interaction of the cytoplasmic influenza virus proteins such as M1 may help in cluster formation and stabilization of lipid rafts.

Even VSV G, the model basolateral protein, becomes palmitoylated (Brown *et al.*, 1989) and associates with raft-like structures present in basolateral membrane. In fact VSV G protein facilitates the formation of cholesterol- and sphingomyelin-rich lipid domain in vesicles and in VSV particles (Harder *et al.*, 1998). Therefore, initially viral components are concentrated at the localized lipid raft like regions, the assembly site. Subsequently, lateral interaction among the viral components amplify the changes in local lipid composition, which in turn facilitate further concentration of viral components leading to bud formation.

For retroviruses, particularly HIV, interaction among env protein (gp160, gp120/gp41) and MA protein play critical roles for association with lipid rafts and budding. As indicated earlier, the env protein although a basolateral protein, can independently associate with lipid rafts in the absence of MA

protein. This interaction of env protein to the lipid raft is due to the palmitoylation of the two cysteine residue in the cytoplasmic tail (CT) of env protein and occurs during the transport of the env protein to the plasma membrane. Thus with HIV env protein, unlike the TMD influenza virus HA or NA, the palmitoylation of the cytoplasmic tail is critical for its interaction of with lipid rafts. Therefore, it is possible that env protein may be passively incorporated into the virus particles because it is present in lipid rafts, the site of virus budding and that interaction of env proteins with MA is not required for virus budding. HIV gag protein (cytoplasmic protein) become myristoylated, independently interacts with lipid raft and bud efficiently in the absence of HIV env protein producing VLPs. However, whether these VLPs containing Gag only have envelope with similar lipid raft composition as the virus env protein remains to be determined. It is known that budding of virus particles in polarized epithelial cells in the absence of env protein is random compared to basolateral budding of HIV in the presence of env protein. This would suggest that the presence of env proteins in lipid rafts and its interaction with Gag protein further facilitate the accumulation of Gag proteins in the lipid rafts leading Gag-Gag lateral clustering and multimerization. Furthermore, interaction of env with Gag protein may prevent internalization and promote budding. Unlike the assembly and budding of C-type viruses which occur only at the plasma membrane, the B- and D-type viruses, preassemble the viral core in the cytoplasm. A single mutation in the MA protein of Mason-Pfizer monkey virus (M-PMV; type-D retrovirus), abolishes cytoplasmic assembly and renders the mutant virus to assemble its core at the plasma membrane like the C type virus.

Although envelope proteins and the interaction of envelope proteins with matrix proteins facilitate budding of virus particles, matrix proteins of most viruses alone can become membrane-bound and bud efficiently. This is particularly true for VSV, a basolateral virus, which does not associate with typical lipid rafts. However, VSV virions possess a lipid composition which is different from that of the host plasma membrane. Therefore, it is likely that VSV G and M protein interaction as well as interaction among M proteins would lead to clustering of lipids binding to G proteins and formation of patches in the plasma membrane. These patches although different from typical lipid rafts as demonstrated by high TX-100 solubility have a high phosphatidic acid, phosphatidylserine, cholesterol, sphingomyelin and phosphatidyl ethanol amine (Luan *et al.*, 1995). G and M protein interaction may alter the lateral distribution of lipids in the plasma membrane. Furthermore, since VSV M may exist in phosphorylated and unphosphorylated forms, unphosphorylated forms are likely to favor lipid patch formation whereas phosphorylation of M leads to loss of lipid patch and dispersion of M into the membrane lipids.

As indicated above, plasma membrane proteins associate with lipid raft or affect the lipid order by interacting with the outer leaflet of the lipid bilayer. These protein-lipid interactions are mediated by GPI in integral membrane proteins without TMD or acyl modification of the amino acids in the TMD and cytoplasmic tail or by direct amino acid/lipid interaction with TMD. However, lipid rafts possess asymmetric distribution of lipids in both outer and inner leaflets of the lipid bilayer. It is known that in the host cell, a number of proteins in the cytoplasmic side interact with the inner leaflet prominently by the acyl groups such as acylated Src kinases. Viral proteins, particularly the matrix proteins comprising the protein component of the inner core can interact with the inner leaflet of the lipid bilayer. Some of these proteins like M1 of the influenza virus or the M protein of VSV and other negative strand viruses appear to directly interact with the inner leaflet of the lipid bilayer by protein/lipid interaction. For some viral matrix proteins, the lipid/proteins interactions are believed to occur by the hydrophobic domains or the exposed hydrophobic surface of the amphipathic helix or by the electrostatic interactions with the positive charges present in the matrix proteins. The lipid/protein interactions are however not lipid raft specific as determined by TX-100 resistance. Only when these matrix proteins interact with integral viral envelope proteins present in the lipid rafts, these matrix proteins become indirectly raft-associated (Ali *et al.*, 2000a). In many cases, these membrane-associated viral matrix proteins interact with each other, form complexes and bud as VLP. The lipid composition of the envelope of VLPs is not known and may aid in understanding the role of lipid raft in the budding process. Matrix proteins of some viruses like HIV and other retroviruses undergo acyl group modification, therefore interact with the inner leaflet of the lipid bilayer with the acyl groups. These matrix proteins like Src kinases are likely to interact with the inner leaflet of the lipid raft-like domain.

Gag protein (p55) of HIV has been shown to bud efficiently from the plasma membrane. p55 after proteolytic cleavage generates a number of proteins: MA (p17) – CA (p24) – p2 – NC (p7) – p6. Several reports have shown that Gag is targeted to lipid raft microdomains (Nguyen and Hildreth, 2000; Rousso *et al.*, 2000; Lindwasser and Resh, 2001; Ono and Freed, 2001; Zheng *et al.*, 2001; Holm *et al.*, 2003). MA is a homologue of the matrix proteins of other virus and possesses a M domain which undergoes myristoylation. However, the interaction of different protein components of p55 with inner leaflet of the lipid bilayer is complex. It has been reported that the M domain possesses determinants within its N terminal region which mediates Gag/membrane interaction. It has also been shown that the interaction domain (I domain) present in NC, functions as an assembly domain for Gag multiimer formation and is also involved in plasma membrane targeting of the Gag proteins (Sandefur *et al.*, 2000). Furthermore, the C terminal position of Gag is

also required from lipid raft-association (Lindwasser and Resh, 2001; Ono and Freed, 2001) suggesting that multimerization as well as myristoylation of Gag proteins are required for the assembly and association of Gag with lipid raft-like domains. However, it has been recently reported that although HIV Gag protein interacts with plasma membrane and forms raft-like detergent resistant barges, these microdomains are different from typical lipid rafts. These detergent resistant lipid/Gag protein complexes were much denser than lipid rafts and did not incorporate classical lipid raft proteins such as GPI-anchored PLAP and were not disrupted by cholesterol extraction. It appeared that Gag protein complexes did not function as a raft-patching agents from the cytoplasmic leaflet of the membrane since typical raft proteins were not associated with Gag associated raft-like membranes. However, it is possible that Gag binds to a different class of lipid rafts since the existence of the different classes of lipid rafts with varying protein and lipid composition has been reported (Röper *et al.*, 2000).

4.2.4.2. Bud formation and release

Bud formation and release require bending of membrane, incorporation of the cargo into the bud, growth of the bud and eventually, pinching off of the bud from the parent membrane. Bending of the membrane requires the pulling and pushing forces at the site of bud. Release of bud requires fusion of apposing membrane of the bud and the donor cell with each other causing fission of the bud from the donor cell and releasing of the particles into the environment. Although this overall mechanism and the physical forces for budding may be same, the contributing factors that govern these processes vary. Clearly, the requirements for budding intracellular transport vesicles are different from that for virus budding and virus release. Clathrin, COPI or COPII required for budding of transport vesicles are not involved in virus budding. In addition, the mechanism of fission of virus buds are also clearly different from that of intracellular transport vesicles. Although budding and vesicle formation go hand in hand, we will analyze these two processes separately.

*4.2.4.2.1. Formation of virus buds.* Bending of membrane is an absolute requirement for bud formation (Farsad and De Camilli, 2003). This will in general involve a transition from more planar structure to a curved structure. Although the physical forces that cause the curvature to occur at the budding site remains unclear, both lipids and proteins can contribute to causing the bending of membrane. Asymmetry in lipid bilayers can cause intrinsic curvature of one monolayer relative to other monolayer leading membrane bending. Selective transfer of lipids between the lipid bilayers, interaction of cholesterol into the budding leaflet as well as hydrolytic cleavage of phosphocholine head group of sphingomyelin by sphingomyelinase generating ceramides with smaller head

group have been proposed to cause membrane deformation and budding (Holopaineu *et al.*, 2000). Specific lipid microdomains such as lipid rafts have been shown to be a contributing factor in virus budding. Depletion of cholesterol, a major component for lipid raft formation, often suppresses virus budding indicating the role of lipid raft in bud formation.

In addition, to specific lipid microdomain, virus bud formation requires specific viral proteins. However, interaction TM viral proteins which partition with and stabilizes lipid rafts may not be sufficient for bud formation although their absence may affect budding partially. It has been observed that the expression of the TM viral proteins such as influenza HA, NA, VSV G or HIV env protein on the cell surface does not lead to either bud formation or extracellular release of bud containing these proteins. Similarly, other observations clearly suggest that lipid rafts with or without viral membrane proteins as well as the pulling force of glycoproteins are not sufficient for initiating bud formation. On the other hand, matrix proteins of many viruses often expressed alone are sufficient for bud formation and cause efficient release of buds such as VLPs. Matrix proteins which binds to the cytoplasmic leaflet of lipid bilayers, can cause bending of membrane and lead to generating the pushing force to initiate virus buds. Interaction of matrix proteins with each other at the cytoplasmic side will also mimick asymmetric lipid distribution and lipid to lipid interaction causing membrane bending. However, it is not clear if in these budding processes involving matrix protein alone, lipid microdomains play any role. Since it has been shown that matrix proteins of influenza and SV unlike that of HIV expressed alone do not interact with lipid rafts, it would suggest that lipid rafts are not required for virus budding to occur. However, lipid component of released particles containing matrix protein alone has not been analyzed to show whether budding of these VLP particles occurred from lipid microdomains or randomly from plasma membrane. In addition, cytoskeletal components like actin filaments, membrane tethered myosin motors also cause membrane deformation in the formation of cell surface tubular microvilli. However, their role in virus budding remains unknown at present.

*4.2.4.2.2. Release of virus particles.* Subsequent to bud formation, buds are released by a mechanism of fusion of the apposing membrane and fission of the bud from the cell. These processes determine the size and shape of the particles. It appears that a number of factors both virus and host may affect this process. Among the viral factors both glycoproteins as well as nucleocapsids and matrix proteins are critical components. Virus particles released in the medium can be either spherical or filamentous of varying sizes. Icosahedral nucleocapsids such as found with Semliki Forest virus clearly determines the spherical shape of the released virus particles. Similarly, helical VSV nucleocapsid is critical in determining the bullet shape and the length of

the virus particles. Defective interfering VSV particles contain smaller nucleocapsids which is responsible for producing small virus particles. These observations indicate that at least with these viruses, separation of virus buds from the host membranes depends on the cargo nucleocapsid and occurs immediately after the enclosure of the nucleocapsid. However, many viruses such as influenza viruses are flexible and pleomorphic and can produce spherical or filamentous particles. With these viruses, a number of factors may play critical roles in causing the fusion and fission processes and determining the size and shape of released virus particles.

Among the viral factors, matrix proteins as well as glycoproteins have been shown to affect virus shape and size. Deletion of cytoplasmic tail of HA and NA was shown to generate bizarre filamentous shape of virus particles. Reduced lipid raft-association of tail minus HA and NA was proposed to be the cause of such abnormal virus particles suggesting the role of lipid raft in both budding and fission of virus particles (Zhang *et al.*, 2000). Secondly, some influenza virus strains exhibited filamentous morphology and matrix proteins contributed to this strains specific filamentous shape (Roberts *et al.*, 1998; Bourmakina and Garcia-Sastre, 2003; Hui *et al.*, 2003). We have recently shown that influenza virus matrix protein like many other viruses possesses "late" domain (L domain) which affects fission of virus buds. It remain to be seen if this mechanism i.e. lack of fission caused by M1 L domain, was also responsible for filamentous form of A/Udorn virus (Roberts *et al.*, 1998).

In addition to viral factors, a number of host components have been shown to be involved in the budding process. A number of host proteins including ubiquitin, Tsg101, Vps4, Nedd4, and other members of vacuolar protein sorting pathway, which are involved in the formation multivesicular bodies in the endosome have been shown to be involved in the budding process of a number of virus (Luban, 2001; Perez and Nolan, 2001; Cimarelli and Darlix, 2002; Freed 2002). They possess WW domain and interact with the L domain of virus matrix protein. All of these host proteins in some way facilitate the fusion and fission process so that any defect in the interaction of these virus and host components will lead to defective or incomplete virus release, often forming multiple particles joined together. However, how the host proteins or host protein/M interactions will help in the process of fusion and fission is not clear. Furthermore, these particles were not completely filamentous or tubular but exhibited clover leaf-like structure suggesting incomplete fusion and fission. It will be interesting to determine if these particles represented a state similar to hemifusion in which only the inner leaflet undergoes fusion and therefore can not undergo complete fission and release from the host membrane and separation from each other.

In addition, cytoskeletal components particularly actin microfilaments have been shown to contribute to filamentous form of influenza virus (Roberts and Compans, 1998). It has been proposed that microfilaments which bind to

vRNP may provide outward pushing force in bud formation. However, if actin is involved the budding process, the fusion of membrane in the bud and fission of particles will require disassembly of actin filaments at the last stage of the budding process. Enhanced release of virus particles from influenza virus-infected HeLa cells with actin disruptive agents (Gujuluva *et al.*, 1994) as well as increase in production of spherical over filamentous particles in influenza- and parainfluenza virus infected polarized MDCK cells (Roberts and Compans, 1998) support the role of actin in the budding process. Bud formation and virus release have been shown to be an energy dependent process requiring ATP (Hui and Nayak, 2001; 2002). However, specific role of ATP in the budding process remains to be elucidated.

Finally, lipid rafts can affect both bud formation and fusion and fission process at multiple steps. As indicated earlier, asymmetry in the lipid bilayer can cause membrane curvature leading to the formation of buds. Assembly of lipid rafts at the budding site will affect physical properties of the membrane including lipid heterogeneity, lipid/protein interaction, increased viscosity and rigidity, slow diffusion. Presence of lipid heterogeneity could cause increased budding, increased fission and release of buds. For example, if one leaflet of the lipid microdomain is in raft and the other in nonraft state, one leaflet of the lipid microdomain will be higher in density and viscosity. This would lead to the formation of curvature provided the asymmetry in lipid leaflets are stably maintained by interaction with proteins in and around the raft. Heterogeneity in viscosity in the budding site due to the presence of both raft and nonraft lipids will facilitate fusion and fission. However, although we know that many viruses bud from lipid raft microdomain and possess lipid raft microdomain in their envelope, we do not know about the topology of microheterogeneity in lipid composition, viscosity in budding site for assessing the role of lipid raft, specifically in the budding process itself such as membrane bending, bud growth, fusion and fission leading to bud release. However, it is evident that lipid raft plays an important role in the budding process since destabilizing lipid raft by depleting cholesterol affects virus budding.

## 5. CONCLUSION

Lipid microdomains, particularly lipid rafts, play important roles in many aspects of virus biology including receptor binding, entry, uncoating including fusion, RNA replication, protein transport, protein sorting, protein targeting, budding, fusion, fission and release of buds as well as maintaining the integrity and stability of virus envelope and virus structure. Furthermore, lipid rafts provide the critical elements for signal transduction by many viral components. However, specific aspects and functions of lipid rafts in virus biology

are poorly understood. One of the problems in defining the association and involvement of lipid rafts in virus biology is the heterogeneity among the membrane lipid rafts as determined by varying TX-100 insolubility. It will be therefore important to define the classes of lipid rafts and determine the association of a specific class of lipid rafts with a specific viral membrane and viral function. Secondly, lipid rafts, on the one hand, increase selective inclusion of viral proteins and exclusion of nonviral host components from the budding site whereas, on the other hand, they provide platforms for pseudotype formation by bringing the foreign proteins to the budding site and thereby facilitating their incorporation in viral envelope. The latter property of lipid rafts aids in pseudotype formation and will be an important area of research for targeting enveloped virus vectors to specific cells and tissues either by broadening or narrowing their host specificity. Finally, involvement of lipid rafts in virus replication may provide a novel antiviral approach. Topical application of β-CD, a lipid raft destabilizer, shows the promise of antiviral effect in HIV transmission (Khanna *et al.*, 2002) possibly by preventing virus entry and affecting virus budding and virus structure as well.

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