Studying Spatial Distributions of Influenza Hemagglutinin on the Plasma Membrane of Fibroblasts: A Work in Progress

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Summary: The major envelope protein of influenza virus, hemagglutinin (HA), mediates the fusion of virus to cell for infection, and can mediate cell-cell fusion. It has been studied as a "raft" protein, as it is found in detergent-resistant membranes (DRM) and trafficks apically in polarized epithelia. Moreover, the viral envelope of influenza itself is rich in sphingomyelin and cholesterol. Using both immunogold electron microscopy and fluorescence resonance energy transfer (FRET) microscopy, we are examining the distribution of HA on the surface of fibroblasts expressing wild-type HA.

Keywords: cholesterol; fibroblasts; FRET; microdomains; raft; sphingomyelin

The complexities of biomembrane structure are of great interest because membranes are involved ubiquitously in biological function. In particular, the spatial organization of membrane lipids and proteins into sub-micron sized domains enriched in cholesterol, sphingolipids, and saturated lipids ("rafts") is controversial because of the paucity of methods to visualize membrane domains directly on appropriate length scales (Anderson and Jacobson, 2002a). Detergent-insolubility at low temperatures has been used as the operational definition of "raft" constituents (Brown and London, 2000) but also provides, at best, only indirect information. Nonetheless, there is a correlation between detergent-insolubility of proteins and many biological functions: assembly and budding of HIV, Ebola, and influenza (Ali et al., 2000; Bavari et al., 2002; Nguyen and

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Hildreth, 2000; Ono and Freed, 2001; Scheiffele et al., 1999; Zhang et al., 2000) appear to occur in or depend on microdomain lipids, and membrane fusion catalyzed by certain proteins such as influenza hemagglutinin (HA), appears to be disrupted by removal of membrane cholesterol by methyl-β-cyclodextrin (MβCD) (Sun and Whittaker, 2003). These effects are thought to result from redistribution of the HA and lipid after disruption of putative "raft" domains which presumably restrict lateral diffusion and thereby maintain a high local concentration of HA (Takeda et al., 2003).

Microdomains are also proposed to play a role in protein sorting and trafficking, cell signaling, toxin and pathogen binding, and potentially the faulty steps that lead to protein aggregation in Alzheimer's disease (Brown and London, 2000; Simons and Ikonen, 1997). Extraction of cholesterol and lipids by MβCD treatment has been shown to disrupt aggregation and function of microdomain-associated proteins such as Lyn kinase and its signal transduction pathway with the IgE receptor (Sheets et al., 1999). However, the unperturbed structure of membrane microdomains has not yet been visualized, nor have many of their physical properties such as lipid phase state been established in living cells at physiological temperatures. Visualization and physical characterization of such domains will almost certainly help illuminate how they perform their numerous biological functions.

The structure of membranes on the molecular level is clearly far more complex than the traditional view of the fluid mosaic model.(Simons and Ikonen, 1997; Singer and Nicolson, 1972) However, current models do not find a consensus on membrane organization (Anderson and Jacobson, 2002b; Brown and London, 2000; Edidin, 2001; Edidin, 2003; Fujiwara et al., 2002; Kwik et al., 2003; Maxfield, 2002; Sharma et al., 2004; Simons and Ikonen, 1997); how proteins and lipids orchestrate the large number of biological functions that occur in cellular membranes, on what spatial scale, and in what lipid phase various functions are performed, remains largely unclear. Distinction among these various models would provide fundamental insight into how cells and viruses organize their membranes.

The *lipid shell* model (Anderson and Jacobson, 2002b), similar to the boundary lipid model introduced in the late 1970s (Moore et al., 1978), depicts lipids preferentially associating with certain proteins in a layer of a few molecules (~7 nm) in thickness. Thus, rafts would be defined by direct interactions between proteins and their nearest protein or lipid neighbors, and would constitute a small total number of molecules. The *fluid phase* raft model depicts liquid ordered

membrane domains (Brown and London, 2000; Ge et al., 1999; Ge et al., 2003; Maxfield, 2002; Munro, 2003; Rietveld and Simons, 1998) composed of lipids enriched in cholesterol and sphingomyelin which preferentially associate with each other and certain proteins (Brown and London, 2000; Ge et al., 1999). In this model, raft-associated proteins and lipids partition preferentially into raft (liquid ordered) domains (Brown and London, 2000; Harder and Simons, 1997; Rietveld and Simons, 1998). However, the proteins may not necessarily bind lipid molecules directly, in contrast with the lipid shell model. For example, it has been proposed that glycosyl-phosphatidyl-inositol (GPI)-linked proteins may be largely unclustered within domains despite preferential partitioning into those domains (Brown and London, 2000). In this model, diffusion or flow of proteins within fluid domains may occur independently of other proteins and lipids. The *solid phase* raft model depicts solid crystalline (also called gel) phase (Munro, 2003) membrane domains which consist of preferentially associating lipids, also enriched in cholesterol and sphingomyelin, in which certain proteins are preferentially soluble. However, solid phase domains are not expected to allow relative motion of domain constituents and have been largely dismissed as incompatible with cellular function (Brown and London, 2000) and with changes in lateral mobility of membrane constituents (Kenworthy et al., 2004). The *picket fence* model (Fujiwara et al., 2002; Nakada et al., 2003) depicts proteins and lipids as free to diffuse laterally within domains contained by barriers composed of stationary membrane proteins, but confined by these barriers on millisecond timescales. Traversal of the barriers ("hopping") is possible but may require multiple attempts. On the other hand, membrane heterogeneity may be maintained by continual site-dependent *endo- and exocytosis*, rather than by specific lateral interactions between membrane components(Edidin, 2003). Finally, it is possible that a combination of the above (*multiple domain models*)(Maxfield, 2002) best describes the organization of cell membranes.

Recently, we have focused our attention on testing the predictions of the fluid domain raft model for several reasons: 1. the fluid domain raft model has recently received considerable attention in studies of cell membranes and biomembrane models (Baumgart et al., 2003; Brown and London, 2000; Rietveld and Simons, 1998; Veatch and Keller, 2002; Veatch and Keller, 2003); (Rietveld and Simons, 1998) 2. the domains of HA we observe (see below) are much larger than a few times the molecular dimension, eliminating domain models which predict organization exclusively on molecular length scales 3. fluid domains are expected to have distinct, testable

properties, including rounded boundaries, preferential partitioning of certain proteins and lipids into those domains, and a uniform density of constituents within the domains. We are in the process of testing the predictions of the numerous raft models described in literature, and in particular the fluid phase raft domain model, for consistency or inconsistency with our observations of the lateral organization of HA on fibroblast cell plasma membranes.

The lipid-dependent clustering of HA in cellular membranes has been implicated previously by detergent-extraction studies (Melkonian et al., 1999; Tatulian and Tamm, 2000) and electron microscopy (Takeda et al., 2003). However, mutations in HA which alter its fractionation after detergent treatment (*i.e.* DRM or "raft" association) (Melkonian et al., 1999) do not necessarily reduce fusion competence (Melikyan et al., 1997), and microdomain association does not depend on viral infection (Skibbens et al., 1989). Thus the connection between microdomain association and function could be clarified by direct one-dimensional and (especially) two-dimensional visualization of the clusters. For example, if the function of microdomain association is to assist influenza virus in exiting from an infected cell, as has been suggested in the case of vesicular stomatitis virus glycoprotein (Brown and Lyles, 2003), the fusion-associated domain could function independently. Hence, lateral clustering of HA may play a role in its infectivity at two points in its life cycle: 1. membrane fusion which mediates viral entry into the host cell, 2. concentration of HA and other viral proteins into domains which will eventually bud from the cell as new viruses. Thus, we propose that the transmembrane domain sequence of HA may have evolved to optimize the protein and lipid composition of the viral envelope, which is formed in the cell membrane prior to budding. We suggest that the mechanism of concentration of viral proteins by rafts (see above) may be widespread in enveloped viruses, since other viral glycoproteins have been shown to aggregate into 100-150 nm membrane domains that might serve as viral budding sites (Brown and Lyles, 2003; Takeda et al., 2003).

Here, electron microscopy has proven useful because it provides access to crucial nanometer length scales (Fig. 1). We are currently determining whether HA expressed on fibroblast cell plasma membranes is distributed randomly or non-randomly, to determine whether the distribution is lipid-dependent, and to determine which models of membrane "raft" organization are consistent or inconsistent with the observed distribution. Because constraint of lipid motion is also closely linked to the steps leading to fusion of influenza virus with host cells (Chernomordik

et al., 1998), the question of the spatial distribution of HA on cell surfaces has many important consequences.

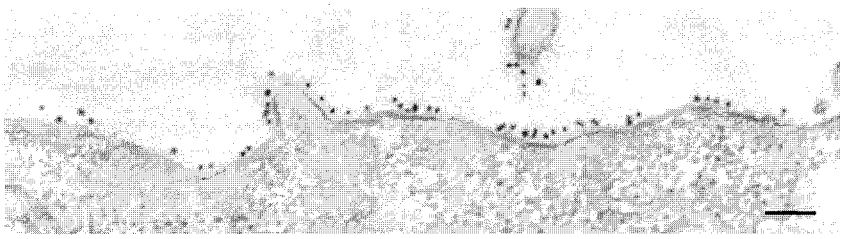

Fig. 1. HAb2 fibroblast immunostained for HA with 10nm goat anti mouse secondary antibody and then fixed. Bar = 10nm.

Quantitative methods exist for analysis of arbitrary particle distributions and can extract information about inter-particle interactions (Prior et al., 2003; Ripley, 1977; Ripley, 1979). Quantification of clustering using multiple types of statistical analysis is crucial since many factors can mislead the eye, and each statistical method will have its strengths and weaknesses. For example, a reduction in the antibody binding probability (capture ratio) can erroneously appear to suggest a reduction in clustering and significantly affect the shape and peak of the frequency distribution of nearest neighbor distances, even when the pattern of clustering has not actually changed. We are currently unifying a number of useful methods from diverse fields to probe the spatial pattern of clustering of influenza HA and its lipid-dependence. Furthermore, many of these methods are applicable to both one-dimensional (serial section) and two-dimensional (*en face*) visualizations of particles. *En face* images typically have the advantage of larger numbers of particles given the same linear dimension of the visualized membrane patch, and provide additional types of information such as biases in the angular orientation of particles and their neighbors. In particular, the methods we have chosen are relatively model-independent and allow for the possibility of either a random or a clustered membrane distribution. We hope to distinguish between membrane models which result in uniform density within domains due to preferential partitioning of molecules into those domains, and models of domains resulting from molecular aggregation by direct molecule-molecule interactions.

This approach may help determine if the measured HA distribution is consistent or inconsistent with partitioning of trimers into domains with uniform intra-domain density, which will help to explain the general inconsistency within the raft field in reported domain sizes, depending on the specific experimental method and its corresponding, accessible range of length scales (Anderson and Jacobson, 2002b). For such purposes, electron microscopy is advantageous compared to other methods which probe a narrower range of length scales, such as fluorescence resonance energy transfer (FRET) which can access from 1 to ~10 nm, or confocal microscopy, which is barely capable of resolving such domains, which are apparently smaller than <1 μm in lateral dimension in most cells at physiological temperatures. The ability of immunogold electron microscopy to probe such a wide range of length scales (~10 nm to > 1 μm) places it in an unusual category of techniques which bridge between the molecular and the microscopic regimes. The combination of immunoelectron microscopy with FRET can yield relevant information over more than three orders of magnitude in length (~10-9 m to 10-6 m). This is exactly the range of distances that will be important in unraveling the various contributions of protein-protein and protein-lipid energetics to microdomain-based augmentation of enzyme kinetics, signaling cascades, and pathogenesis by infectious agents.

[1] Ali, A., R.T. Avalos, E. Ponimaskin, and D.P. Nayak. 2000. *In* J Virol. Vol. 74. 8709-19.
[2] Anderson, R.G.W., and K. Jacobson. 2002a. Cell biology *Science*. 296:1821-1825.
[3] Anderson, R.G.W., and K. Jacobson. 2002b. *Science*. 296:1821-1825.
[4] Baumgart, T., S.T. Hess, and W.W. Webb. 2003. *Nature*. 425:821-824.
[5] Bavari, S., C.M. Bosio, E. Wiegand, G. Ruthel, A.B. Will, T.W. Geisbert, M. Hevey, C. Schmaljohn, A. Schmaljohn, and M.J. Aman. 2002 *In* J Exp Med. Vol. 195. 593-602.
[6] Brown, D.A., and E. London. 2000. 275:17221-17224.
[7] Brown, E.L., and D.S. Lyles. 2003. *Journal of Virology*. 77:3985-3992.
[8] Chernomordik, L.V., V.A. Frolov, E. Leikina, P. Bronk, and J. Zimmerberg. 1998. *Journal of Cell Biology*. 140:1369-1382.
[9] Edidin, M. 2001. 11:492-496.
[10] Edidin, M. 2003. *Nat Rev Mol Cell Biol*. 4:414-8.
[11] Fujiwara, T., K. Ritchie, H. Murakoshi, K. Jacobson, and A. Kusumi. 2002. *Journal of Cell Biology*. 157:1071-1081.
[12] Ge, M., K.A. Field, R. Aneja, D. Holowka, B. Baird, and J.H. Freed. 1999. *Biophysical Journal*. 77:925-933.
[13] Ge, M., A. Gidwani, H.A. Brown, D. Holowka, B. Baird, and J.H. Freed. 2003. *Biophys. J.* 85:1278-1288.
[14] Harder, T., and K. Simons. 1997. *Current Opinion in Cell Biology*. 9:534-542.
[15] Kenworthy AK, B.J. Nichols, C.L. Remmert, G.M. Hendrix, M. Kumar, J. Zimmerberg, and J. Lippincott-Schwartz. 2004. *J. Cell Biol.* Jun 7;165(5):735-46.
[16] Kwik, J., S. Boyle, D. Fooksman, L. Margolis, M.P. Sheetz, and M. Edidin. 2003. Membrane cholesterol, lateral mobility, and the phosphatidylinositol 4,5-bisphosphate-dependent organization of cell actin. *Proc Natl Acad Sci U S A*. 100:13964-9.
[17] Maxfield, F.R. 2002. *Current Opinion in Cell Biology*. 14:483-487.
[18] Melikyan, G.B., H. Jin, R.A. Lamb, and F.S. Cohen. 1997. *Virology*. 235:118-128.
[19] Melkonian, K.A., A.G. Ostermeyer, J.Z. Chen, M.G. Roth, and D.A. Brown. 1999. *Journal of Biological Chemistry*. 274:3910-3917.
[20] Moore, B.M., B.R. Lentz, and G. Meissner. 1978. *Biochemistry*. 17:5248-55.
[21] Munro, S. 2003. *Cell*. 115:377-388.
[22] Nakada, C., K. Ritchie, Y. Oba, M. Nakamura, Y. Hotta, R. Iino, R.S. Kasai, K. Yamaguchi, T. Fujiwara, and A. Kusumi. 2003. *Nature Cell Biology*. 5:626-U3.
[23] Nguyen, D.H., and J.E. Hildreth. 2000. *In* J Virol. Vol. 74. 3264-72.
[24] Ono, A., and E.O. Freed. 2001. *Proc Natl Acad Sci U S A*. 98:13925-30.
[25] Prior, I.A., C. Muncke, R.G. Parton, and J.F. Hancock. 2003. *Journal of Cell Biology*. 160:165-170.
[26] Rietveld, A., and K. Simons. 1998.. *Biochimica et Biophysica Acta (BBA) - Reviews on Biomembranes*. 1376:467-479.
[27] Ripley, B.D. 1977. *Journal of the Royal Statistical Society Series B-Methodological*. B39:172-192.
[28] Ripley, B.D. 1979. *Journal of the Royal Statistical Society Series B-Methodological*. 41:368-374.
[29] Scheiffele, P., A. Rietveld, T. Wilk, and K. Simons. 1999. *Journal of Biological Chemistry*. 274:2038-2044.
[30] Sharma, P., R. Varma, R.C. Sarasij, Ira, K. Gousset, G. Krishnamoorthy, M. Rao, and S. Mayor. 2004. *Cell*. 116:577-89.
[31] Sheets, E.D., D. Holowka, and B. Baird. 1999. *Journal of Cell Biology*. 145:877-887.
[32] Simons, K., and E. Ikonen. 1997. *Nature*. 387:569-572.
[33] Singer, S.J., and G.L. Nicolson. 1972. *Science*. 175:720-731.
[34] Skibbens, J.E., M.G. Roth, and K.S. Matlin. 1989. *Journal of Cell Biology*. 108:821-832.
[35] Sun, X., and G.R. Whittaker. 2003.. *In* J Virol. Vol. 77. 12543-51.
[36] Takeda, M., G.P. Leser, C.J. Russell, and R.A. Lamb. 2003. *Proc Natl Acad Sci U S A*. 100:14610-7.
[37] Tatulian, S.A., and L.K. Tamm. 2000. *Biochemistry*. 39:496-507.
[38] Veatch, S.L., and S.L. Keller. 2002. *Physical Review Letters*. 89:art. no.-268101.
[39] Veatch, S.L., and S.L. Keller. 2003. *Biophysical Journal*. 84:725-726.
[40] Zhang, J., A. Pekosz, and R.A. Lamb. 2000. *In* J Virol. Vol. 74. 4634-44.