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Biochimica et Biophysica Acta 1610 (2003) 208–216
BIOCHIMICA ET BIOPHYSICA ACTA
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Review

Structural information about organized cholesterol domains from specific antibody recognition

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Received 11 April 2002; received in revised form 31 October 2002; accepted 31 October 2002

Abstract

Cholesterol-rich domains have been observed to exist in cell membranes under physiological and pathological conditions. Their compositions and the microenvironment of their formation vary over a wide range. Very little information is however available on the molecular structure and organization of these domains. The techniques available to provide such structural information are reviewed here first. The possibility of using tailor-made antibodies as reporters of molecular organization in membranes is then considered. The concept of antibodies recognizing molecular organization rather than single molecular epitopes is established, reviewing the existing works on antibody and protein recognition of crystalline molecular arrays. The information that such antibodies could provide in cells is finally examined together with a proof of application.

Keywords: Cholesterol-rich domain; Antibody; Molecular recognition; Cell membrane; Structure

1. Statement of the problem

Until the late 1990s, the classical 'fluid mosaic' model was accepted as the best description of the molecular organization in cell membranes. In this model, phospholipids and cholesterol molecules constitute the tassels of a disordered mosaic, in which proteins are embedded, homogeneously distributed and freely diffusing. The lipid bilayer was conceived as a neutral two-dimensional solvent, which does not interact with the proteins dissolved in it, and only functions to separate internal from external space.

The possibility of segregation of cholesterol-rich domains of different composition had been considered in the 1980s to explain the distribution of cholesterol in the membranes of eye lens fiber cells, where the cholesterol/phospholipid ratio reaches values as high as 4 [1]. Such a high ratio required reconsideration of the fluid mosaic model because cholesterol is immiscible with phospholipids in those ranges of composition. Eye lens fiber cells are specialized, terminally differentiated cells, and the formation of domains in their membranes may not be representative of other cells. The basic idea of segregation of distinct domains in the plasma membrane was however supported by studies performed on epithelial cells [2]. The plasma membrane of these cells is polarized into apical and basolateral domains, with the former domain being enriched in sphingolipids, while the latter is enriched in phosphatidylcholine.

Once evidence for the existence of organized cholesterol-rich domains in other cells started to accumulate, the classical 'fluid mosaic' model was definitely questioned. In 1997, Simons and Ikonen [3] summarized ideas that had been evolving for a number of years, establishing a new concept of membrane organization. The central tenet is that sphingolipids and cholesterol cluster dynamically to form rafts that move within the lipid bilayer as distinct units. It was proposed that these rafts function as platforms for the attachment of proteins when membranes are moved around inside the cell and during signal transduction.

Involvement of rafts has been implied, to date, in all kinds of physiological and pathological processes including signal transduction during the allergic immune response and in the assembly of immunological synapses [4]. The formation of amyloid plaques in Alzheimer disease [5], HIV and other retrovirus assembly and release [6], sperm capacitation [7]

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0005-2736/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0005-2736(03)00019-1

and platelet activation [8] are just a few other examples where raft involvement has been indicated. The suggested structural model of lipid rafts involves a liquid ordered assembly of sphingolipids associated laterally with one another, with the voids between the large head-groups filled by cholesterol molecules. The latter function as spacers between the predominantly saturated lipid chains, which have a lower cross-sectional area relative to the large sphingolipid heads [3,9]. Proteins anchored to saturated lipids (such as GPI), specific transmembrane proteins or proteins that carry hydrophobic modifications partition into rafts owing to preferential packing of their membrane anchors. The fluid regions in between the close packed sphingolipid–cholesterol clusters are occupied by phospholipids.

To date, however, there is no direct structural information to confirm or disprove the raft model. It would be advantageous to determine some molecular parameters of the component arrangement within the domains. Do all lipid rafts have the same molecular organization, and what is this organization? Are the components juxtaposed in any regular alternation in the plane of the membrane? Is the distribution of the components within one domain homogeneous? These are only a few questions that come to mind, which are essential to our understanding of the functioning of lipid rafts and cholesterol-rich domains in general. Once accepted that cholesterol is not homogeneously distributed in cell membranes, one would expect a spectrum of different organization motifs, dependent on cell or domain function, on cholesterol concentration and on the physiological or pathological conditions which the cell is experiencing.

The purpose of this report is to review the existing techniques that have been used or potentially can provide structural information about organized cholesterol-rich domains in cell membranes. In particular, the use of specialized antibodies that are capable of two-dimensional and three-dimensional structural pattern recognition will be considered.

2. Available techniques for structural characterization of cholesterol-rich domains in membranes

The number of examples where organized cholesterol-rich domains were found in cell membranes has enormously increased within the last few years. Thus, only some indicative examples will be mentioned here for the purpose of illustrating the various techniques and the information that can be derived from them. No attempt will be made at a comprehensive review of the subject, which is much more effectively described in the various manuscripts in this issue. Moreover, all types of cholesterol-rich domains will be examined within the same framework. Thus, atherosclerotic cells or eye lens fiber cells, and cholesterol/sphingolipid rafts will be considered side by side, disregarding the differences in composition or the specific conditions of formation. This is because the conceptual difficulties in determining structural parameters within membranes in living cells are expected to be basically similar, even in very different environments.

Sphingolipid–cholesterol rafts were first detected because they are insoluble in the detergent Triton X-100 at 4 °C. Because of their high lipid content, these detergent-insoluble, glycolipid-enriched complexes float to a low density during gradient (sucrose) centrifugation, together with any associated proteins. The proteins are thus identified as raft-associated. These same proteins, once identified as raft-associated, were subsequently exploited to study the spatial distribution, the size and the properties of rafts through the clustering of fluorescence, gold or other immunolabels. A wide range of imaging techniques was used to study rafts [4], including fluorescence spectroscopy, transmission electron microscopy (TEM), near-field scanning optical microscopy (NSOM), atomic force microscopy (AFM) and photonic force microscopy. Each technique has advantages and disadvantages; each provides information at a different resolution. A few examples are given below.

Varma and Mayor [10] used fluorescent resonance energy transfer (FRET) between GPI-anchored proteins in living cells to show that these proteins are clustered in lipid domains likely to be smaller than 70 nm, thus explaining why they cannot be detected by light microscopy.

Friedrichson and Kurzchalia [11] showed that GPI-anchored proteins exist in clusters by using short cross-linkers. Harder et al. [12] used antibodies against raft-associated proteins to co-cluster the small rafts into super rafts that could be detected by light microscopy.

In photonic force microscopy [13] a laser trap is used to confine the motion of a bead bound to a raft protein through an antibody. The bead's fluctuations inside the trapping potential are tracked with sub-nanometer and microsecond resolution by high-resolution single particle tracking. The method measures the viscous damping of the membrane domain in the lipid bilayer. Considering that raft-associated protein diffusion is significantly reduced compared to that of non-raft proteins, the measurements agree with lipid rafts being cholesterol-stabilized complexes of 26–40 nm in diameter diffusing as one entity for minutes.

Signaling molecules were localized on the cytoplasmic face of native membranes by immunogold labeling and high-resolution TEM [14]. The high affinity IgE receptor, which is clustered in rafts, and two of its associated tyrosine kinases were mapped in membrane sheets prepared from mast cells. One of the kinases was found associated in small clusters, co-localized with small- and medium-sized clusters of receptor. The other kinase is dramatically recruited in large clusters of the receptor. TEM measurements may thus in principle provide some direct information about the organization of micro- or nanocrystalline domains in cell membranes. The option of performing cryo-microscopy in vitrified samples would furthermore guarantee the absence of artifacts due to drying of the biological membrane.

NSOM [15] exploits the near-field interaction of light with substrates to image surfaces at resolutions of tens of nanometers, well beyond the resolution of conventional light microscopy. NSOM was applied to image the plasma membrane of skin fibroblasts, using a fluorescently labeled phospholipid analog and fluorescent antibodies to membrane proteins. Both fluorophores showed the presence of distinct patches, tens to hundreds of nanometers in size, in fixed and dried cell membranes.

AFM provides topographic images of surfaces of materials as soft as cells, under liquid, with a lateral resolution of up to 10 nm. AFM recently provided an interesting contribution to the understanding of the artifacts possibly arising from detergent extraction of rafts from cell membranes [16]. Observed in situ after Triton X-100 treatment, the detergent-resistant plasma membrane (DRM) fragments form domains whose size exceeds 15–20 μm^2, orders of magnitude larger than the largest domains observed in living cells, strongly suggesting that membrane microdomains rearrange into larger DRMs during Triton X-100 treatment.

Ocular lens cell plasma membranes were shown to contain immiscible cholesterol domains, using small angle X-ray diffraction [17]. Small angle X-ray diffraction allows the detection of relatively large (nanometer scale) periodicity in oriented structures. The periodicity of 3.4 nm, characteristic of the cholesterol monohydrate unit cell, was observed in ocular lens cell plasma membranes. Spanning the long dimension of two cholesterol molecules, it coincides with the thickness of the membrane in the direction perpendicular to the bilayer. This demonstrates that there are domains in the membrane with structure akin to that of cholesterol monohydrate crystal structure, at least in that direction. The domains are thus not only cholesterol-rich, but also close to crystalline, if not fully crystalline.

The same technique was applied to study the plasma membrane of smooth muscle cells in cholesterol-fed rabbits, also showing a periodicity of 3.4 nm [18], and consequently the presence of crystalline or almost crystalline cholesterol domains. This is the only technique, among those cited above, that provided until now real structural information from in vivo or ex vivo cell membranes.

Clearly, none of these techniques provides any structural characterization of the lateral organization of the components within the cholesterol-rich domains.

Much information, albeit indirect, was derived from studies performed on membrane models in vitro [19–21]. Much of this is described in other manuscripts in this issue (Bach and Wachtel, this issue; McConnell, this issue), and will thus not be detailed here. Only the studies that potentially contain structural information at the molecular level will be mentioned below.

AFM was used to provide images of cholesterol/sphingomyelin domains in supported bilayers at nanometer resolution [22]. The technique can, in principle, provide images at molecular resolution of crystalline layers.

Another potentially interesting technique that has been applied in a proof-of-concept type of study is time-of-flight imaging secondary ion mass spectrometry (TOF-SIMS) [23]. It involves a unique development of mass spectrometry for molecule-specific imaging of frozen hydrated samples at micrometer resolution.

Neutron grazing incidence diffraction (GID) [24] was used to provide evaluations of the size of domains in model binary lipid mixtures. The information does not reach yet the molecular packing level. GID has, however, the very definite potential to provide molecular level information on the structural organization of thin films such as membranes. X-ray GID, combined with X-ray reflectivity, has been applied to determine the structure of phospholipid monolayers [25,26], and recently, the packing arrangement of a glycosphingolipid–phospholipid monolayer mixture [27]. The nucleation of crystalline films of cholesterol on water in the presence and absence of phospholipids has been monitored [28]. The crystallization of cholesterol at the air–water interface was recently described as the dynamic formation of an ordered trilayer of cholesterol with structure akin to that of cholesterol monohydrate, mediated through the transformation of other less ordered structures [29].

3. Specific antibodies can provide information on the organization of crystalline layers: proof of concept

Clearly, a complete in situ X-ray structure determination would be the ultimate goal for those interested in the molecular organization inside cholesterol-rich domains in cells. Such a goal is however well beyond our possibilities for various reasons: the concentration of the domains is low in most cells (not in eye lens cells or atherosclerotic cells), their size is in the tens of nanometers and their crystallinity may well be not high enough to provide interpretable diffraction signals. Furthermore, the biological milieu is often difficult to preserve during extraction procedures, and the X-ray radiation itself may modify the organization of the microdomains. Thus, reaching this ultimate goal will require much additional technical development.

With this realization, we have to consider other approaches, albeit less informative. One conceivable possibility is to create a battery of molds that are sensitive each to the molecular organization of one type of domain, by virtue of shape-complementarity and chemical complementarity to some or all the components. These ‘‘recognition patterns’’ might then be tested on the arrays, looking for one that fits. The information on the appropriate mold could then be directly translated into structural information on the domains (Fig. 1). With this idea in mind, we embarked 10 years ago in a series of studies using antibodies as the recognition tool and crystals as substrates.

It has been repeatedly shown, especially in biomineralization research, that specific structural recognition can occur between specialized proteins and targeted crystal surfaces

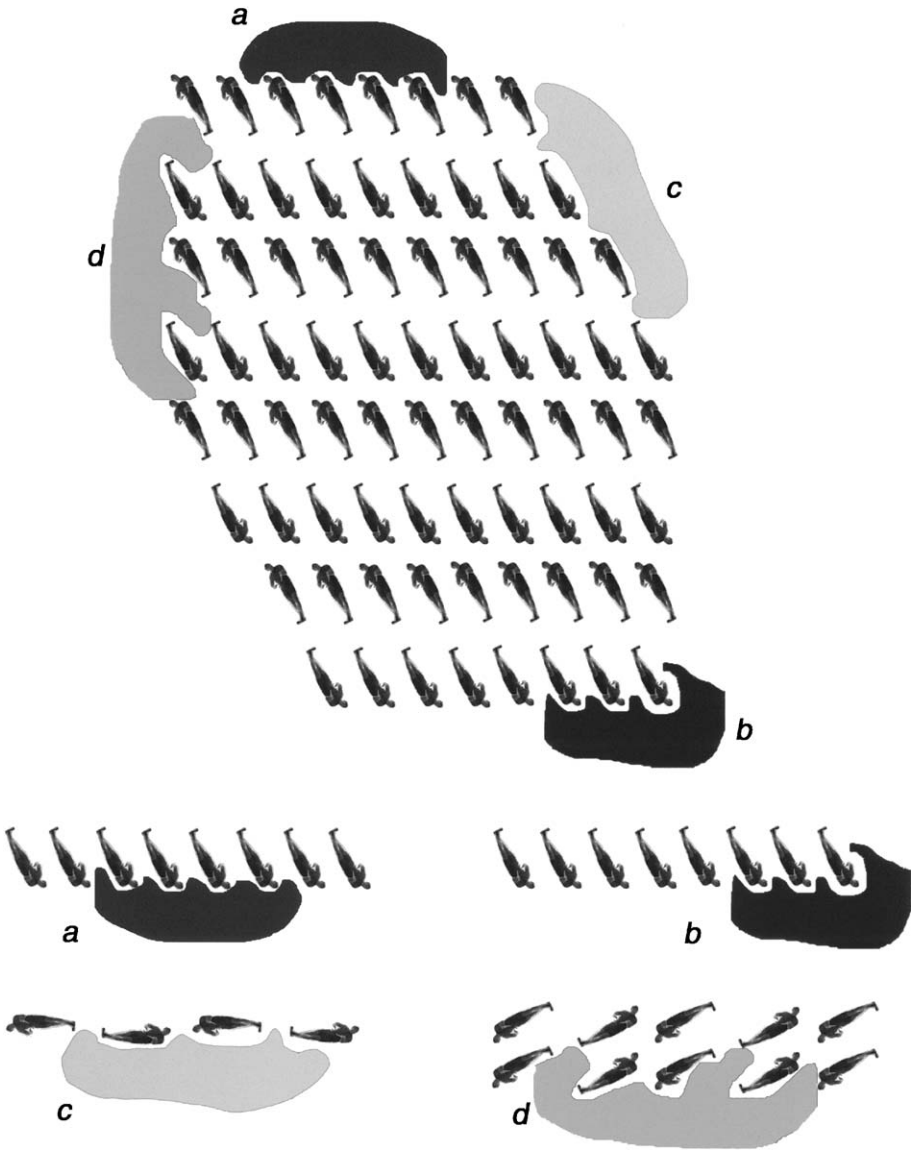

Fig. 1. Schematic representation of the possible complementarity between antibody variable regions (a, b, c, d) and organized crystal surfaces. The arrangement of the components in the crystal is inspired by the bilayer structure of cholesterol monohydrate. The recognition of the same antibody variable regions for the specified schematic arrangements is reproduced below, illustrating possible membrane arrangements with molecular organizations similar or identical to those delimiting the crystal faces.

[30,31]. Interestingly, the recently determined structures of an antifreeze protein from eel pout and one from a beetle proved to be highly complementary to the specific surfaces of ice crystals to which they bind [32,33]. Can a similar situation arise between antibodies and crystals in pathological conditions?

There is no good reason to assume that the immune system should relate to crystals in a manner different from other invaders because of their intrinsic nature as crystals. Antibody binding sites generally show a good geometrical and chemical fit to the surface domains of bound macromolecules. This surface-to-surface recognition may occur with any surface, including crystal surfaces. The substantial difference between conventional antigens, such as proteins, and crystal surfaces is that crystals are formed of small molecules that would not interact with antibodies in a dispersed solution. When organized in a three-dimensional array, their surfaces may well be expected to have characteristics akin to those of a macromolecular surface. In antibody complexes with protein antigens, the buried interfacial area typically ranges between 600 and 1000 $Å^2$ [34]. This would correspond, in a molecular crystal, to an array of 12–20 molecules with a cross section of 5×10 Å. The establishment of high affinity interactions would imply the recognition of a repetitive series of molecular moieties in the correct arrangement.

The use of specific antibodies as reporters of the structure of organized arrays of small molecules was initiated when we discovered in the synovial fluid of gout patients the presence of a population of antibodies that accelerated in

vitro the formation of crystals of monosodium-urate monohydrate (MSUM) [35]. To explain these data, MSUM crystals, which are symptomatically associated with the disease, were postulated to elicit a specific immune response, rather than the general inflammation that was believed to be involved in the response of the organism to the crystals.

In order to characterize the molecular nature of the antibody–crystal interactions, we subsequently isolated monoclonal antibodies that interact strongly with crystals of cholesterol monohydrate and dinitrobenzene [36], and recently of the tripeptide leucine–leucine–tyrosine [69].

Two of the antibodies that selectively interact with cholesterol monohydrate crystals were studied in depth [37]. One of these, antibody 36A1, was shown to interact preferentially with one set of faces in the cholesterol crystals. These faces are characterized by long hydrophobic stretches exposing the cholesterol backbones, separated by hydrophilic steps where water molecules and the 3β-hydroxyls of the cholesterol molecules emerge. The second antibody, 58B1, is highly specific to cholesterol monohydrate crystals. It interacts with more than one set of crystal faces, all exposing the hydrophobic backbone and the hydrophilic hydroxyls of cholesterol in various arrangements. Most importantly, neither antibody interacts or recognizes the isolated cholesterol molecule, which exposes a surface completely different from that exposed by the array of moieties at the cholesterol crystal surfaces. The variable regions of the two antibodies, as well as of antibodies specific to other crystals were sequenced, and the structures of their binding sites were modeled [38]. The model is highly reliable for antibody 36A1 because its problematic H3 loop is very short and rigid. It is less defined for antibody 58B1, which has an extremely long H3 loop and is consequently supposed to be more flexible. Docking of the step-shaped binding site model of antibody 36A1 on the molecular step of the recognized face resulted in an excellent geometrical and chemical match with cholesterol hydroxyls and hydrophobic cholesterol backbones, respectively (Fig. 2).

We subsequently showed that both antibodies interacting specifically with cholesterol crystals are able to recognize cholesterol arrays organized in the form of monolayers at the air–water interface, while antibodies that are not specific to cholesterol crystals do not [39]. In monolayers, the cholesterol molecules are aligned with each other in an arrangement geometrically akin both to that of cholesterol in the crystal structure and in cell membrane bilayers. Moreover, antibody 36A1 does not interact with monolayers of epicholesterol, showing fine stereochemical/structural discrimination between arrays of isomeric steroids [40]. The antibody affinity to the cholesterol monolayers is so high that labeling by the antibody is observed in equilibrium with a residual concentration in solution at least as low as 10^{-11} M [41]. It is evident that stereochemical recognition operates at interfaces of organized surfaces as well as, or even better than, on separated molecules.

Within the above conceptual framework, it is interesting to consider whether the anti-cholesterol antibodies reported in the literature are responsive to cholesterol molecules or to cholesterol arrays. Anti-cholesterol antibodies were first reported in 1988 by Swartz et al. [42], after immunizing mice with liposomes containing 71% cholesterol, together with lipid A as adjuvant. They were isolated by their ability to react with liposomes containing 71% cholesterol, but not with liposomes containing 43% cholesterol. The antibodies were reported to bind to cholesterol crystals [42]. Naturally occurring autoantibodies to cholesterol were subsequently reported in miniature pigs [43] and humans [44]. The apparent paradox of antibodies being raised against a self-molecule could be satisfied if the antibodies were generated not against isolated cholesterol molecules, but against molecular arrays that are not present at the time when the immune system is defined.

The concept of antibodies as reporters of molecular organization is assuming more and more credibility. In a

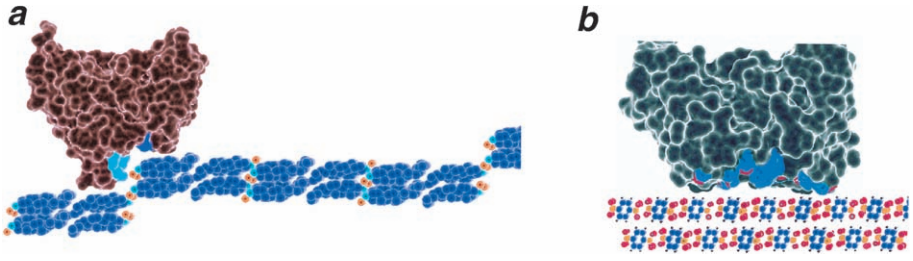

Fig. 2. Proposed docking models for (a) antibody 36A1 on the cholesterol monohydrate crystal surface that it recognizes. Blue, cholesterol backbone in the crystal; cyan, hydroxyl groups of cholesterol; orange, water molecules; brown, model of the antibody variable region, with the hydrophilic and hydrophobic regions in the binding-site color-matched to the respective epitope targets. (b) Antibody 122B1 on the dinitrobenzene crystal surface that it recognizes. Blue, aromatic ring; orange, N and red, oxygen of the nitro-group; grey, model of the antibody variable region with the aromatic and polar groups in the binding-site color-matched to the respective epitope targets. The shape complementarity is accompanied in both cases by chemical complementarity between the amino acid side-chains of the antibody and the functional groups of the recognized surfaces. The two models of antibodies, 122B1 and 36A1, are similar to the arrangements schematically represented in Fig. 1 by (b) and (a), respectively. An additional antibody, specific to crystals of the tripeptide leucine–leucine–tyrosine, has been found whose model is very similar to that represented in Fig. 1 by (d) [69].

very recent manuscript, O'Nuallain and Wetzel report the isolation of antibodies that bind an Alzheimer peptide in its amyloid fibril state, but not in its soluble, monomeric state [45].

In principle, one could thus imagine to generate different specific antibodies against epitopes of known organization, characterize them in the artificial system and then use them as reporters of different organizations of cholesterol and other components in cells, as suggested at the beginning of the section.

The extant question is whether the antibodies can be used in cells. The following section is a proof of application.

4. Application of specific antibodies to the detection of cholesterol-rich domains in cells

The application of anti-cholesterol monoclonal antibodies to the detection of cholesterol in cells could present new opportunities for learning about the trafficking and functioning of cellular cholesterol, besides its organization. Agents for the detection of cholesterol in cells include filipin, a polyene antibiotic, digitonin, a saponin, and thiol-activated cytolysin toxin polypeptides [46–51]. Each reagent binds to unesterified cholesterol, reacting with the 3β-hydroxyl group on cholesterol, and has been successfully used to localize cholesterol in cells. The use of digitonin is problematic in that the detergent properties of this agent tend to extract cholesterol from cells contemporaneous to binding it, forming insoluble crystalline complexes that can be identified with the electron microscope. Filipin has been a very useful probe of cellular cholesterol that can be visualized with both fluorescence and electron microscopy. Filipin shows extensive labeling of plasma membrane and intracellular cholesterol. However, filipin does not detect cholesterol in clathrin-coated regions of the plasma membrane, possibly due to steric hindrance from the clathrin coat on these vesicles [52].

Anti-cholesterol monoclonal antibodies have been successfully applied to label cholesterol in crystals, liposomes and plasma lipoproteins [42,53]. One limitation of anti-cholesterol antibodies in their possible use to label cholesterol in cultured cells is that while they are expected to label plasma membrane cholesterol, they cannot be readily used to label intracellular cholesterol. This is because immunoglobulin proteins do not penetrate an intact plasma membrane, as do the much smaller cholesterol probes filipin (m.w. 655) and digitonin (m.w. 1229). To allow penetration of antibodies, the plasma membrane must be permeabilized. This is usually done by extracting lipids from the plasma membrane with solvents such as acetone, ethanol, methanol or detergents such as Triton X-100. Of course, these permeabilizing agents will extract or significantly disturb cholesterol in the cell. However, it is possible to label cryosections prepared from frozen cells or tissue as a means to access the intracellular space without permeabilizing the cell. Recently, this has been done for the labeling of intracellular cholesterol with a cholesterol-binding toxin and an anti-cholesterol antibody [54,55].

The interaction of an anti-cholesterol antibody with cholesterol in the plasma membrane of a cell would be expected to be limited to one specific condition of cholesterol molecules. An antibody that reacts with a defined epitope on the cholesterol molecule would not be expected to easily detect cholesterol in the plasma membrane because there, cholesterol associates closely with phospholipids. It is thus likely that under most circumstances the cholesterol epitopes would not be exposed sufficiently to react with a nonpenetrating molecule such as IgM [56].

We found that antibody 58B1, which was selected for its reactivity with cholesterol monohydrate crystals, does not react with the surface of cultured cells such as macrophages and fibroblasts under normal conditions. It is only when the plasma membrane is enriched with cholesterol that this antibody shows reactivity with the cell surface [57]. Maximal cholesterol labeling occurs when fibroblasts or macrophages are incubated with low-density lipoprotein (LDL) or acetylated LDL, respectively, as a source of cholesterol. With this cellular cholesterol enrichment, antibody 58B1 shows labeling of the plasma membrane. These cells take up LDL and acetylated LDL by receptor-mediated endocytosis that transports these lipoproteins to lysosomes [58]. The lipoproteins are degraded in the lysosomes releasing unesterified cholesterol. Some cholesterol traffics to the plasma membrane and to intracellular membranes, the latter where acyl-CoA:cholesterol acyltransferase (ACAT) esterifies the cholesterol for storage in cellular lipid droplets [59]. Pharmacological inhibition of ACAT causes further buildup of unesterified cholesterol within intracellular and plasma membranes. With ACAT inhibition, the intensity of antibody 58B1 plasma membrane labeling and the number of cells labeled are greater than when cells are incubated with lipoproteins without ACAT inhibition.

The pattern of antibody 58B1 labeling is punctate (Fig. 3), suggesting the presence of cholesterol microdomains, the predicted epitope requirement for reactivity of this antibody. This staining pattern is very different from that of filipin, which shows a diffuse staining of the plasma membrane, consistent with the more widespread distribution of isolated cholesterol molecules that this smaller penetrating cholesterol probe can label. In addition to labeling of plasma membrane cholesterol microdomains, antibody 58B1 also labels small extracellular particles surrounding macrophages. These stained extracellular particles could be shed plasma membrane microdomains or other forms of cholesterol excreted by the cultured cells.

When labeling any antigen with antibody, it is necessary to show that the labeling is specific; in the present case, it is necessary to show that cholesterol microdomains are detected. Specificity of cholesterol staining can be inferred from a number of control tests. For antibody 58B1 labeling of cells [57], the labeling could be eliminated when cellular

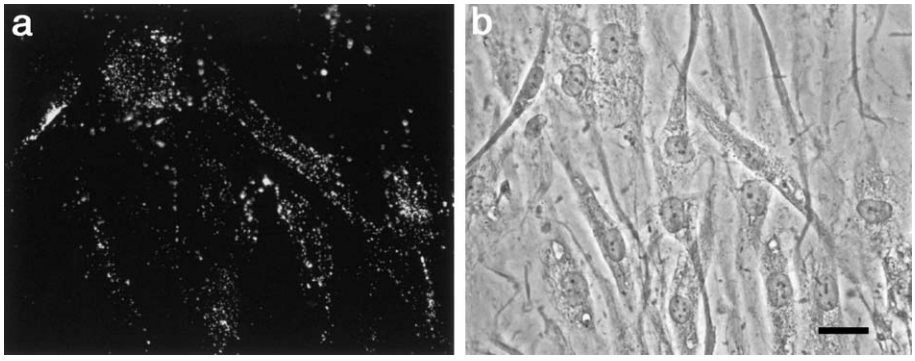

Fig. 3. Antibody labeling of plasma membrane cholesterol microdomains in cholesterol-enriched fibroblasts. Normal fibroblasts were grown following a procedure that enriches the fibroblasts with unesterified cholesterol, fixed, and labeled with anti-cholesterol antibody 58B1. A biotinylated secondary antibody coupled with fluorescently labeled avidin was used for detection. Fluorescence (a) and phase (b) photomicrographs of the same microscopic field are shown. Bar: 20 μm. Reproduced from Ref. [57].

cholesterol was removed from the plasma membrane by incubation with cholesterol acceptors such as HDL or cyclodextrin, or by extraction with organic solvents such as methanol. Oxidative modification of the 3-β-OH group on cholesterol by exposure of cells to cholesterol oxidase also eliminated labeling, presumably because of the importance of this group for preserving epitope structure [49,60,61]. Lastly, the cholesterol microdomains labeled by antibody 58B1 responded to agents that modulate cholesterol trafficking in living cells. Mainly, plasma membrane labeling was decreased when cells were treated with ketoconazole, an agent that impairs trafficking of cholesterol from lysosomes to the plasma membrane. Plasma membrane labeling also was decreased in mutant NPC fibroblasts with a known defect in trafficking of cholesterol from lysosomes to plasma membrane. Thus, antibody 58B1 detects a plasma membrane pool of cholesterol, responsive to agents that modulate cholesterol trafficking to and from the plasma membrane.

Not all antibodies raised and selected against cholesterol crystals may meet these types of specificity tests. Antibody 36A1, which has been characterized much more extensively (see Section 3), shows extensive labeling of cellular plasma membranes even when these membranes are not enriched with cholesterol. Concomitantly, cyclodextrin-mediated removal of cholesterol from the plasma membrane [57,62] did not eliminate labeling by antibody 36A1 (unpublished data). Neither did treatment of the test cells with cholesterol oxidase decrease antibody 36A1 labeling. Thus, we conclude that this antibody detects something, in addition to cholesterol, that occurs in the plasma membrane of cells. This behavior is in agreement with experiments performed on monolayers of triacontanol, an alcohol with a long saturated aliphatic chain, where labeling with antibody 36A1 was observed, albeit with a much lower affinity than cholesterol [41].

The exact structural nature of the plasma membrane cholesterol microdomains detected by antibody 58B1 remains to be determined. However, the characteristics of antibody 58B1 binding to cholesterol crystals and monolayers, but not to isolated molecules, guarantee that the recognized epitope is organized in crystalline-like microdomains [63]. Such cholesterol microdomains form in model membranes when the membranes are enriched with cholesterol [18,64–67]. However, the detergent-insoluble lipid microdomains enriched in sphingomyelin and cholesterol, associated with rafts and caveolae, presumably have a different structure. Accordingly, the cholesterol microdomains we detected in the plasma membrane with antibody 58B1 were sensitive to extraction with ice-cold 1% Triton X-100, while lipid rafts and caveolae are not extracted under these conditions. In conclusion, the conceptual procedure of applying antibodies, raised and isolated against crystals, to detect the same or similar arrangements in cells is operational, provided caution is applied in the interpretation of the results especially in relation to the uniqueness of the detected epitope.

Because of the epitope specificity of antibodies, the potential exists for the development of new monoclonal antibodies that can detect other pools of cholesterol within the plasma membrane. It may be possible to expand the reactivity of cholesterol antibody 58B1 with cells by enzymatically digesting phospholipids in the plasma membrane, thereby unmasking additional cholesterol labeling [68]. Antibody 58B1 should be generally useful for studying gene products that regulate cholesterol trafficking and efflux in cells. For example, because antibody 58B1-labeled cholesterol microdomains do not form in mutant NPC fibroblasts [57], this antibody could be useful in screening for drugs that normalize defective cholesterol trafficking in these cells.

5. Concluding remarks

The existence of cholesterol-rich microdomains in cell membranes is well established and accepted. Very little information is however available yet on the molecular

structure and organization of these domains. Even the most sophisticated structural techniques, which provide information on thin films, cannot yet be readily applied to cells in vivo or even to membranes ex vivo. The possibility of using tailor-made antibodies as reporters of molecular organization in membranes is suggested. These antibodies could be raised and selected against specific molecular organizations of cholesterol, or complexes of cholesterol with sphingolipids or other plasma membrane components. It might thus be conceivable to create a library of antibodies, each recognizing different and well-characterized organized arrays. The functioning of these antibodies would not be conceptually different from that of conventional antibodies, besides their performing pattern recognition on a family of targets, which may have identical composition, but differ from each other by their organization.

Acknowledgements

We thank all the members of the laboratory who have contributed to this work by providing data, discussions and comments during the years.

This work was supported by a grant from the Israel Science Foundation to LA. LA is the incumbent of the Dorothy and Patrick Gorman Professorial Chair. MG is the recipient of the Jeanine Kluger Scholarship.

References

[1] L.K. Li, L. So, A. Spector, J. Lipid Res. 26 (1985) 600–609.
[2] K. Simons, G. Van-Meer, Biochemistry 27 (1988) 6197–6202.
[3] K. Simons, E. Ikonen, Nature 387 (1997) 569–572.
[4] K. Simons, D. Toomre, Nat. Rev., Mol. Cell Biol. 1 (2000) 31–39.
[5] D.R. Riddell, G. Christie, I. Hussain, C. Dingwall, Curr. Biol. 11 (2001) 1288–1293.
[6] A. Ono, E.O. Freed, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 13925–13930.
[7] A.J. Travis, T. Merdiushev, L.A. Vargas, B.H. Jones, M.A. Purdon, R.W. Nipper, J. Galatioto, S.B. Moss, G.R. Hunnicutt, G.S. Kopf, Dev. Biol. 240 (2001) 599–610.
[8] S. Bodin, S. Giuriato, J. Ragab, B.M. Humbel, C. Viala, C. Vieu, H. Chap, B. Payrastre, Biochemistry 40 (2001) 15290–15299.
[9] K. Simons, E. Ikonen, Science 290 (2000) 1721–1726.
[10] R. Varma, S. Mayor, Nature 394 (1998) 798–801.
[11] T. Friedrichson, T.V. Kurzchalia, Nature 394 (1998) 802–805.
[12] T. Harder, P. Scheiffele, P. Verkade, K. Simons, J. Cell Biol. 141 (1998) 929–942.
[13] A. Pralle, P. Keller, E.L. Florin, K. Simons, J.K.H. Horber, J. Cell Biol. 148 (2000) 997–1007.
[14] B.S. Wilson, J.R. Pfeiffer, J.M. Oliver, J. Cell Biol. 149 (2000) 1131–1142.
[15] J. Hwang, L.A. Gheber, L. Margolis, M. Edidin, Biophys. J. 74 (1998) 2184–2190.
[16] M.C. Giocondi, V. Vie, E. Lesniewska, J.P. Goudonnet, C. Le Grimellec, J. Struct. Biol. 131 (2000) 38–43.
[17] R.F. Jacob, R.J. Cenedella, R.P. Mason, J. Biol. Chem. 276 (2001) 13573–13578.
[18] T.N. Tulenko, M. Chen, P.E. Mason, R.P. Mason, J. Lipid Res. 39 (1998) 947–956.
[19] D.A. Brown, E. London, J. Membr. Biol. 164 (1998) 103–114.
[20] A. Rietveld, K. Simons, Biochim. Biophys. Acta MR, Rev. Biomembr. 1376 (1998) 467–479.
[21] T.-Y. Wang, R. Leventis, J.R. Silvius, Biophys. J. 79 (2000) 919–933.
[22] H.A. Rinia, M.M.E. Snel, J.P.J.M. van der Eerden, B. de Kruijff, FEBS Lett. 501 (2001) 92–96.
[23] D.M. Cannon, M.L. Pacholski, N. Winograd, A.G. Ewing, J. Am. Chem. Soc. 122 (2000) 603–610.
[24] C. Gliss, H. Clausen-Schaumann, R. Gunther, S. Odenbach, O. Randl, T.M. Bayerl, Biophys. J. 74 (1998) 2443–2450.
[25] C.A. Helm, H. Mohwald, K. Kjaer, J. Als-Nielsen, Biophys. J. 52 (1987) 381–390.
[26] C.A. Helm, P. Tippmann-Krayer, H. Mohwald, J. Als-Nielsen, K. Kjaer, Biophys. J. 60 (1991) 1457–1476.
[27] J. Majewski, T.L. Kuhl, K. Kjaer, G.S. Smith, Biophys. J. 81 (2001) 2707–2715.
[28] S. Lafont, H. Rapaport, G.J. Somjen, A. Renault, P.B. Howes, K. Kjaer, J. Als-Nielsen, L. Leiserowitz, M. Lahav, J. Phys. Chem., B 102 (1998) 761–765.
[29] H. Rapaport, I. Kuzmenko, S. Lafont, K. Kjaer, P.B. Howes, J. Als-Nielsen, M. Lahav, L. Leiserowitz, Biophys. J. 81 (2001) 2729–2736.
[30] L. Addadi, S. Weiner, Angew. Chem., Int. Ed. Engl. 31 (1992) 153–169.
[31] L. Addadi, S. Weiner, M. Geva, Z. Kardiol. 90 (2001) 92–98.
[32] Z. Jia, C.I. DeLuca, H. Chao, P.L. Davies, Nature 384 (1996) 285–288.
[33] Y.C. Liou, A. Tocilj, P.L. Davies, Z.C. Jia, Nature 406 (2000) 322–324.
[34] I.A. Wilson, R.L. Stanfield, Curr. Opin. Struct. Biol. 3 (1993) 113–118.
[35] M. Kam, D. Perl-Treves, D. Caspi, L. Addadi, FASEB J. 6 (1992) 2608–2613.
[36] N. Kessler, D. Perl-Treves, L. Addadi, FASEB J. 10 (1996) 1435–1442.
[37] D. Perl-Treves, N. Kessler, D. Izhaky, L. Addadi, Chem. Biol. 3 (1996) 567–577.
[38] N. Kessler, D. Perl-Treves, L. Addadi, M. Eisenstein, Proteins: Struct. Funct. Genet. 34 (1999) 383–394.
[39] D. Izhaky, L. Addadi, Adv. Mater. 10 (1998) 1009–1013.
[40] D. Izhaky, L. Addadi, Chem. Eur. J. 6 (2000) 869–874.
[41] M. Geva, D. Izhaky, D.E. Mickus, S.D. Rychnovsky, L. Addadi, ChemBioChem 2 (2001) 265–271.
[42] G.M. Swartz, M.K. Gentry, L.M. Amende, E.J. Blanchette-Mackie, C.R. Alving, Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 1902–1906.
[43] N. Wassef, S. Johnson, G. Graeber, G.J. Swartz, C. Schultz, J. Hailey, A. Johnson, D. Taylor, R. Ridgway, C. Alving, J. Immunol. 143 (1989) 2990–2995.
[44] C.R. Alving, G.M. Swartz, N.M. Wassef, Biochem. Soc. Trans. 17 (1989) 637–639.
[45] B. O'Nuallain, R. Wetzel, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 1485–1490.
[46] M. Palmer, Toxicon 39 (2001) 1681–1689.
[47] P.M. Elias, D.S. Friend, J. Goerke, J. Histochem. Cytochem. 27 (1979) 1247–1260.
[48] H.S. Kruth, M. Vaughan, J. Lipid Res. 21 (1980) 123–130.
[49] H.S. Kruth, J. Blanchette-Mackie, J. Avigan, W. Gamble, M. Vaughan, J. Lipid Res. 23 (1982) 1128–1135.
[50] M. Iwamoto, I. Morita, M. Fukuda, S. Murota, S. Ando, Y. Ohno-Iwashita, Biochim. Biophys. Acta 1327 (1997) 222–230.
[51] R. Bittman, Lipids 13 (1978) 686–691.
[52] D.J. McGookey, K. Fagerberg, R.G.W. Anderson, J. Cell Biol. 96 (1983) 1273–1278.
[53] J. Dijkstra, G.M.J. Swartz, J.J. Raney, J. Aniagolu, L. Toro, C.A. Nacy, S.J. Green, J. Immunol. 157 (1996) 2006–2013.
[54] W. Mobius, Y. Ohno-Iwashita, E.G. van Donselaar, V.M.J. Shimada, Y. Shimada, T. Fujimoto, H.F.G. Heijnen, H.J. Geuze, J.W. Slot, J. Histochem. Cytochem. 50 (2002) 43–55.

[55] M.S.F. Clarke, C.R. Vanderburg, M.M. Bamman, R.W. Caldwell, D.L. Feeback, J. Appl. Physiol. 89 (2000) 731–741.
[56] C.R. Alving, G.M. Swartz, Crit. Rev. Immunol. 10 (1991) 441–453.
[57] H.S. Kruth, I. Ifrim, J. Chang, L. Addadi, D. Perl-Treves, W.Y. Zhang, J. Lipid Res. 42 (2001) 1492–1500.
[58] M.S. Brown, J.L. Goldstein, Ann. Rev. Biochem. 52 (1983) 223–261.
[59] L. Liscum, N.J. Munn, Biochim. Biophys. Acta 1438 (1999) 19–37.
[60] G. Kellner-Weibel, Y.J. Geng, G.H. Rothblat, Atherosclerosis 146 (1999) 309–319.
[61] Y. Barenholz, E.J. Patzer, N.F. Moore, R.R. Wagner, Adv. Exp. Med. Biol. 101 (1978) 45–56.
[62] A.E. Christian, M.P. Haynes, M.C. Phillips, G.H. Rothblat, J. Lipid Res. 38 (1997) 2264–2272.
[63] D. Perl-Treves, M. Kam, L. Addadi, Mol. Cryst. Liq. Cryst. 278 (1996) 1–15.
[64] Y. Ohno-Iwashita, M. Iwamoto, S. Ando, S. Iwashita, Biochim. Biophys. Acta 1109 (1992) 81–90.
[65] M. Bloom, J.L. Thewalt, Mol. Membr. Biol. 12 (1995) 9–13.
[66] F. Schroeder, A.A. Frolov, E.J. Murphy, B.P. Atshaves, J.R. Jefferson, L. Pu, W.G. Wood, W.B. Foxworth, A.B. Kier, Proc. Soc. Exp. Biol. Med. 213 (1996) 150–177.
[67] S.W. Hui, in: P.L. Yeagle (Ed.), Biology of Cholesterol, CRC Press, Boca Raton, 1988, p. 213.
[68] E.J. Patzer, R.R. Wagner, Nature 274 (1978) 394–395.
[69] M. Geva, F. Frolow, M. Eisenstein, L. Addadi, J. Am. Chem. Soc. 125 (2003) 696–704.