Benchmarks

Effects of freezing and protein cross-linker on isolating membrane raft–associated proteins

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Since the discovery of cellular membrane rafts, the defining of these domains has remained ambiguous due to a great number of isolation procedures proposed for the extraction of the rafts from cells. Characterization of membrane rafts using Triton X-100 insolubility is limited by the fact that weak interactions between proteins and lipids within the membrane rafts cannot be detected. In order to study the role of membrane rafts in cell signal transduction, it is crucial that weak membrane raft–associated proteins are detected. In this report, we demonstrate that by incorporating 3,3′-dithiobis(sulfosuccinimidyl propionate) (DTSSP) crosslinking and freezing at -80°C into the membrane raft isolation procedure of HaCaT cells, both membrane raft–associated proteins caveolin-1 and Fas receptor are able to be reproducibly isolated into a single fraction containing the membrane rafts of the cells.

Membrane rafts, or lipid rafts, are small, heterogeneous, dynamic microdomains with lower fluidity and a higher degree of saturation than the bulk membrane. Rafts form by self-aggregation of cholesterol and glycosphingolipids, and they participate in compartmentalization of certain cellular processes (1–5). Since the discovery of membrane rafts, the defining of these domains has remained elusive and controversial (6). One major factor contributing to the ambiguity of membrane rafts is a great number of isolation procedures documented for the extraction of the rafts from cells (7).

The operational definition of membrane rafts defines these entities by the traditional method used to isolate them: "A lipid raft is a membrane domain that is resistant to extraction in cold 1% Triton X-100 and floats in the upper half of a 5–30% sucrose density gradient" (2,8). Differing published procedures deviate from the traditional method for membrane raft isolation (modifications over the years include changes in the buffer composition, form of homogenization of cells, gradient composition, and centrifugation time and speed), since membrane rafts of different cell types have required different isolation procedures. Consequently, those membrane rafts isolated using different procedures differ in their composition of proteins and lipids. Furthermore, most of these procedures lack critical details needed for reproducibility, adding to the perplexity of the definition of membrane rafts.

It has been shown that the characterization of membrane rafts using Triton X-100 insolubility is limited by the fact that weak interactions between proteins and lipids within the membrane rafts cannot be detected (9,10). We used caveolin-1 and Fas receptor (FasR) (Cat. nos. SC-894 and SC-715, respectively; Santa Cruz Biotechnology, Santa Cruz, CA, USA) as indicators for strong and weak membrane raft–associated proteins, respectively, in order to demonstrate that protein isolation could be significantly improved by using a simple chemical crosslinking procedure prior to freezing the cells at -80°C before cell lysis. Caveolae, morphologically identifiable components of membrane rafts known to be associated with cholesterol, are the only unanimously accepted members of the membrane raft family (1,11). They are stabilized by caveolin-1, a protein component, which is also commonly used in the identification of membrane rafts (1). FasR, a transmembrane death receptor, is able to translocate to and accumulate in the membrane rafts of cells upon certain stimuli in order to trigger downstream apoptotic signaling (12–15). Evidence of membrane raft isolation includes the exclusive presence of caveolin-1 as one isolatable fraction of the density gradient (Figure 1, Panel D).

3,3′-dithiobis(sulfosuccinimidyl propionate) (DTSSP; Pierce Protein Research Products, Thermo Fisher Scientific, Rockford, IL, USA) is a water-soluble, membrane-impermeable crosslinker that works by N-hydroxysulfosuccinimide (sulfo-NHS)–ester crosslinking primary amines of membrane proteins. Chemical crosslinking of HaCaT cell membrane proteins using DTSSP reduced the diffusion of caveolin-1 and enabled us to isolate caveolin-1 and weakly raft-associated FasR together, which would otherwise be dissociated from one another during the lysis and isolation process (Figure 1, A and B). Our data showed that caveolin-1 was isolated in fractions 2 and 3 after treating the cells with DTSSP (Figure 1B). Without the use of the chemical crosslinking, caveolin-1 protein was present throughout the density gradient; therefore, the membrane rafts had not been exclusively isolated, although most of the caveolin-1 was found in fraction 2 (Figure 1A). The FasR protein mainly existed in the cytosolic protein fraction 5 without the use of DTSSP crosslinking, but was detected in fraction 2 after treating HaCaT cells with the protein crosslinker (Figure 1, A and B).

The weakly raft-associated FasR could also be isolated to fraction 2 by a simple step of freezing the cells at -80°C before lysis. The membrane raft isolation procedure is lengthy, usually requiring a 5-h ultracentrifugation for density gradient flotation in addition to the time needed to treat the cells prior to raft isolation; because of this, the cells are often frozen overnight. However, the step of freezing at -80°C is most often not recorded in the descriptions of the methods. Not mentioning freezing during the membrane isolation procedure

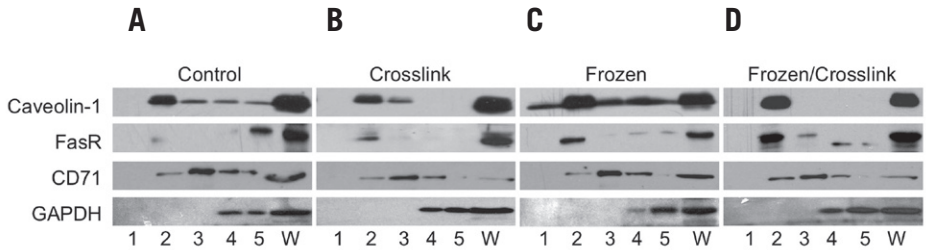

Figure 1. Western blot analysis of differential membrane raft protein isolation by freezing at -80°C and membrane chemical crosslinking. Western blots of membrane raft proteins caveolin-1 and Fas, cytoplasmic GAPDH, and non-raft membrane protein CD71 in (A) control (not crosslinked or frozen), (B) crosslinked, (C) frozen, (D) frozen and crosslinked cell samples. Membrane proteins were crosslinked by applying cold 1.25 mM DTSSP to cells on ice for one hour. Cells were scraped from plates and centrifuged at 11,200× *g*. The cell pellet was frozen at -80°C for >4 h. Cells were washed and sheared in TNET buffer (50 nM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100). Cell lysates were then placed at the bottom of a 40–30–5% step gradient of OptiPrep medium in a swing bucket rotor tube and ultracentrifuged at 4°C at 132,000× *g* for 5 h. A portion of each whole cell lysate was saved for analysis. Five fractions of equal volume were retrieved from the tube after ultracentrifugation. Sample fractions (lanes 1–5) and whole cell lysates (W) were buffered and SDS-PAGE was performed on 15% acrylamide/bisacrylamide gels followed by semi-dry transfer to nitrocellulose membrane. Membranes were blocked with dry milk and then probed with antibodies specific to the caveolin-1, FasR, GAPDH, and CD71 proteins.

ignores a critical detail needed in order to ensure reproducibility in this isolation method. Freezing the HaCaT cell lysates overnight at -80°C hinders the exclusive isolation of caveolin-1 to fraction 2 of the density gradient (Figure 1C), which could be due to the diffusion during the longer period of storage. Our data showed that caveolin-1 is present throughout the density gradient, with more of the protein found in non-raft fractions (fractions 1, 3, 4, and 5) than in the samples that were not frozen (Figure 1, A and C). In contrast, freezing the cells promotes the affinity of FasR to the membrane rafts (Figure 1, A and C). The amount of cytoplasmic protein GAPDH (Cat. no. SC-25778; Santa Cruz Biotechnology) in the raft fraction was not noticeably affected under the same experimental conditions, demonstrating that our isolation procedure does not promote nonspecific relocalization of cytoplasmic proteins into the membrane raft fraction (Figure 1, A–D). However, while non–raft-bound membrane protein CD71 (Cat. no. SC-32272; Santa Cruz Biotechnology) mainly existed in fraction 3 of the density gradient, the fraction 2:fraction 3 CD71 ratio was increased after treating with the crosslinker (Figure 1, A and D, B), indicating that DTSSP might have nonspecifically crosslinked some nonraft proteins to the raft-associated proteins. The fraction 2:fraction 3 CD71 ratio was not noticeably changed after freezing (Figure 1, A and C).

In summary, we have shown that the membrane-impermeable protein crosslinker DTSSP promotes the affinity of membrane raft–associated proteins to the isolatable membrane rafts of HaCaT cells. Interestingly, we are the first to show that freezing the HaCaT cells prior to density gradient flotation also promotes the affinity of the weakly raft-associated protein FasR to the isolated raft fraction. Adversely, freezing hinders the exclusive isolation of the membrane raft–identifying protein, caveolin-1. By incorporating the steps of DTSSP crosslinking and freezing at -80°C into the membrane raft isolation procedure of HaCaT cells, both the caveolin-1 and FasR proteins are reproducibly isolated into a single fraction containing the membrane rafts of the cells.

A detailed protocol of the membrane raft isolation procedure is available online.

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Competing interests

The authors declare no competing interests.

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