

Separation of Lipid “Rafts” by Sucrose Gradient in the Thermo Scientific Sorvall® WX Ultracentrifuge

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Introduction

Certain constituents of the plasma membrane domain form an ordered liquid phase with reduced membrane fluidity¹. These membrane compartments were termed lipid “rafts”, glycolipid-enriched membrane domains (GEMs) or detergent-insoluble glycolipid-enriched domains (DIGs). These rafts maintain a distinct composition of lipid, sphingolipid and cholesterol^{1,2} and are shown to be involved in many cell biology processes (reviewed in^{3,4}), including protein sorting, membrane trafficking, virus infection and signal transduction⁴.

Because of distinct composition of lipid and proteins localized in it, rafts are resistant to mild detergent extraction at low temperature (4°C). Additionally, they float to the low density fraction on sucrose gradients^{3,4} and can be easily isolated by sucrose gradient ultracentrifugation. This method is the most commonly used approach to study the lipid rafts biochemically.

In the present study, we isolated the lipid rafts using an AH-650 swinging rotor and the Thermo Scientific Sorvall WX ultracentrifuge.



Key Words

- Lipid Rafts
- Sucrose Gradient
- Ultracentrifugation
- Swinging Bucket Rotors

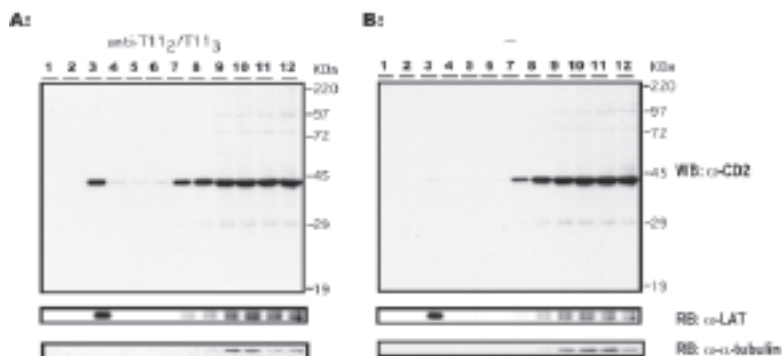


Figure 1: CD2 inducibly associates with lipid rafts. 8×10^7 activated human T cells were either crosslinked by a combination of anti-T11₂ + anti-T11₃ mAbs (ascites, 1/100) (A) or left untreated (B) at 37°C for 10 min. Total cell lysates were fractionated by sucrose gradient then 0.4 ml of each fraction was collected (columns 1-12), treated with PNGase F and resolved by SDS-PAGE. CD2 was blotted with polyclonal M32B antibody and visualized. Membranes were subsequently stripped and re-blotted with anti-LAT polyclonal mAbs or anti- α -tubulin mAb to verify positions of raft and soluble fractions, respectively.

Methods

Cell culture: Human peripheral blood mononuclear cells (PBMCs) were isolated from leukopaks of healthy blood donors by Ficoll gradient centrifugation and nylon wool column. The purified PBMCs were cultured in RPMI-1640 containing 10% human AB serum (Nabi, FL), 1% penicillin/streptomycin (Gibco/BRL, Rockville, MD), and 2 mM glutamine (Gibco/BRL). Activated T cells were obtained by culturing the PBMCs as described above plus a 1:200 dilution of 2Ad2 (Anti-CD3₃ mAb) ascites and 25 ng/ml PMA (phorbol 12-myristate 13-acetate). Cells were then diluted 1:5, maintained in culture and used within 1-3 days. The human Jurkat cell line J77 was maintained in RPMI 1640, 10% FCS with 1% penicillin/streptomycin and 2 mM glutamine in 5% CO₂ at 37°C.

Lipid raft separation: 5×10^7 - 1×10^8 J77 or activated human T cells, or 2.5×10^8 resting human T cells were individually harvested, resuspended in

1 ml complete medium and stimulated by antibody crosslinking (in our case, anti-CD2). After crosslinking, cells were rinsed once with ice cold PBS, and then resuspended in 1 ml of ice-cold 1 x MBS (25 mM MES, 150 mM NaCl [pH6.5]), 1% Triton X-100 supplemented with 1 mM PMSE, 0.35 TIU aprotinin, and 5 μ g/ml leupeptin. Following 30 min incubation on ice, the lysates were homogenized with 10 strokes in a Dounce homogenizer (Wheaton, Millville, NJ), gently mixed with an equal volume of ice-cold 80% sucrose (Fisher) (w/v) in 1 x MBS, and loaded in the bottom of a centrifuge tube. Then, the sample was overlaid with 2 ml of 30% sucrose and 1 ml of 5% sucrose (w/v), both prepared in 1 x MBS. The sucrose gradient samples were spun at 39,000 rpm (180,000 x g) in a Thermo Scientific AH-650 rotor in a WX Ultracentrifuge at 4°C for 20-21 h. DIG domains were harvested by collecting 0.4 ml fractions, beginning at the top of the gradient. Western blotting was performed to check the protein distribution in each fraction.

Results and Discussion

Consistent with previous reports, detergent insoluble glycolipid-enriched microdomains (DIGs) are found in sucrose gradient fractions 2-4^{5,6} where the palmitoylated LAT molecule is predominantly located⁵. Other proteins including α -tubulin are not associated with lipid rafts⁶, but rather are located in the soluble cellular fractions. As shown in Figure 1, following CD2 crosslinking, substantial amounts of CD2 are recruited to the lipid raft fraction, whereas in the absence of CD2 crosslinking, little if any CD2 is localized to the rafts. This result indicates that CD2 association with lipid rafts is inducible⁷.

Recently, many reports suggest that lipid rafts can serve as a platform for lymphocyte signaling, because:

1) numerous signaling molecules essential for immunoreceptors were found localized in the DIG^{3,4}. These include Src family protein tyrosine kinases^{8,9}, heterotrimeric and monomeric Ras-like G proteins¹⁰, molecules involved in Ca²⁺ flux¹¹ as well as the small signaling molecule phosphatidylinositol bisphosphate (PIP²)¹⁰.

2) multiple chain immunoreceptors such as T cell receptors (TCRs)^{6,10}, B cell receptors (BCRs)^{12,13} and high affinity IgE receptors (Fc ϵ RI)¹⁴ are recruited to rafts and that raft integrity is required for effective signal transduction (reviewed in¹⁵).

The inducible association of human CD2 with lipid rafts offers an explanation of many CD2 signaling properties. This process may account for synergistic T cell stimulation by CD2 and TCR⁷.

Besides the western blotting detection of raft associated proteins, the redistribution of labeled antibodies (biotinylated or iodinated) against surface protein can also be used to indirectly indicate the protein – lipid raft inter-

action. Moreover, each fraction of sucrose gradient can be used to do immuno-precipitation or kinase assay.

Membrane isolation by ultracentrifugation is increasing in popularity. Thermo Scientific ultracentrifuges and swinging bucket rotors isolate distinct membrane fractions using sucrose gradients.

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References

1. Brown, D.A. and E. London, Structure of detergent-resistant membrane domains: does phase separation occur in biological membranes? *Biochem Biophys Res Commun*, 1997. 240(1): p. 1-7.
2. Harder, T. and K. Simons, Caveolae, DIGs, and the dynamics of sphingolipid-cholesterol microdomains. *Curr Opin Cell Biol*, 1997. 9(4): p. 534-42.
3. Brown, D.A. and E. London, Functions of Lipid Rafts in Biological Membranes. *Annu Rev Cell Dev Biol*, 1998. 14: p. 111-136.
4. Simons, K. and E. Ikonen, Functional rafts in cell membranes. *Nature*, 1997. 387(6633): p. 569-72.
5. Zhang, W., R.P. Tribble, and L.E. Samelson, LAT palmitoylation: its essential role in membrane microdomain targeting and tyrosine phosphorylation during T cell activation. *Immunity*, 1998. 9 (2): p. 239-46.
6. Montixi, C., C. Langlet, A.M. Bernard, J. Thimonier, C. Dubois, M.A. Wurbel, J.P. Chauvin, M. Pierres, and H.T. He, Engagement of T cell receptor triggers its recruitment to low-density detergent-insoluble membrane domains. *Embo J*, 1998. 17(18): p. 5334-48.
7. Yang, H. and E.L. Reinherz, Dynamic recruitment of human CD2 into lipid rafts: linkage to T cell signal transduction. *J. Biol. Chem.*, 2001. in press.
8. Kabouridis, P.S., A.I. Magee, and S.C. Ley, S-acylation of LCK protein tyrosine kinase is essential for its signalling function in T lymphocytes. *Embo J*, 1997. 16(16): p. 4983-98.
9. Van't Hof, W. and M.D. Resh, Dual fatty acylation of p59(Fyn) is required for association with the T cell receptor zeta chain through phosphotyrosine-Src homology domain-2 interactions. *J Cell Biol*, 1999. 145(2): p. 377-89.
10. Xavier, R., T. Brennan, Q. Li, C. McCormack, and B. Seed, Membrane compartmentation is required for efficient T cell activation. *Immunity*, 1998. 8(6): p. 723-32.
11. Fujimoto, T., Calcium pump of the plasma membrane is localized in caveolae. *J Cell Biol*, 1993. 120⁵: p. 1147-57.
12. Cheng, P.C., M.L. Dykstra, R.N. Mitchell, and S.K. Pierce, A role for lipid rafts in B cell antigen receptor signaling and antigen targeting. *J Exp Med*, 1999. 190 (11): p. 1549-60.
13. Aman, M.J. and K.S. Ravichandran, A requirement for lipid rafts in B cell receptor induced Ca(2+) flux. *Curr Biol*, 2000. 10(7): p. 393-6.
14. Field, K.A., D. Holowka, and B. Baird, Compartmentalized activation of the high affinity immunoglobulin E receptor within membrane domains. *J Biol Chem*, 1997. 272 (7): p. 4276-80.
15. Langlet, C., A.M. Bernard, P. Drevot, and H.T. He, Membrane rafts and signaling by the multichain immune recognition receptors. *Curr Opin Immunol*, 2000. 12 (3): p. 250-5.

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