

# 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 fluidity<sup>1</sup>. 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<sup>1,2</sup> and are shown to be involved in many cell biology processes (reviewed in<sup>3,4</sup>), including protein sorting, membrane trafficking, virus infection and signal transduction<sup>4</sup>.

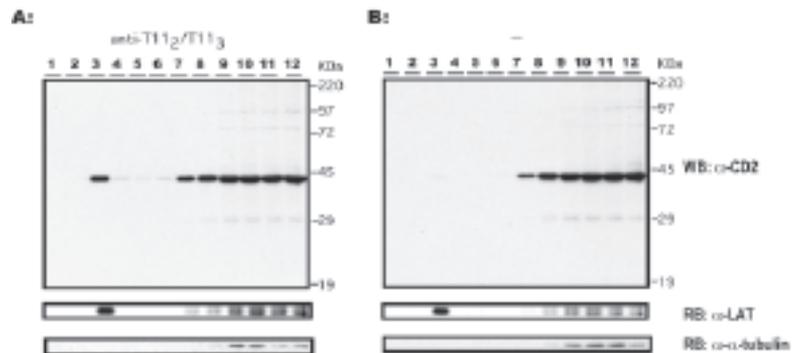
Because of distinct composition of lipid and proteins localized in it, rafts are resistant to mild detergent extraction at low temperature (4°C). Additionally, they float to the low density fraction on sucrose gradients<sup>3,4</sup> and can be easily isolated by sucrose gradient ultracentrifugation. 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.  $8 \times 10^7$  activated human T cells were either crosslinked by a combination of anti-T11<sub>2</sub> + anti-T11<sub>3</sub> 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- $\alpha$ -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-CD3<sub>3</sub> 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% CO<sub>2</sub> at 37°C.

**Lipid raft separation:**  $5 \times 10^7$  -  $1 \times 10^8$  J77 or activated human T cells, or  $2.5 \times 10^8$  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  $\mu$ 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<sup>5,6</sup> where the palmitoylated LAT molecule is predominantly located<sup>5</sup>. Other proteins including  $\alpha$ -tubulin are not associated with lipid rafts<sup>6</sup>, 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 inducible<sup>7</sup>.

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<sup>3,4</sup>. These include Src family protein tyrosine kinases<sup>8,9</sup>, heterotrimeric and monomeric Ras-like G proteins<sup>10</sup>, molecules involved in Ca<sup>2+</sup> flux<sup>11</sup> as well as the small signaling molecule phosphatidylinositol bisphosphate (PIP<sup>2</sup>)<sup>10</sup>.

2) multiple chain immunoreceptors such as T cell receptors (TCRs)<sup>6,10</sup>, B cell receptors (BCRs)<sup>12,13</sup> and high affinity IgE receptors (Fc $\epsilon$ RI)<sup>14</sup> are recruited to rafts and that raft integrity is required for effective signal transduction (reviewed in<sup>15</sup>).

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 TCR<sup>7</sup>.

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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