GPI-anchored proteins and glycoconjugates segregate into lipids rafts in Kinetoplastida

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Keywords: lipid rafts, Kinetoplastida, GPI-anchor, sphingolipid, sterol.

Abstract

The plasma membranes of the divergent eukaryotic parasites, *Leishmania* and *Trypanosoma,* are highly specialised, with a thick coat of glycoconjugates and glycoproteins playing a central role in virulence. Unusually, the majority of these surface macromolecules are attached to the plasma membrane via a GPI anchor. In mammalian cells and yeast, many GPI-anchored molecules associate with sphingolipid and cholesterol-rich detergent resistant membranes, known as lipid rafts. Here we show that GPI-anchored parasite macromolecules (but not the dual acylated *Leishmania* surface protein, HASPB, or a subset of the GPI-anchored GIPL glycolipids) are enriched in a sphingolipid/sterol-rich fraction resistant to cold detergent extraction. This observation is consistent with the presence of functional lipid rafts in these ancient, highly polarised organisms.

1. Introduction

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Abbreviations: GPI, glycosylphosphatidylinositol; DRM, detergent resistant membrane; VSG, variant surface glycoprotein; LPG, lipophosphoglycan; GIPL, glycoinositol phospholipid; HASP, hydrophilic acylated surface protein; PI, phosphatidylinositol; IPC, inositol phosphorylceramide; PIP, phosphatidylinositol phosphate.

The plasma membrane of eukaryotes is typically partitioned into functional domains. In addition, specialised micro-domains exist which harbour small subsets of proteins. In higher eukaryotic systems, several plasma membrane proteins, particularly those anchored by a glycosylphosphatidylinositol (GPI) moiety, are associated with sphingolipid/cholesterol-rich micro-domains termed rafts [reviewed 1,2]. These domains are thought to be analogous to detergent resistant membranes (DRMs) [3] and possibly also to plasma membrane structures termed caveolae [1]. Rafts, which form within the Golgi complex, have been implicated in polarised trafficking in epithelial cells, specifically to the apical plasma membrane, and also in the assembly of supramolecular complexes involved in signal transduction [1,2]. A recent comprehensive study in *Saccharomyces cerevisiae* demonstrated the presence of similar lipid micro-domains in yeasts [4]. Interestingly, unlike in mammalian cells, rafts in yeast are first detected in the endoplasmic reticulum (ER), leading to the suggestion that they are involved in the biosynthetic delivery of protein to the cell surface.

Protozoan parasites of the phylum Kinetoplastida cause a spectrum of tropical and sub-tropical diseases. Much research has focused on the unusually high levels of GPI-anchored molecules present on the surfaces of these organisms [5]. These are essential for survival of mammalian bloodstream-form *Trypanosoma brucei* [6]. The most intensively studied GPI-anchored molecules are the variant surface glycoprotein (VSG) of bloodstream-form *T. brucei* [7] and in *Leishmania,* the metalloprotease GP63 (or leishmanolysin) [8] and the glycoconjugates, lipophosphoglycan (LPG) and the glycoinositol phospholipids (GIPLs) [9]. All of these molecules have been implicated in virulence: VSG in evasion of both the cellular and humoral host immune response [7]; LPG and GP63 in resistance of insect stage (promastigote) *Leishmania* to hydrolytic enzymes in the vector gut and to mammalian complement [10]; LPG in invasion of and survival within macrophages [11]; GIPLs for viability of both the insect and mammalian (amastigote) stages of the life cycle [12]. These molecules are suggested to form a dense, homogenous and protective coat at the parasite cell surface [13]. Given the predisposition of these organisms to traffic large amounts

of GPI-anchored molecules through the specialised flagellar pocket to the cell surface [14,15], it is of interest to investigate whether lipid rafts exist in these cells and to determine the role they play in secretion and lipid/protein transport. The presence, or otherwise, of kinetoplastid rafts may also influence our view of the surface architecture of these protozoans; for example, whether micro-domains involved in signal transduction are present at the plasma membrane. Additional cell surface components of *Leishmania* are the unusual hydrophilic acylated surface proteins (HASPs) [16]. These polypeptides are anchored to the plasma membrane via a myristoylated/palmitoylated N-terminus [17]; similarly modified proteins are found associated with the cytoplasmic leaflet of lipid rafts in mammalian cells [18]. The presence, or otherwise, of the HASPs in lipid rafts may help elucidate their route to the cell surface and their cryptic function.

Recent work has suggested that lipid micro-domains may form in *T. brucei* [19]. In this study, we show that DRMs are present in both *Leishmania* and *T. brucei*, suggesting that they occur throughout the Kinetoplastida. We define lipid rafts in *L. major* as being enriched in sphingolipid (inositol phosphosphingolipid) and sterol (probably ergosterol, as in yeast), in addition to GPI-anchored glycoconjugate (LPG) and glycoprotein (GP63). Surprisingly, the dual acylated HASPB appears to be largely excluded from these domains, and the members of the GIPL family are differentially sequestered.

2. Materials and Methods

2.1 Cell culture and radio-labelling. *L. major* promastigotes (MHOM/IL/81/Friedlin) were maintained as previously described [17]. For protein analyses, cells were pretreated for 30 min in Dulbecco's MEM (DMEM) (ICN) lacking methionine and cysteine, then metabolically labelled for 90 min in the same medium containing 50 µCi/ml of TRAN[^{35}S]-label™ (>1000 Ci/mmol) (ICN). For LPG analyses, cells were pretreated for 30 min in glucose-free (DMEM) (Gibco BRL) containing 0.8 µg/ml tunicamycin (Sigma) and 1% bovine serum albumin (Sigma), then metabolically labelled for 3 hours in the same medium containing 400 µCi/ml of D-[2-^{3}H] mannose (21 Ci/mmol) (NEN). For lipid

analyses, cells were labelled for 24 hours in DMEM (ICN) supplemented with 10% foetal calf serum (Gibco BRL) and 10-20 μCi/ml of myo-[^{3}H] inositol (102 Ci/mmol) (Amersham) or 10 μCi/ml R-[2-^{14}C] mevalonic acid lactone (48.0 mCi/mmol) (Amersham). For pulse-chase analysis, 10^8 *Leishmania* promastigotes were starved for 45 min at 26˚C in 1 ml DMEM lacking methionine and cysteine. Cells were then labelled in 0.5 ml of the same medium with 100 μCi/ml of TRAN[^{35}S]-label™ for 5 min, diluted in 10ml of DMEM containing 10% foetal calf serum and chased for 40 min. Culture adapted 427 trypomastigote *Trypanosoma brucei brucei* were grown in HMI9 medium, with 10% foetal calf serum at 37˚C in a 5% CO_2 atmosphere and maintained at a culture density of <1 x 10^7ml.

2.2 Detergent extraction. *Leishmania* cell pellets (5x10^7) were washed with PBS and extracted with 1ml 1% TX-100 in PBS plus protease inhibitors (Complete™ Mini (Roche) and 1μg/ml pepstatin (Sigma)) for 10 min at 37ºC or on ice. Detergent soluble and insoluble molecules were separated by centrifugation for 2 min at 14000 rpm in an Eppendorf 5402 centrifuge at room temperature or 4ºC. 1 x 10^7 *T. brucei* trypomastigotes, expressing VSG 221, were extracted with 50μl 0.35% TX-100 in PBS, supplemented with 20mM $ZnCl_2$ to inhibit endogenous GPI-PLC [20], at 4˚C or room temperature.

2.3 Protein extraction and analyses. *Leishmania* cell pellets were lysed in 50μl 1% SDS PBS, adjusted to 1ml with 1% TX-100 PBS and supplemented with 5mM each of $CaCl_2$ and $MnCl_2$. The TX-100 extract was supplemented similarly and both fractions incubated with 10μl agarose-bound concanavalin A (Sigma) for 1 hour at room temperature, to precipitate glycosylated proteins (primarily GP63). The subsequent fractions were heated in loading buffer and separated by SDS-PAGE.

2.4 Lipid extraction and analyses. *Leishmania* glycolipids were extracted in chloroform/methanol/water (1:2:0:8, v/v) and analysed on Silica Gel 60 aluminium-backed HPTLC plates (Merck) developed in chloroform/methanol/1M ammonium acetate/13M NH_4OH/water (180:140:9:9:23, v/v) as previously described [21]. For sterol analysis, total lipids were extracted in

chloroform/methanol (2:1, v/v) overnight at room temperature, phase separated and the lower organic phase dried in a Speed Vac® (Savant). Samples were developed in chloroform/methanol/water (65:35:8, v/v) on Kieselgel 60 TLC plates (Merck) [4] and exposed directly to Kodak Biomax MS film. Cholesterol (Sigma) was used as a marker.

2.5 LPG extraction and analysis. After glycolipid extraction as above from [^{3}H] mannose-labelled cells, the remaining pelleted material (proteins and LPG) was heated in loading buffer and separated by SDS PAGE.

2.6 Isolation of detergent resistant membranes. 1-2x10^{8} *Leishmania* cells resuspended in 500 µl PBS (plus protease inhibitors) were disrupted with 500 µm glass beads (Sigma). The resultant lysate was adjusted to 1% TX-100 PBS (plus protease inhibitors) and extracted for 10 min at 4°C. Samples were loaded at the bottom of Optiprep® step gradients as previously described [4]. After centrifugation at 50,000rpm for 3 hours and 25,000rpm for 15 hours in a SW60 rotor, 6 fractions were taken from the top of the gradient. Fractions were either subject to lipid/LPG analysis as above or precipitated with trichloroacetic acid [4] for protein analyses.

2.7 Electrophoresis and Western blotting. Cell or fractionation pellets were resuspended in running buffer, boiled and separated by SDS PAGE. Proteins were either silver stained (Biorad) or electrophoretically transferred to Immobilon P membranes (Millipore). Metabolically labelled proteins and LPG were detected using the Kodak BioMax TranScreen intensifying screen system. GP63 was detected using a polyclonal antibody (provided by Rob McMaster, University of British Columbia). HASPB was detected using a polyclonal antibody [16]. VSG 221 was isolated by hypertonic lysis of trypomastigotes in 20mM Tris-HCl, purified by ion exchange chromatography on DEAE-Sephadex [20] and used to raise rabbit-antiserum.

3. Results

3.1 Temperature-dependent detergent extraction of the major Leishmania surface macromolecules.

One of the defining properties of DRMs/lipid rafts is their differential behaviour when extracted with 1% TX-100 at varying temperatures. Specifically, raft lipids and proteins are resistant to extraction at 4ºC, but are effectively solublised at a higher temperature [3]. The predominant macromolecules on the surface of *Leishmania* promastigotes, LPG and GP63, are both GPI-anchored. Fig. 1 shows the results of extraction of *Leishmania* cells with 1% TX-100 PBS at 4ºC and 37ºC. Both GP63 (Fig. 1a) and LPG (Fig. 1b) are clearly non-extractable at 4ºC, but are completely solublised at 37ºC. These data provide preliminary evidence that the major parasite GPI-anchored macro-molecules are segregated into detergent resistant membranes, which may be analogous to lipid rafts.

3.2 Temperature-dependent detergent extraction of Leishmania lipids.

In mammalian cells and yeast, specific lipids (sphingolipids and sterols) as well as GPI-anchored proteins display differential detergent extractability. In *Leishmania* the behaviour of various classes of lipid was examined under the same specific extraction conditions. As previously described [22], the phosphoglycerides, phosphatidylinositol (PI) and phosphatidylinositol phosphate (PIP), and the sphingolipid, inositol phosphorylceramide (IPC), can be identified as the major species in *L. major* after labelling with myo-[^{3}H] inositol and separation by HPTLC. Fig. 2a clearly demonstrates that whilst IPC is resistant to cold extraction with 1% TX-100, both PI and PIP are solubilised. Again, raising the temperature leads to complete extraction of the labelled species. Similarly, *de novo* synthesised sterols (ergosterol rather than cholesterol in *Leishmania* promastigotes [23]) labelled with R-[2-^{14}C] mevalonic acid lactone are non-extractable with cold TX-100 (Fig. 2c). Thus, by performing a simple detergent extraction, there is an enrichment of components (GPI-anchored molecules, sphingolipids and sterols) that are characteristic of eukaryotic lipid rafts. It should also be noted that the minor inositol-containing species, the GIPLs [22,24], are differentially extracted at 4ºC (Fig. 2b). Those species labelled A, B and C are almost completely solublised; D, E and F are at least partially non-extractable; G

is also soluble. HPTLC separation shows A, B and C to be the least polar labelled GIPLs. By analysis of R_f values and direct comparison of the distinctive profiles with available data [22,24], these probably represent iso-forms of GIPL-1 and GIPL-2. The most polar labelled GIPL (G) is probably P-GIPL-2 or P-GIPL-3; these are the only lyso-alkyl GIPLs [22,24]. Similar comparisons suggest that those GIPLs that demonstrate a degree of insolubility (D, E and F) are probably iso-forms of GIPL-3 and possibly GIPL-2 [22,24]. The implication of these results is that the GIPLs inhabit different lipid environments. This could reflect differential localisation to sub-cellular compartments, either during biosynthesis [21] or at steady state [25], or segregation into distinct lipid domains at the plasma membrane.

3.3 Temperature-dependent detergent extraction of T. brucei VSG.

The presence of lipid micro-domains has been reported in *T. brucei* [19]. Fig. 3 shows that, like *Leishmania* GP63 and LPG, *T. brucei* VSG is detergent-extracted from cells in a temperature-dependent manner. This suggests that VSG localises within DRMs, and illustrates that such lipid micro-domains are probably a widespread feature of the phylum Kinetoplastida. However, this differential extractability is only observed when the detergent concentration is reduced from 1% to 0.35% TX-100, indicating that the lipid composition of *T. brucei* rafts is somewhat different to that in *Leishmania*, and indeed mammalian and yeast cells. VSG has previously been demonstrated to be detergent-extracted with 1% TX-100 at 4ºC from synthetic membranes with a mammalian lipid content [26].

***3.4 Kinetics of GP63 DRM/lipid raft association*.**

It is known that mammalian lipid rafts form in the Golgi complex [3]. However, analogous *S. cerevisiae* micro-domains can be detected in the ER [4]. The kinetics of GP63 association with DRMs were analysed by pulse chase analysis, using the same cell extraction conditions as previously. From these data, it is clear that GP63 becomes detergent- insoluble very rapidly: at time 0 min (after a 5 min pulse), only a small minority of the protein is detected as soluble. This soluble fraction then begins to disappear over time in a reproducible manner. This suggests that as in yeast, *Leishmania* rafts form in the ER or another early

compartment of the secretory pathway, and may act as biosynthetic centres and/or in the delivery of proteins to the plasma membrane.

3.5 Further characterisation by direct isolation of DRMs/lipid rafts.

Membrane fractions representing DRMs were first isolated as low density material by gradient centrifugation [3]. Using Optiprep® step gradients, putative lipid rafts have been isolated from *Leishmania* after cold TX-100 treatment. Fig. 5 illustrates the gradient, the fractions taken and their analysis. Silver staining of proteins following SDS-PAGE (Fig. 5b) shows the majority of proteins to be in the higher density fractions towards the bottom of the gradient (fractions 5 and 6). However, a single, intense band (*) is clear in low density fraction 2. Note that the quantity of material in fraction 6 (the bottom of the gradient) is variable and has previously been proposed to contain non-specifically pelleted protein, including raft-associated molecules that have failed to be fully released by the cell disruption procedure [3]. By immuno-staining, the silver-stained band in fraction 2 is confirmed as GPI-anchored GP63; this protein is only found in this low density fraction and with non-specifically pelleted material in fraction 6 (Fig. 5c). Similarly, D-[2-^{3}H] mannose-labelled LPG segregates solely with this low density material (Fig. 5e). In contrast, the dually acylated HASPB is found largely in fractions 4-6 (high density material), with a minor component in fraction 2 (Fig. 5d). Thus, contrary to expectations based on results in mammalian systems [18], myristoylated/palmitoylated HASPs are, at best, only partially associated with lipid rafts in *Leishmania.* It has been previously demonstrated by fractionation that this surface protein is tightly associated with membranous material [17]. In summary, then, GPI-anchored protein (GP63) and glycoconjugate (LPG) appear to be tightly associated with the low density lipid raft fraction, while di-acylated HASPB and GPI-anchored GIPLs are at least partly solublised under our experimental conditions. These observations suggest that the GPI-anchored macromolecules, GIPLs and HASPB exist within separate membrane domains.

The lipid composition of mammalian [3] and *S. cerevisiae* [4] rafts has been documented as rich in sphingolipid. By labelling with myo-[^{3}H] inositol, followed by density gradient separation of the detergent-treated cell extracts, we

examined the PI and IPC composition of each fraction. Fig. 6 clearly demonstrates that whereas IPC behaves as raft-associated material, being enriched in low density fractions, PI is concentrated towards the bottom of the gradient indicating that it has been detergent-solubilised. In conclusion, the low density fractions (1 and 2) isolated on the gradients are enriched in IPC and GPI-anchored molecules, whereas PI and the majority of cell proteins are detergent-solublised and therefore found in the high density fractions. These data demonstrate that DRMs can be isolated from *Leishmania* as low density fractions that share the characteristics of lipid rafts in higher eukaryotes.

4. Discussion

In a simple cold detergent treatment of insect-stage *Leishmania* and bloodstream form *T. brucei,* the major classes of GPI-anchored molecules (GP63, LPG, some GIPLs and VSG) are resistant to extraction. This suggests that DRMs/lipid rafts are a feature of the divergent Kinetoplastida and are therefore likely to be conserved in eukaryotic evolution. In *Leishmania, de novo* synthesised sphingolipid (IPC) and sterols behave in a similar detergent-resistant manner, whereas the phosphoglycerides, PI and PIP, and most GIPLs are solubilised, indicating that distinct lipid domains exist in these organisms. Further analyses, employing density gradient centrifugation, isolated the detergent-resistant membranes (DRMs) as a low density fraction rich in GPI-anchored macromolecules and sphingolipid. The lipid composition of these DRMs – enriched in IPC and sterol – mirror those in yeast [4]. Yeast, protozoa and plants synthesise IPC-based sphingolipids, rather than sphingomyelin-based sphingolipids as in mammalian cells [27]. Another parallel with yeast is that *Leishmania* rafts form in an early secretory compartment. Whether this is related to their compositional similarity or not is unclear, but it may implicate *Leishmania* rafts in protein delivery to the cell surface, as in *S. cerevisiae* [4].

It is hypothesised that these assemblies of sphingolipids and sterols form a discrete liquid-ordered phase in the liquid-disordered (or liquid crystalline) membrane due to the presence of long, saturated hydrocarbon chains [1,2].

Proteins associating with this ordered environment tend to possess saturated acyl chain anchors, being either GPI-anchored or modified with myristate and/or palmitate. As might be predicted, GPI-anchored GP63 and LPG segregate into *Leishmania* DRMs. However, it appears that only some species of GPI-anchored GIPLs behave in a similar manner. This could be due to the different head group composition of the GIPLs or because individual GIPL species have different hydrocarbon chain anchors in *L. major* [22,24]. It is possible that the length/degree of saturation will affect whether a GIPL enters the liquid-ordered or liquid-crystalline phase in the lipid bilayer [26]. DRM-associated GP63 [28] and LPG [29,30] are both modified with a very long (C_{24} or C_{26}) saturated alkylglycerol moiety. The relatively short acyl chain length of myristate (C_{12}) and palmitate (C_{14}) may prevent HASPB associating stably with *Leishmania* rafts. Although lyso-alkyl P-GIPL-2 and 3 have very similar anchors to LPG, they appear to be soluble under the same conditions. However, these species probably act as intermediates in LPG biosynthesis [22,24] and therefore may occupy a sub-cellular compartment of a specific lipid composition.

Our previously published data on HASPB localisation have demonstrated that only a proportion (20-30%) of the protein may be located on the external face of the plasma membrane, the rest being on the cytosolic side [17]. It is notable that a similar proportion was detected in the lipid raft fraction (Fig. 5d). Although rafts do form on the inner face of the mammalian plasma membrane, their composition is unknown [1,2]. It is possible that the DRM-associated HASPB detected is that associated with the outer leaflet of the plasma membrane.

What are the implications for the presence of lipid rafts on the surface architecture of protozoan parasites? They may form functional micro-domains at the plasma membrane involved in, for example, signal transduction [31] or the formation of specific adhesion/recognition sites. Alternatively, in *Leishmania*, they may serve as intracellular biosynthetic centres for the manufacture of LPG and GIPLs [32]. As described above, the major GPI-anchored surface molecules are thought to form a protective barrier in promastigote *Leishmania* and bloodstream form *T. brucei* [13]. Obviously the concept of lipid micro-domains rich in such

molecules does not fit easily into such a simple model. However, it is possible to take an alternative view in which the plasma membrane of the parasite exists largely in a liquid-ordered phase with liquid-crystalline micro-domains or rafts [33]. In this model, sphingolipid/sterol-rich platforms could serve in the delivery of GPI-anchored molecules to the plasma membrane in a “default” polarised manner via the flagellar pocket. The early formation of rafts in the secretory pathway of *Leishmania* would support a role in transport of GPI-anchored molecules and/or biosynthetic centre formation, but does not preclude other functions. The functions of mammalian lipid rafts remain unclear even though their presence at the plasma membrane is becoming ever less contentious.

As yet, we have no indication of raft distribution in the Kinetoplastida, but it should be noted that eukaryotic cilia and flagella have distinct lipid compositions [34]. It would thus be of interest to investigate the distribution of IPC/ergosterol micro-domains in these flagellated protozoa, particularly given recent demonstrations of differential protein distribution between the cell body and flagellum in these organisms [35,36].

Acknowledgements

We thank Robert McMaster, for the generous provision of antibodies to GP63, and Gareth Morgan and Tim Jeffries for critical reading of this manuscript. This work was supported by the Wellcome Trust (grant ref. no. 045493/Z/95/Z, 061343/Z/00/Z).

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Figure Legends

Fig. 1. Temperature-dependent detergent extraction of *Leishmania* GP63 and LPG. Insoluble (I) and soluble (S) fractions after cell extraction with 1% TX-100 at 4ºC or 37ºC and separation by SDS-PAGE. Size markers are shown on the left of each image (kDa). **a**, [^{35}S] methionine/cysteine labelled GP63. **b**, [^{3}H] mannose-labelled LPG.

Fig. 2. Temperature-dependent detergent extraction of *Leishmania* lipids. Insoluble (I) and soluble (S) fractions after cell extraction with 1% TX-100 at 4ºC or 37ºC. O, origin; F, solvent front. **a**, HPTLC of [^{3}H] inositol-labelled lipids. **b**, longer exposure of (a) allows visualisation of minor glycolipid species (labelled A-G). The PIP signal obscures D in S. The relevant region of the plate has been expanded for clarity. **c**, TLC of [^{14}C] mevalonate-labelled lipids. Sterols migrate to the top of the plate under these conditions.

Fig. 3 Temperature-dependent detergent extraction of *Trypanosoma brucei* VSG*.*

Immunoblot of *T. brucei* extracts obtained using 0.35% TX-100 at 4˚C or room temperature (rt) in the presence of Zn^{2+}. If Zn^{2+} is omitted from the extraction buffer, the VSG is fully solubilised under these conditions due to cleavage of the GPI-anchor (data not shown).

Fig. 4. Pulse-chase analysis of detergent-extracted *Leishmania* GP63. Insoluble (I) and soluble (S) fractions after cell extraction with 1% TX-100 at 4ºC. Cells were pulse-labelled with ^{35}S methionine/cysteine and chased for 40 min, with samples taken at 0, 10, 20 and 40 min.

Fig. 5. Density gradient fractionation of *Leishmania* DRMs.

a, schematic of gradient, illustrating the concentration of Optiprep® in each step. **b,** proteins isolated from 6 gradient fractions, separated by SDS-PAGE and silver-stained. **c**, immunoblot of (b) using anti-GP63 polyclonal antibody. **d**, immunoblot of (b) using anti-HASPB polyclonal antibody. **e**, SDS-PAGE analysis of material extracted from a density gradient of [^{3}H] mannose-labelled cells.

Fig. 6. Density gradient fractionation of inositol-labelled *Leishmania* extracts.

Lipid extracts of fractions from [^{3}H] inositol-labelled cells were separated by HPTLC. The major labelled species, PI and IPC, are shown. PI and IPC were exposed to film for different times (3 and 21 days respectively) to enhance clarity.

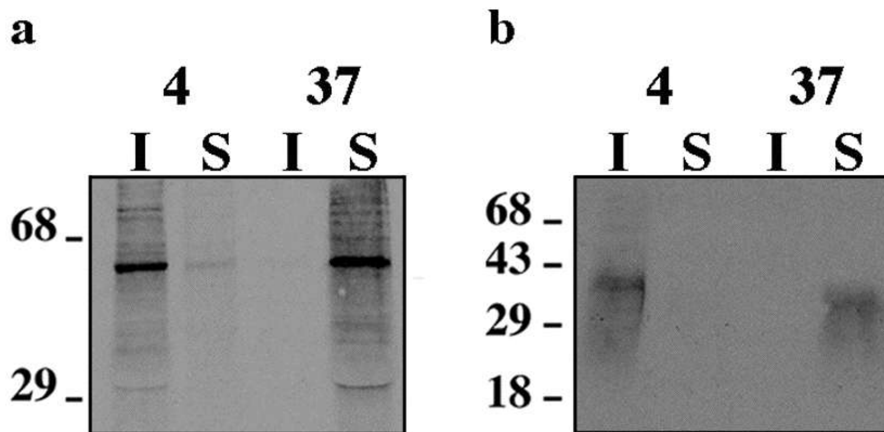

Figure 1, Denny et al 2001

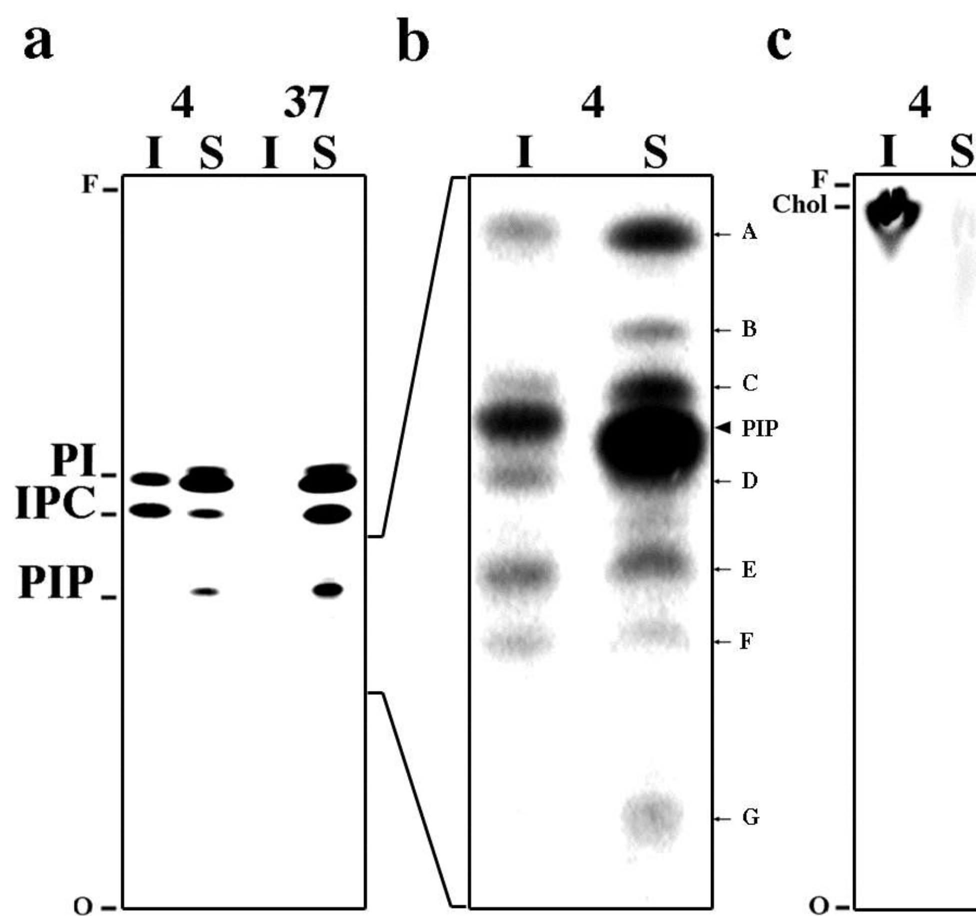

Figure 2, Denny et al 2001

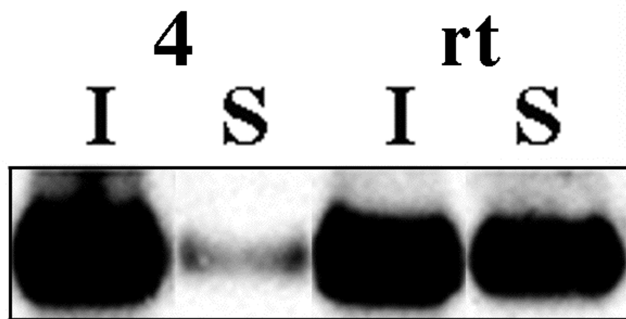

Figure 3, Denny et al 2001

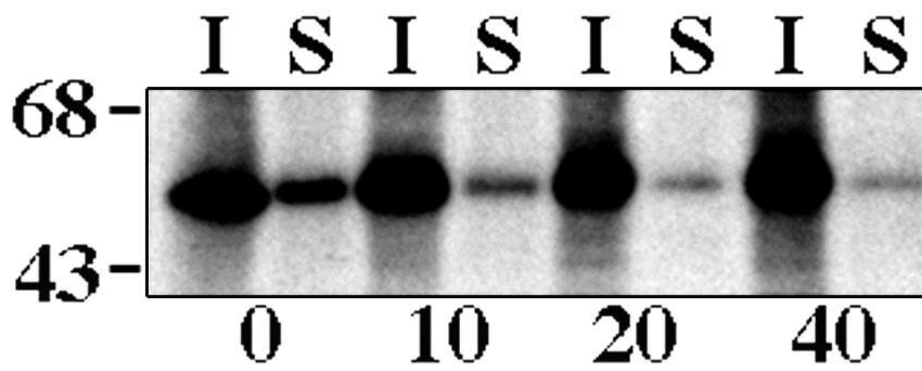

Figure 4, Denny et al 2001

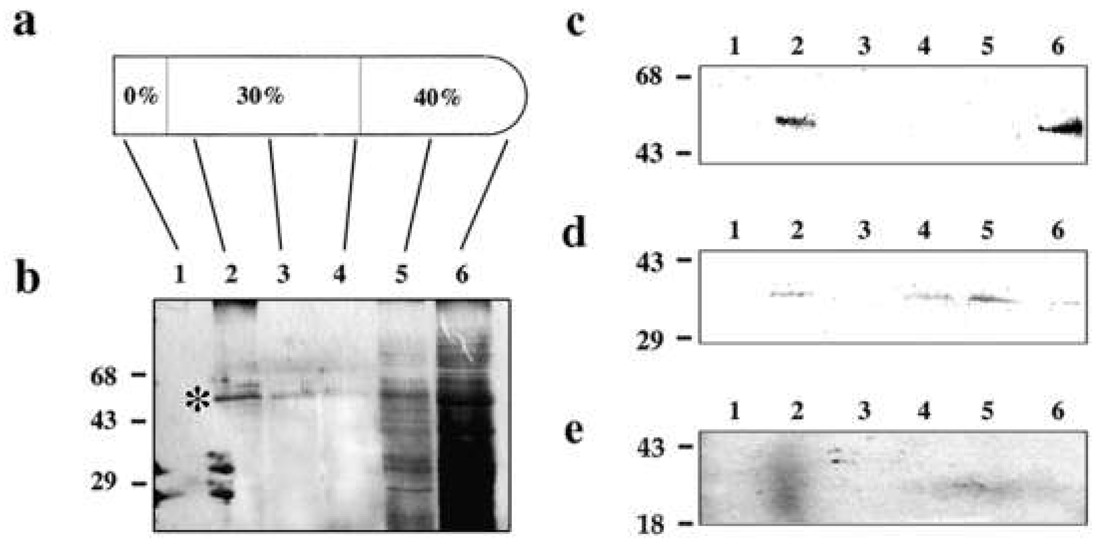

Figure 5, Denny et al 2001

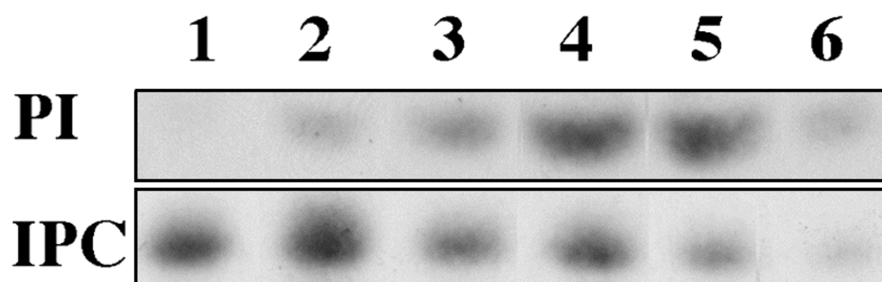

Figure 6, Denny et al 2001