Methyl- β -Cyclodextrin Increases Permeability of Caco-2 Cell Monolayers by Displacing Specific Claudins from Cholesterol Rich Domains Associated with Tight Junctions

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Key Words

Cholesterol • Claudin • Lubrol WX • Occludin • Tight junctions • Triton X100

Abstract

In a previous study we demonstrated that depletion of Caco-2 cell cholesterol results in the loss of tight junction (TJ) integrity through the movement of claudins 3 and 4 and occludin, but not claudin 1, out of the TJs [1]. The aims of this study were to determine whether the major tight junction (TJ) proteins in Caco-2 cells are associated with cholesterol rich, membrane raft-like domains and if the loss of TJ integrity produced by the extraction of cholesterol reflects the dissolution of these domains resulting in the loss of TJ organisation. We have demonstrated that in Caco-2 cells claudins 1, 3, 4 and 7, JAM-A and occludin, are associated with cholesterol rich membrane domains that are insoluble in Lubrol WX. Co-immunoprecipitation studies demonstrated that there is no apparent restriction on the combination of claudins present in the rafts and that interaction between the proteins is dependent on cholesterol. JAM-A was not co-immunoprecipitated with the other TJ proteins indicating that it is resident within a distinct

population of rafts and therefore is likely not directly associated with the claudins/occludin present in the TJ complexes. Depletion of Caco-2 cell cholesterol with methyl- β -cyclodextrin resulted in the displacement of claudins 3, 4 and 7, JAM-A and occludin, but not claudin 1, out of the cholesterol rich domains. Our data indicate that depletion of cholesterol does not result in the loss of the TJ-associated membrane rafts. However, the sterol is required to maintain the association of key proteins with the TJ associated membrane rafts and therefore the TJs. Furthermore, the data suggest that cholesterol may actually directly stabilise the multi-protein complexes that form the TJ strands.

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Introduction

Tight junctions (TJs) consist of a series of multi-protein complexes that associate to form a series of continuous interconnected strands that encircle the apical end of the lateral plasma membrane in epithelial and endothelial cells [2-4]. The TJs not only physically link adjacent cells within an epithelium/endothelium but also form a semi-

permeable barrier between the cells that defines the permeability characteristics of the paracellular pathway. The main physical structure of the tight junctions is composed of tetra-spanning integral membrane proteins that are members of either the claudin or occludin families of proteins [2-4]. Currently little detail is known about the molecular organisation of these proteins within the junctional strands and what factors influence the gross architecture and stability of the junctional network.

Cholesterol is an important structural lipid that is primarily located in the plasma membrane. The bulk of the membrane cholesterol is sequestered with sphingomyelin and glycosphingolipids in dynamic membrane microdomains which were called lipid rafts [5-7] but are now termed membrane rafts [8]. Due to this very distinctive lipid composition these domains are highly ordered compared to the bulk of the membrane rendering them resistant to solubilisation by non-ionic detergents such as Triton X100 [5-7]. This characteristic has been employed to isolate the membrane rafts from cells. The detergent resistant membranes (DRMs) isolated after detergent extraction are an *in vitro* representation of the membrane rafts present within the cell membranes and may not correspond exactly to the pre-existing rafts in cells. Nevertheless, DRMs have proved very useful tools in understanding the characteristics and function of membrane rafts. Because of their unique biophysical characteristics membrane rafts attract and sequester specific classes of membrane associated proteins. These include GPI-anchored membrane proteins (e.g. alkaline phosphatase), double-acylated proteins, such as Src-family of kinases or the α subunits of heterotrimeric G-proteins and transmembrane proteins, in particular ones that has been palmitoylated [5, 6]. Membrane rafts therefore act as platforms to facilitate the association and assembly of specific sets of proteins and as such have been linked with a number of critical intracellular processes including intracellular signalling, vesicular transport and sites of actin assembly [7, 9-11].

The first indication that membrane cholesterol is critical for TJ integrity came from a series of morphological studies that demonstrated the region of plasma membrane immediately surrounding the TJ network is rich in cholesterol [12]. Subsequent research has shown that depletion of membrane cholesterol results not only in the removal of cholesterol from the region of the plasma membrane around the TJ network but also the disruption of the TJs leading to a loss of junctional integrity [13, 14]. We have shown that depletion of membrane cholesterol with methyl- β -cyclodextrin (MCD)

in Caco-2 cells causes the redistribution of claudins 3 and 4 and occludin, but not claudin 1, out of the TJs resulting in the loss of TJ integrity [1]. Although cholesterol is required to maintain TJ organization the exact function of this sterol is currently unknown. Research indicates that occludin, and in particular the hyperphosphorylated form of occludin, is associated with Triton X100 resistant DRMs that are thought to correspond to membrane rafts associated with the TJs [1, 14-18]. Whether claudins are associated these rafts is still a matter of conjecture as there is evidence to support both the inclusion and exclusion of claudins from Triton insoluble DRMs [1, 14-18]. It is also unclear how these domains relate to the tight junctions *in situ* and whether the decrease in junctional integrity and the redistribution of the major TJ proteins produced by depletion of cholesterol reflects the dissolution of these domains.

In this study we have demonstrated that the major TJ proteins in Caco-2 cells are associated with DRMs that can be isolated using the detergent Lubrol WX. Furthermore we present evidence that the DRMs represent the cholesterol rich membrane rafts associated with the TJs *in situ*. We also demonstrate that in Caco-2 cells depletion of cholesterol with MCD results in the displacement of claudins 3, 4 and 7, and occludin, but not claudin 1, out of the tight junction associated rafts. Our data indicate that membrane cholesterol is not required to maintain the TJ-associated membrane rafts but is critical to preserve the association of key integral membrane proteins with these structures and therefore the TJs. Furthermore, the data suggest that cholesterol may actually directly stabilise the multi-protein complexes that form the TJ strands.

Materials and Methods

Materials

Tissue culture media and supplements were purchased from Invitrogen (Paisley, UK). All protease inhibitors were obtained from Calbiochem (Nottingham, UK) and the Lubrol WX was from Serva (Oxford, U.K.). The claudin and occludin antibodies were purchased from Zymed (San Francisco, USA); the flotillin antibody was from Transduction Laboratories (San Diego, USA) whereas the horseradish peroxidase conjugated secondary antibodies were from Bio-Rad (Hemel Hempstead, UK). All other reagents were obtained from Sigma-Aldrich (Poole, UK) unless stated otherwise.

Caco-2 Cell Culture Caco-2 cells were maintained in Modified Eagles Medium (MEM) supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine, 50units/ml penicillin G, 50 μ l streptomycin and 1x non-essential amino acids at 37°C, 5%

CO₂ Cells were fed every 3 to 4 days and subcultured every 7 days. For experimentation, cells (passage 41 to 60) were seeded on Transwell (Corning, USA) permeable polyester filters with a surface area of 4.7cm². The cells on the filters were cultured for 21 days until a fully differentiated confluent monolayer had been formed, which was characterized by a transepithelial electrical resistance (TEER) of between 3500 and 4000 ohms.cm².

Treatment of Cells with MCD, Sphingomyelinase and MCD/Sphingomyelinase

Cells were harvested from 3 Transwells and suspended in MEM which did or did not contain 25mM MCD at 4°C for 30min or were exposed to 1U/ml SM'ase for 1hr at 37°C. In some experiments cell were exposed to 1U/ml SM'ase prior to being treated with 25mM MCD (4°C for 30min). The cells were then solubilised with 0.5% Lubrol and DRMs isolated as described below.

Treatment of Caco-2 Cell Monolayers with MCD

To extract membrane cholesterol from the Caco-2 monolayers the tissue culture media in both the apical and basolateral chambers was carefully replaced with MEM containing 5mM MCD. The monolayers were then incubated at 37°C, 5% CO₂ for 5hr. The cells were then solubilised with 0.5% Lubrol and DRMs isolated as described below.

Extraction of Caco-2 monolayers with Different Detergents

Cells were harvested from Transwell filters and solubilised in DRM isolation buffer (150mM NaCl, 25mM Tris (pH7.4), 2mM EGTA, 2mM NaF, 10mM NaVO₄, 10mM Na pyrophosphate) containing protease inhibitors (10μ/ml aprotinin, leupeptin and pepstatin) and either Triton X100 (1%), Brig 58 (1%), Brij 96 (1%) or Lubrol WX (0.5%) for 30min at 4°C. The cell extract was then centrifuged for 30min at 16,000 x g in an Eppendorf microfuge. Following centrifugation the supernatant was carefully removed and the pellet resuspended in 250μl DRM buffer containing 1% Triton X100 and 0.1% SDS and sonicated. The supernatant (soluble material) and the resuspended pellet (insoluble material) were then analysed by western blot analysis.

Isolation of DRMs

Cells from three transwell filters were scraped into 800μl ice-cold DRM isolation buffer. To this was added 800μl of 1.0% Lubrol WX (in DRM buffer) and the cell suspension left on ice for 30min. Using this protocol the protein to Lubrol mass ratio was approximately 1:5. In the experiments where Triton X100 was used to extract the cells the amount of Triton was adjusted to give the desired protein to detergent mass ratio. The resulting cell extract was then brought to 40% sucrose by addition of 80% sucrose. 3ml of this was placed in the bottom of an ultracentrifuge tube and overlaid with 6ml of 30% sucrose then 3ml of 5% sucrose. All the sucrose solutions were made in DRM buffer and contained 0.1% Lubrol or Triton X100. The

gradients were then centrifuged in a SW41 rotor at 200,000xg for 20hr. After centrifugation the gradient was divided up into twelve 1ml fractions and the pellet suspended in 1ml of DRM buffer.

Immunoprecipitation

The sucrose gradient fractions containing the DRMs (fractions 3, 4 and 5) were pooled, diluted to 12ml with DRM buffer and then centrifuged at 150,000xg for 90min. The resulting DRM pellet was resuspended in 300μl of DRM buffer. For immunoprecipitation, 50μl aliquots of DRM suspension were incubated with or without (control) 5μl of antibody overnight at 4°C. The following morning 50μl of recombinant protein A agarose beads and 50μl of DRM buffer containing 10% (w/v) BSA was added to the incubations. After a further 2hr incubation at 4°C, the protein A beads were pelleted by centrifugation at 2,500xg for 2.5min and washed five times by resuspension in 0.5ml of DRM buffer before being resuspended in 100μl of laemmli sample buffer and boiled for 5min. In some experiments, the immuno-complexes were incubated with 25mM MCB or 25mM MCD complexed to cholesterol (control) for 1hr immediately before addition of the protein A beads.

Preparation of cholesterol/methyl β cyclodextrin complexes

The cholesterol/MCD complexes were produced as described by Pike and Miller [19]. In brief, 6mg of cholesterol was dissolved in 80μl of isopropanol:CHCl₃ (2:1) and MCD (200mg) was dissolved in 2.2ml of water. The MCD solution was then heated to 80°C and the cholesterol added in 20μl aliquots with stirring. The solution was kept at 80°C and continuously stirred until the solution was clear.

Western Blot Analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously [1]. For western blotting, the cell extracts were separated on either 12% or 10% SDS-PAGE gels and electrophoretically transferred onto PVDF membranes, these were then incubated with the appropriate primary antibody. The bound primary antibodies were subsequently detected using a horseradish peroxidase conjugated secondary antibody in conjunction with the ECL detection system (Amersham Pharmacia Biotech). Typical exposure times for the blots were 1-5min.

Measurement of Transepithelial Resistance and Paracellular Permeability: These were measured as described previously [1].

Measurement of Protein and Cholesterol: These were measured as described previously [1].

Statistical Analysis

Statistical analysis was performed by unpaired two-tailed Student's t-test with P<0.05 considered statistically significant.

Results

Influence of Extraction Conditions on DRM Isolation

We began by examining whether it was possible to use Triton X100 to isolate TJ protein containing DRMs from Caco-2 cells (Fig1 A and B). To set the location of the DRMs within the sucrose gradients used to isolate these domains we employed flotillin, a membrane raft associated protein, and cholesterol as markers. Using a Triton to protein mass ratio of 1:10 it was possible to isolate DRMs as demonstrated by the presence of flotillin and cholesterol at the top of the sucrose gradient (gradient fractions 3 through 5). However, no claudin 1 or claudin 4 was present in this region of the gradient, reaffirming that using Triton X100 under these conditions it was not possible to isolate claudin/occludin containing DRMs [1]. When the protein to Triton ratio was decreased to 1:2.5 the amount of flotillin and cholesterol in fractions 3 through 5 increased indicating that the yield of DRMs had improved. In addition, a small proportion of claudin 1 and claudin 4 was now present in the DRM containing fractions. Therefore under these conditions it appears that it is possible to isolate claudin 1 and 4 containing DRMs; however, the yield was poor. As an additional control to monitor the solubilisation of the cell membranes we also examined the distribution of Na/K ATPase a protein known not to associate with membrane rafts in enterocytes [20]. This protein was fully solubilised demonstrating that under the extraction conditions employed we did obtain efficient solubilisation of the cell membranes.

Solubilisation of Claudin 4 with Different Detergents

To try and improve the yield of TJ associated DRMs we examined whether detergents other than Triton X100 might be employed to extract these domains. In order to obtain some indication as to which detergents might prove useful we determined the solubility of claudin 4 after extraction of the Caco-2 cells with a range of detergents that had been successfully used to isolate DRMs in other cell types (Fig. 2). Whilst Triton X100 solubilised the majority of claudin 4 the other detergents examined (Lubrol WX, Brij 58 and Brij 96) only partially solubilised the claudin. Brij 58 and Brij 96 solubilised 55.5% and 47.1% (means from two experiments) of the claudin 4, respectively. In comparison Lubrol WX only solubilised 39.5% of the claudin 4 (mean from two experiments). As Lubrol solubilised the least amount of claudin 4 it was

Fig. 1. Isolation of Claudin Containing DRMs Using Triton: Caco-2 cell monolayers were extracted with Triton X100 at a protein to detergent mass ratio of either 1:10 or 1:2.5 and the DRMs isolated as described in the Methods. Following centrifugation each gradient was divided into 12 x 1ml fractions and each fraction analysed for cholesterol (A) or by Western Blot (B).

chosen for further investigation.

Isolation of TJ Protein Containing DRMs using Lubrol WX

Caco-2 cells were solubilised with Lubrol WX and the extracted DRMs separated by sucrose density centrifugation (Fig. 3). As previously, the location of the DRMs within the gradients was determined by examining the distribution of flotillin and cholesterol. A significant proportion of both flotillin and cholesterol was found to be insoluble and float at the top of the gradients in fractions 3 through 5, indicating that DRMs are present in this region of the gradients. Na/K ATPase was fully

Fig. 2. Solubility of Claudin 4 after Detergent Solubilisation of Caco-2 Cells: Caco-2 cells were solubilised with either Triton X100 (TX), Brij 58 (B58), Brij 96 (B96) or Lubrol WX (LWX) (protein to detergent mass ratio of approximately 1: 5) for 30min before the insoluble material was pelleted by centrifugation. The supernatant (S) and insoluble material (I) were then analysed by Western Blot.

solubilised and remained at the bottom of the gradients demonstrating that the extraction conditions used (mass ratio of protein to detergent of approximately 1:5) were sufficient to solubilise the Caco-2 cell membranes. We then examined the distribution of a range of TJ associated proteins. These proteins can be divided into two distinct groups: integral membrane proteins that either form the TJ barrier (claudins 1, 3, 4, 7 and occludin) or regulate TJ assembly (Jam-A) and cytosolic adaptor proteins (ZO1, ZO2 and ZO3). A significant proportion of claudins 1, 3, 4, and 7, occludin and JAM-A was insoluble (43.2%, 72.5%, 63.4%, 42.9%, 48.1% and 28.2% respectively; means from three three experiments) and present in fractions 3 through 5. In contrast, all the three ZO isoforms were fully solubilised and remained at the bottom of the gradients (data is only shown for ZO1). The co-localisation of the claudins, occludin and JAM-A with the DRM markers is a strong indication that these proteins are associated with DRMs and, unlike Triton X100, Lubrol can be employed to isolate the TJ associated DRMs from Caco-2 cells. It should be noted that we did screen the distribution other claudin isoforms (claudins 2, 5, 7, 8, 15). However, claudins 2, 5 and 15 were not expressed in the Caco-2 cell line used for these studies. Claudin 8 was also expressed but at extremely low levels, which were at the limits of detection by Western Blot, so was not examined further. In the initial experiments the pellets obtained following the density gradient centrifugation were analysed alongside the gradient fractions. Typically less than 1% of the total amount of each claudin isoform or occludin loaded onto a gradient was detected in the pellet (data not shown). As insignificant amounts of the tight junction proteins were present in the pellet, this fraction was not analysed in subsequent studies.

Association of Claudins/Occludin with the DRMs is Sensitive to Depletion of Cholesterol and Sphingomyelin

Next to confirm that the claudins and occludin are associated with DRMs we determined how depletion of cell cholesterol and/or sphingomyelin prior to Lubrol

Fig. 3. TJ Proteins are Associated with Lubrol Insoluble, Cholesterol Rich, Domains. Caco-2 cell monolayers were solubilised in 0.5% Lubrol WX and then centrifuged on a discontinuous sucrose density gradient to isolate the DRMs. Following centrifugation the gradients were divided into 12 x 1ml fractions. Each fraction was then analysed for cholesterol (A) or by Western Blot (B). The amount of cholesterol in each fraction is expressed as a percentage of the total amount cholesterol present in all 12 fractions. The data shown are the means for two separate isolations.

Fig. 4. Retention of Claudins within DRMs is Dependent on Cholesterol and Possibly Sphingomyelin. Caco-2 cell monolayers were harvested and pretreated with either 25mM MCD or 1.0U/ml SM'ase and 25mM MCD before being solubilised in 0.5% Lubrol WX. The cell extracts were then separated on a discontinuous sucrose density gradient. Following centrifugation the gradient was divided into 12 x 1ml fractions. Fractions 3, 4 and 5 were pooled to generate a DRM containing fraction (D) and fractions 9 through 12 pooled to produce a soluble non-DRM fraction (ND). The D and ND fractions were then analysed by Western Blot. The blots shown are representative of four independent experiments.

Fig. 5. Influence of MCD on the Association of TJ Proteins with the DRMs. Caco-2 cell monolayers were treated with 5mM MCD for 5hrs being solubilised in Lubrol. The DRMs were extracted and isolated as described in the Methods. Following centrifugation each gradient was divided into 12 x 1ml fractions and each fraction analysed by western blot. The blots shown are representative of three independent experiments.

extraction influenced the solubility of the proteins (Fig. 4). Cell cholesterol and sphingomyelin were reduced by pretreating the cells with 25mM MCD or 1.0U/ml sphingomyelinase (SM'ase). To reduce both cholesterol and sphingomyelin the MCD and SM'ase treatments were combined. MCD alone totally solubilised occludin and claudins 3, 4, and 7 but only partially solubilised claudin 1. In contrast, SM'ase had no detectable effect on the solubility occludin or claudins 3, 4 and 7. However, the SM'ase did solubilise a proportion of claudin 1. When MCD was combined with SM'ase all the TJ proteins were solubilised. These findings confirm that the TJ proteins reside in the DRMs within Caco-2 cells. Moreover they demonstrate that the association of occludin and claudins

3, 4 and 7 with the DRMs is dependent on cholesterol, whilst the association of claudin 1 is dependent on cholesterol and sphingomyelin.

MCD Depletion of Cholesterol Displaces Certain TJ Proteins from the DRMs

We have previously shown that depletion of Caco-2 cell membrane cholesterol with MCD produces a movement of claudin 3, claudin 4 and occludin, but not claudin 1, out of the TJs resulting in a loss of junctional integrity [1]. We therefore examined whether these changes could result from the loss of claudin/occludin association with the DRMs. Incubation of Caco-2 monolayers for 5 hrs with 5mM MCD resulted in the

Fig. 6. Influence of MCD on the Distribution of TJ Proteins within the Sucrose Gradients. Caco-2 cell monolayer were treated with 5mM MCD for 5hrs being solubilised in Lubrol. The DRMs were extracted and isolated as described in the Methods. Following centrifugation each gradient was divided into 12 x 1ml fractions and each fraction analysed by Western Blot. Following densitometric analysis of the western blots the amount of each protein present in (a) fractions 3 to 5 (i.e protein associated with DRMs), (b) fractions 6 to 8 and (c) fractions 9 through 12 (i.e. soluble non-DRM associated protein) was calculated and then expressed as a percentage of the total amount of each protein present in the whole gradient, i.e. all twelve gradient fractions. The data shown are the mean $\pm$ SEM from three separate experiments. * $p < 0.05$, ** $p < 0.01$ versus untreated controls.

extraction of 40 to 45% of the cell membrane cholesterol and a drop in the monolayer TEER from 4050 $\pm$ 40 ohms.cm² (controls) to 250 $\pm$ 30 ohms.cm² in treated monolayers (means $\pm$ SEM from three experiments). When we examined the Lubrol solubility of the TJ proteins (Figs 5 and 6), pretreatment with MCD was found to cause a significant reduction in the amount of claudins 3, 4, and 7, occludin and JAM-A in fractions 3, 4 and 5 (DRM containing fractions). This was accompanied by a significant increase in the amount of the three proteins in the fractions 9, 10, 11 and 12 (non-DRM, soluble, fractions). Cholesterol removal therefore caused a loss of association with the DRMs. It should also be noted that the MCD treatment also appeared to produce a pronounced reduction in the amount of claudin 7, no similar response was noted for the other TJ proteins examined. MCD only produced a minor redistribution of claudin 1. There were insignificant drops in the amount of claudin 1

in both the DRM and non-DRM fractions that was accompanied by a small but significant increase in the intermediate fractions. These data indicate that MCD treatment of the Caco-2 cell monolayers leads to a significant displacement of JAM-A, claudins 3, 4, 7 and occludin, but not claudin 1, out of the DRMs. The retention of claudin 1 within the DRMs after MCD treatment also demonstrates that the depletion of cholesterol does not result in the dissolution of these domains.

Organisation of the TJ Proteins within the DRMs

The data presented in Figs 4, 5 and 6 demonstrate that, unlike the other three claudin isoforms, the association of claudin 1 with the DRMs is not solely dependent on cholesterol. One explanation for this disparity is that there are two populations of DRMs, one that harbours claudin 1 the other claudins 3, 4 and 7, which respond differently to MCD. To address this we

Fig. 7. Co-immunoprecipitation of Claudin/Occludin from the DRMs. DRMs were immunoprecipitated as described in the Methods before being analysed by Western Blot (A). In other experiments the immunocomplexes were exposed to 25mM MCD (+) or 25mM MCD complexed to cholesterol (-) for 2hr before being analysed by Western Blot (B) The blots shown are representative of 3 experiments.

conducted a series of immunoprecipitations (Fig. 7A). These studies showed that one always observed the co-immunoprecipitation of the four claudin isoforms and occludin. Although the signal obtained with claudin 7 appeared significantly less than the other 4 TJ proteins. In addition, no JAM-A co-immunoprecipitated with the claudins (data not shown). This indicates that claudins 1, 3, 4 and 7 and occludin, but not JAM-A, can all reside within the same population of DRMs.

Finally, in order to gain insight into the role of cholesterol in the maintenance of TJ protein interaction and TJ integrity we determined what, if any, influence MCD (25mM for 1hr) treatment of the immunoprecipitated DRMs had on the co-immunoprecipitation of the TJ proteins. Representative western blots of the immunoprecipitation are shown in Fig. 7B. In brief, treatment of the DRMs immunoprecipitated with claudin 1 had no effect on the amount of claudin 1 in the immunocomplexes but dramatically reduced the amounts of the other three TJ proteins. The same pattern of changes was observed for the DRMs immunoprecipitated with claudin 4. MCD treatment had no effect on the amount of claudin 4 in the immuno-complexes, however, in this instance depletion of cholesterol did result in the total loss of the other three TJ proteins. The situation with the DRMs immunoprecipitated with claudin 3 is slightly more complex. In this case MCD treatment did produce a reduction in the amount of claudin 3 in the immunocomplexes. This was accompanied by a disproportionately larger decrease in the amounts of claudins 1 and 4 and occludin. These results demonstrate that removing cholesterol from the DRMs results in a reduction in the interaction between the TJ proteins. It should be noted that in the controls for these studies the immunocomplexes were incubated with 25mM MCD bound to

cholesterol to eliminate any non-specific influence of the MCD.

Discussion

In a previous study we demonstrated that the detergent Triton X100 could not be employed to isolate TJ associated DRMs from Caco-2 cells [1]. This observation was somewhat unexpected given that Triton X100 had been successfully used to isolate similar domains from both MDCK and T84 cells [14-18]. We were therefore curious to determine the factors behind this disparity. Since the publication of our original paper, research has demonstrated that the conditions used to isolate a particular population of DRMs are dependent on a number of factors including the cell type being investigated and the extraction conditions, or more specifically the type and concentration of detergent, being employed [21-23]. Consequently our inability to obtain the claudin/occludin containing DRMs from the Caco-2 cell using Triton X100 might reflect the extraction conditions we had employed. Previously we used a protein to detergent mass ratio of approximately 1:10, as has been recommended by recent review articles [21, 22]. If the TJ associated DRMs in Caco-2 cells are particularly sensitive to solubilisation by Triton X100, using such a high protein to detergent ratio could have resulted in the complete solubilisation of the TJ associated DRMs. To test this hypothesis we decreased the stringency of the extraction conditions and dropped the protein to detergent ratio used to 1:2.5. Under these conditions we did observe a small proportion of claudins 1 and 4 associated with DRMs indicating that, as postulated, the TJ associated DRMs in Caco-2 cells are sensitive to solubilisation by Triton X100. The yield of

the DRMs might be improved by further decreasing the protein to Triton mass ratio used for extraction. However, this might prove problematic as this has the potential to generate artifacts due to the inefficient solubilisation of the cells' plasma membranes. Although, the use of Triton X100 is not ideal for isolating the TJ protein associated DRMs from Caco-2 cells it has been employed to isolate these structures from other cells [14-18]. This suggests that in other cell types the TJ associated DRMs are more resistant to solubilisation by Triton X100 than the equivalent domains in Caco-2 cells. This disparity probably reflects the innate differences in the organisation and/or lipid/protein content of the plasma membrane known to exist between cell types.

As demonstrated by the solubility study presented in Figure 2 it may be feasible to use a number of detergents to isolate the TJ associated DRMs from Caco-2 cells. However, based on the results of the solubility study we choose to focus our attention on Lubrol WX. This decision was also influenced by the fact that this detergent has been successfully used to isolate Triton sensitive DRMs from a number of cells [24, 25]. Using Lubrol WX we demonstrated it is possible to isolate TJ protein containing DRMs from Caco-2 cells. We examined a range of TJ proteins, which can be divided into two distinct groupings. The first group are integral membrane proteins and are either involved in the assembly of the TJ network (JAM-A) [2-4] or form the main physical structure of the TJs (claudins 1, 3, 4 and 7 and occludin) [2-4]. The second group consists of three cytosolic adaptor proteins (ZO isoforms) that link the integral membrane proteins that form the TJ strands to either the actin cytoskeleton or the cytosolic protein plaque associated with the TJ network [2-4]. A significant proportion of each of the TJ associated integral membrane proteins was found to be associated with the Lubrol insoluble DRMs. Although we employed flotillin as a marker to indicate the position of the TJ associated DRMs in the sucrose gradients, the data indicate that only a portion of this protein may be associated with these domains. Curiously, the flotillin containing DRMs appear to be extracted most efficiently when TritonX100 (low protein to detergent ratio) rather than when Lubrol WX was employed to solubilise the cells. One explanation for this is that flotillin is actually associated with a number of different classes of DRMs that have different solubilities in Triton X100 and Lubrol WX.

Unlike the claudins, JAM-A and occludin, none of the three ZO isoforms were observed to be associated with the Lubrol insoluble DRMs. As the ZO proteins are

cytosolic, the association of these proteins with the DRMs will depend entirely on their interaction with integral membrane proteins (claudins, JAM-A and occludin) embedded in the DRMs. Obviously if this interaction is broken during the extraction of the DRMs the ZO proteins would no longer be tethered to these structures and would be rendered soluble. Unfortunately this would appear to be the case in this study. Using Triton X100 to isolate the TJ associated DRMs it has been possible to isolate ZO-1 associated with occludin contained DRMs [14]. Therefore by careful modification of the Lubrol extraction buffers it may be possible to maintain the interaction between the ZO proteins and the claudins/occludin and so the association of the ZO proteins with the DRMs. We did not determine whether other adaptor proteins such as MUPP1 and PATJ [2-4] are associated with the DRMs as it is likely that, as observed for the ZO isoforms, the extraction conditions would also result in the loss of interaction between these proteins and the claudins/occludin present in the DRMs. Although none of the ZO protein were associated with the isolated DRMs it is likely that these proteins are associated with the TJ-associated membrane rafts *in situ*, albeit indirectly through the interaction of the adaptor proteins with the claudins/occludin resident within the rafts.

A key question is how do the Lubrol insoluble DRMs relate to the TJ network *in situ*? A number of studies have demonstrated that the TJ network in epithelial cells resides within a region of the plasma membrane that is rich in cholesterol [12, 13]. Furthermore, there is evidence that the cholesterol rich regions of the plasma membrane associated with the TJs are membrane rafts [14-18]. Therefore it is logical to assume that the DRMs containing the TJ proteins isolated from the Caco-2 cells are representative of the membrane raft domains associated with TJs. However, as mentioned in the introduction the use of detergents to isolate membrane rafts from cell membranes can be fraught with problems and one has to be confident that the DRMs isolated after detergent solubilisation do represent raft domains that are actually present in the cell membranes before extraction and not artificially generated by detergent solubilisation of the cells. One concern with using a relatively weak detergent such as Lubrol WX is that artifactual DRMs can be generated through incomplete solubilisation of the cells membranes. To eliminate this possibly we routinely determined the solubility of the Na/K ATPase, as mentioned in the results this is a plasma membrane protein that is not associated with membrane rafts in intestinal epithelial cells [20]. The extraction conditions employed

with Lubrol WX resulted in the solubilisation of the Na/K ATPase demonstrating that we had obtained complete solubilisation of the cells membranes. We also showed that extraction of cholesterol and hydrolysis of sphingomyelin prior to detergent extraction of the Caco-2 cells resulted in the total solubilisation of the TJ proteins and the loss of association with the DRMs indicating that the TJ proteins are associated with cholesterol rich membrane raft domains in the intact cells. Finally, and probably most important, depletion of cholesterol with MCD produced the displacement of the same TJ proteins from the DRMs as we previously observed for the TJs. (i.e. claudins 3 and 4 and occludin are displaced, whilst claudin 1 is retained within the DRMs and TJs) this provides direct evidence that the Lubrol insoluble DRMs are representative of the cholesterol rich membrane environment that surrounds the TJ network in situ [13, 14] and provides further evidence that membrane rafts are associated with the epithelial TJ network in situ.

One interesting and unexpected finding of the cholesterol and sphingomyelin depletion experiments was that the retention of claudin 1 within the DRMs/membrane rafts, unlike the other TJ proteins, is not solely dependant on cholesterol but also requires sphingomyelin. One very simple explanation for this disparity is that claudin 1 is resident within a separate population of membrane rafts that is resistant to MCD treatment. However, this does not appear to be the situation as the results of the co-immunoprecipitation studies demonstrate that claudins 1, 3, 4, 7 and occludin can reside together in the same DRM. If this is the case why is claudin 1, but not claudins 3, 4, 7 retained within a membrane raft after MCD treatment? Given that the claudins represent a very highly conserved family of proteins it is unlikely that the retention of claudin 1 is due to a unique structural element within the protein itself. Alternatively, claudin 1 might be retained through its interaction with an external factor. Studies examining the movement of specific proteins both in and out of membrane rafts in other cell systems have identified several factors, other than retention signals present within the proteins themselves, which influence the association of proteins with the membrane rafts [5-7, 26]. Factors that might aid the retention of claudin 1 include association with the actin cytoskeleton or interaction with other integral membrane proteins resident within the membrane rafts. In this regard the results of our previous study did demonstrate that claudin 1 unlike the other TJ proteins examined did remain associated with a Triton insoluble, cytoskeleton associated, cell fraction following MCD treatment [1]. However, to date there is no indication

that there is any difference in the way claudin 1 and claudins 3, 4 and 7 are associated with the cytoskeleton. Furthermore, if retention of claudin 1 within the rafts is dependent on the cytoskeleton one might expect to observe the movement of claudin 1 out of these domains once the Caco-2 cells had been solubilised and the interaction of claudin 1 with the ZO isoforms and thereby the protein's association with the cytoskeleton had been disrupted. Clearly this does not occur. In addition, pretreatment of the Caco-2 cells with cytochalasin D to disrupt the actin cytoskeleton prior to Lubrol solubilisation had no influence on the DRM association of claudin 1 (data not shown). Therefore it is unlikely that the actin cytoskeleton is a factor in tethering claudin1 to the membrane rafts; a more likely explanation is that claudin 1 is retained within the rafts due to its interaction with an as yet unidentified membrane associated protein or proteins present in the rafts. Further research is required to prove this hypothesis and identify the protein(s) involved.

If, as indicated, claudin 1 is tethered within the membrane rafts and therefore remains associated with the TJs it can be considered as relatively static. In contrast the other TJ proteins would appear to be relatively free to move out of, and possibly back into, the membrane rafts and therefore the TJs and so are relatively dynamic. When first identified TJs were considered inert structures; however, subsequent research has demonstrated that TJs are actually highly active entities [27]. Furthermore rapid changes in the composition of claudins within the TJs may be an important mechanism whereby the permeability of the paracellular pathway of an epithelium/endothelium can be regulated in response to changes in extracellular environment or extracellular stimuli. It appears that the TJ network may actually consist of two components. A relatively static component that is responsible for maintaining the barrier properties (i.e. macromolecular permeability) of the TJs and a dynamic component that controls the ionic permeability properties of the TJs. Our findings seem to support this model. This is even further emphasized when one considers that the main function of claudin 1 appears to be to maintain barrier function by blocking the flux of macromolecules through the paracellular pathway [28], whilst claudins 3, 4 and 7 have all be linked with controlling the ionic permeability properties of the TJs [29, 30]. Our results suggest that the way a TJ protein is associated with the membrane rafts may have a direct impact on its interaction with the TJs. Moreover, the TJ associated membranes rafts may be an important component of the mechanism(s) by which

cells can rapidly modulate the protein composition of TJ junction network, and therefore the permeability of the paracellular pathway, in response to physiological stimuli.

JAM-A was not co-immunoprecipitated with claudins 1, 3 or 4 indicating that this protein is not present in the same population of DRMs/membrane rafts as the other TJ proteins. This finding is not too surprising as JAM-A, unlike claudins 1, 3 and 4, is not a structural component of the TJ network [2-4] but rather controls the assembly of the TJs [31, 32]. Our data suggests that JAM-A resides within a separate compartment of the junctional complex than the TJs and is consistent with the results of a recent study that biochemically dissected the junctional complex in MDCK cells [33]. This fact would also suggest that any influence cholesterol has on the interaction of the claudins/occludin is not mediated through JAM-A. Unlike JAM-A, claudin 7 was co-immunoprecipitated with the other three claudin isoforms; however, the western blot data indicated that the amount of claudin 7 co-immunoprecipitated was much less than for the other claudin isoforms and occludin. If correct this might suggest that in situ only a small proportion of claudin 7 is associated with the other claudin isoforms in the TJ associated membrane rafts and therefore the TJ network. Recent morphological data from both kidney and intestine which has demonstrated that claudin 7 is not concentrated within the TJs but is present along the basolateral plasma membrane [34, 35]. Our results are consistent with this distribution as one would only expect a small proportion of claudin 7 to be associated with the claudins/occludin present in the TJ network.

Although a number of studies have now demonstrated that cholesterol is required to maintain the organization and therefore the integrity of TJs in epithelial cells [1, 13, 14] the exact function of the sterol is still unclear. Our data demonstrate that depletion of cholesterol results in the displacement of specific TJ proteins from both the TJs themselves and the membrane rafts associated with the TJ network. This indicates that association of TJ proteins with the membrane rafts is key in preserving the interaction of the proteins with the TJs. However, cholesterol does not simply act to stabilize the membrane rafts as our data clearly demonstrates that these structures are still present following MCD treatment. Therefore the displacement of the TJ proteins from the TJs does not result from the dissolution of the membrane rafts. Although removal of cholesterol did not result in the loss of the TJ associated membrane rafts the reduction of this sterol will undoubtedly have an impact on the

biophysical characteristics and therefore functionality of these domains. As mentioned in the introduction one of the main functions of rafts is to facilitate the interaction and assembly of specific sets of proteins [5-7]. Therefore cholesterol may be acting to facilitate and/or stabilise the interactions between the integral membrane proteins (i.e. occludin and claudins) within the multi-protein complexes that form the TJ strands. This explanation is consistent with the redistribution data and with the findings from of the co-immunoprecipitation studies examining the influence of MCD on the isolated DRMs. These experiments showed that exposure of the DRMs to MCD resulted in reduction or total loss of interaction between the TJ proteins. In addition, as the claudins/occludin present within the DRMs do not appear to retain their association with the cytosolic adaptor proteins, any influence cholesterol has on the interaction of the claudins/occludin would appear to be direct and not mediated through the cytoskeleton or cytoplasmic protein plaque that is associated with TJs in situ. In addition, as JAM-A is not present in the same population of DRMs as the claudins/occludin it is very unlikely that the cholesterol influence is exerted through this protein. An intriguing possibility is that the TJs are actually akin to Tetraspanin webs [36]. In these structures cholesterol itself promotes/stabilises the interaction of the tetraspanin proteins to help form large multi-protein arrays [36]. In this scenario it is cholesterol and not the rafts *per se* that is the critical element which is responsible for preserving the association of the TJ proteins with the TJs and therefore the integrity of the TJ network. Currently additional studies are underway to test this hypothesis. In addition, if cholesterol is critical to maintain the integrity of the TJ complexes it would explain why it has been extremely difficult to isolate the complexes using standard protocols based on the use of detergents.

In conclusion, the results of this study indicate that the membrane proteins that form the main structural elements of epithelial cell tight junctions reside within cholesterol enriched membrane raft domains. Retention of the proteins within these structures is dependent primarily on cholesterol but, at least in the case of claudin 1, possibly also sphingomyelin. Cholesterol is not required to maintain the TJ associated rafts but is key in preserving the association of key TJ proteins with the membrane rafts and therefore the TJs. Our data suggest that the sterol may directly maintain the TJ complexes by stabilising the interaction between the integral membranes proteins that form these structures.

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