The Lysosomal Trafficking of Prosaposin

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Abstract

Prosaposin is a 65 kDa glycoprotein that can either be targeted to the lysosomal compartment or further glycosylated to a 70 kDa form and secreted from the cell. In the lysosome, prosaposin is cleaved into four individual proteins known as saposins A, B, C and D that function as activators of sphingolipid degradation. Most soluble lysosomal proteins use the mannose 6-phophate receptor to traffic to the lysosome. However, several proteins including prosaposin have been shown not to use this mechanism. Since prosaposin binds several types of sphingolipids and that lipids are involved in the trafficking of proteins to various cellular destinations, we tested the hypothesis that these lipids mediate the trafficking of prosaposin to the lysosomes. Using inhibitors of sphingolipid synthesis in conjunction with mutagenic analysis and dominant negative competition with chimeric constructs, we demonstrated that the D functional domain of prosaposin interacts with sphingomyelin and that the carboxy-terminal region of prosaposin binds a sorting receptor. We also demonstrated that prosaposin binds to sortilin, a recently identified lysosomal sorting receptor. In conclusion, our research unfolded a novel mechanism of lysosomal transport involving a protein-lipid interaction and the sorting receptor sortilin.

Resume

La prosaposine est une glycoprotéine de 65 kDa qui peut être visée au compartiment lysosomale ou au glycosylé supplémentaire à une forme du kDa 70 et être sécrétée de la cellule. Dans le lysosome, le prosaposine est fendu dans quatre différentes protéines connues sous le nom de saposins A, B, C et D qui fonctionnent comme activateurs de dégradation de sphingolipide. La plupart des protéines lysosomale solubles emploient le récepteur du mannose 6-phophate pour trafiquer au lysosome. Cependant, plusieurs protéines comprenant le prosaposine ont été montrées pour ne pas employer ce mécanisme. Puisque le prosaposine lie plusieurs types de sphingolipids et cela des lipides sont impliqués dans le trafic des protéines à de diverses destinations cellulaires, nous avons évalué l'hypothèse que ces lipides négocient le trafic du prosaposine aux lysosomes. En utilisant des inhibiteurs de la synthèse de sphingolipide en même temps que l'analyse mutagénique et la concurrence négative dominante avec les constructions chimériques, nous avons démontré que le domaine fonctionnel de D du prosaposine agit l'un sur l'autre avec des sphingomyélines et que la région de carboxy du prosaposine lie un récepteur assortissant. Nous avons également démontré que le prosaposine lie au sortilin, un récepteur assortissant lysosomale récemment identifié. En conclusion, notre recherche a dévoilé un mécanisme de transport lysosomale impliquant une interaction de protéine-lipide et le récepteur sortilin.

To my wife, who has been supportive for many years. She has been a source of inspiration and love. Thank you.

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Abbreviations

cDNA: complementary DNA

D609: tricyclodecan-9-yl xanthate potassium salt

PDMP: dl-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol-HCL

FB_1: Fumonisin B_1

CHO: Chinese Hamster Ovary Cells

NRK: Normal Rat Kidney Cells

M6P-R: Mannose 6-phosphate Receptor

SL: Sphingolipid

GSL: Glycosphingolipid

Sm: Sphingomyelin

LAP: Lysosomal Acid Phosphatase

LAMP-1: Lysosomal Associated Membrane Protein-1

LAMP-2: Lysosomal Associated Membrane Protein-2

LEP100: Lysosomal Endosomal Protein

LIMP-2: Lysosomal Integral Membrane Protein-2

FITC: fluorescein isothiocynate

TRITC: Tetramethylrhodamine isothiocynate

TGN: Trans-Golgi Network

NK-Lysin: Natural-Killer Cell Lysin

kDa: kiloDalton

$G_{M2}AP$: G_{M2} Activator Protein

MVBs: Multivesicular bodies

GGA: Golgi localized, gamma ear containing, ARF binding protein

COPI: Cytosolic Coat Protein I

PIP2: Phosphatidylinositol 4,5 bisphosphate

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Introduction

Prosaposin is a highly conserved protein involved in a variety of biological processes such as sperm maturation, sphingolipid degradation and cellular differentiation (Morales et al., 1995). Two mature forms of prosaposin have been identified. Both forms are the product of the same gene (Igdoura and Morales, 1995). The 65 kDa form is targeted to the lysosome but can be further glycosylated in the Golgi apparatus to a 70 kDa form that is secreted. The 70 kDa form is found in several body fluids, such as milk (Kondoh et al., 1991), cerebrospinal fluid (O'Brien et al., 1994), seminiferous tubule fluid (Igdoura and Morales, 1995) as well as in the secretion of prostate gland (Morales et al., 1996).

The 65 kDa protein is targeted to the lysosomal compartment and is the precursor of four smaller non-enzymatic proteins that function as activator proteins for sphingolipid degradation (Morimoto et al., 1990). The function of lysosomal saposins is to either solubilize certain membrane glycolipids or to form complexes with lysosomal enzymes and/or their glycolipid substrates to promote their hydrolysis (Kretz et al., 1990).

The trafficking of soluble lysosomal proteins usually depends on the mannose 6-phosphate receptor (M6P-R). However, prosaposin has been shown to interact with membranes in a M6P-R independent manner (Igdoura et al., 1996) and cells from patients with I cell disease have near normal levels of prosaposin as well as other lysosomal proteins despite a mutation in the enzyme that adds phosphate groups to mannose residues of lysosomal proteins (Rijnboutt et al., 1991). This suggests the existence of a novel trafficking mechanism to the lysosomal

compartment.

It has been shown that lipids are able to traffic proteins to various cellular locations including the basolateral compartment (Mays et al., 1995). Prosaposin has been shown to bind several lipids including glycosphingolipids and sphingomyelin (Ciaffoni et al., 2001; Hiraiwa et al., 1992). Based on this fact we have proposed that sphingolipids may participate in the trafficking of prosaposin to the lysosomal compartment. To test this hypothesis, we used compounds that have been shown to block the synthesis of various sphingolipids.

A previous study in our laboratory suggested that the D domain along with the adjacent carboxy-terminal region of prosaposin were required for its transport to the lysosomes (Zhao and Morales, 2000). This lead to the hypothesis that these two regions of prosaposin must bind to a sorting receptor so that prosaposin can be targeted to the lysosomes. Recently, a novel Golgi sorting receptor was identified. This 95 kDa trans-membrane protein was called sortilin. Although no lysosomal proteins have been shown to bind sortilin, the cytosolic tail of this protein contains information that allows it to traffic to the lysosome. Hence we tested the hypothesis that the lysosomal trafficking of prosaposin is mediated by sortilin

Review of the Literature

Objectives

Prosaposin is a resident lysosomal protein; therefore the first objective of this review is to discuss the structure, function and biogenesis of lysosomes. Next, the review of the targeting pathways for lysosomal proteins including soluble hydrolases and lysosomal membrane proteins will be discussed. The trafficking to lysosomes is a complex process involving several proteins. These proteins will be introduced and their mechanism of action reviewed.

Finally, the focus will shift to the sphingolipid activator proteins (SAPs), prosaposin and G_{M2} activator protein. The discussion will include a review on the biosynthesis and intracellular processing of SAPs, tissue distribution and possible roles of these proteins in both health and disease.

Structure and Functions of Lysosomes

Lysosomes are intra-cytoplasmic acidic organelles containing more than 60 hydrolases involved in the degradation of both extracellular and intracellular materials (Morales et al., 1995; Steinman et al., 1983; Suzuki, 1995). Lysosomes are involved in a range of cellular processes including antigen presentation, bone remodeling and regulation of growth factors (Dell'Angelica et al., 2000b). Morphologically, lysosomes are extraordinarily diverse in shape and size but are typically spherical structures of various sizes (0.2-0.4um in diameter), with electron-dense content (Morales et al., 1999), acidic pH (Maxfield, 1982), enriched in acid phosphatases (Bainton, 1981; Steinman et al., 1983) and can be easily identified immunocytochemically (Morales et al., 1999). Endosomes, on the other hand, are

electron-lucent, acid-phosphatase negative elements (Steinman et al., 1983). Multivesicular bodies (MVBs) are intermediate structures between endosomes and lysosomes (Morales et al., 1999) that contain membrane bound structures containing numerous vesicles that pinch off from their delimiting membrane. A clear distinction between late endosomes and early MVBs is sometimes difficult. Lysosomes have a unique surrounding membrane that contains transport proteins that allow the final products of hydrolysis to diffuse to the cytoplasm. The lysosomal membrane also contains a H^+ pump that utilizes ATP to pump H^+ into the lysosome, creating and maintaining an acidic (~ pH 5) environment (Stevens and Forgac, 1997). Other lysosomal membrane proteins are the protective membrane glycoproteins (Fambrough et al., 1988; Ogata and Fukuda, 1994; Rohrer et al., 1996) that are heavily glycosylated to protect the wall of the lysosome and prevent other cellular structures from degradation by lysosomal hydrolases (Sandhoff, 1996). In addition, lysosomes receive directly from the Golgi apparatus, soluble proteins such as hydrolases as well as activator proteins such as the GM2 activator protein and prosaposin (O'Brien and Kishimoto, 1991; Sandhoff, 1996). These proteins function in the degradation of proteins, lipids and carbohydrates within the lysosomes.

Lysosomes are formed by 3 mechanisms: 1) bulk and receptor-mediated endocytosis involving plasma membrane flow by means of pinocytotic vesicles, which mature into endosomes and multivesicular bodies and then finally lysosomes known as endocytic flow (Geuze et al., 1983); 2) phagocytosis of particulate matters such as bacteria, erythrocytes and cellular debris (Morales et al., 1999; Steinman et al., 1983); 3) autophagy involving ER membrane flow through autophagosomes (Tang et

al., 1992). Although endocytosis is a mechanism that exists in most cells (Steinman et al., 1983), phagocytosis occurs mainly in professional phagocytes such as macrophages and Sertoli cells (Steinman et al., 1983). Autophagy occurs in most cells although the formation of lysosomes via this mechanism is not always evident.

The formation of lysosomes occurs most likely by a process of maturation ("maturational model"). The maturational model, proposes that endocytic vesicles fuse with themselves to yield endosomes. The trafficking of proteins from the Golgi apparatus to these endosomes, initiates their transformation from endosomes to lysosomes. This model predicts that endosome, MVBs and lysosomes are transient structures. To test this model, two weak bases, NH_4Cl and leupeptin were used to perturb the progression of these structures. NH_4Cl diffuses to the lysosomes and prevents acidification of the luminal compartment of endosomes, MVBs and lysosomes (Maxfield, 1982; Poole and Ohkuma, 1981). This change in pH prevents the catabolism of molecules and in turn prevents the turnover of MVBs into mature lysosomes (Morales et al., 1999). Leupeptin, which is known to inhibit cysteine proteases such as cathepsin B, H and L (Aoyagi et al., 1969; Kirschke et al., 1976), produced an accumulation of undegraded proteins in lysosomes but not in endosomes (Katunuma and Kominami, 1983). Leupeptin also prevented the turnover of lysosomes and induced morphological changes characterized by the retention of intraluminal membranes suggesting that this compound interfered with the transformation of MVBs to lysosomes (Morales et al., 1999). These two compounds provided support for the maturational model of lysosomal formation. However, conclusive evidence for the maturational model came from three mice models with

genetic defects in sphingolipid metabolism. These mice had defects in β-hexosaminidase A (Tay-Sachs), β-hexosaminidase B (Sandhoff disease) (Phaneuf et al., 1996) or prosaposin (Fujita et al., 1996). The rationale for using cells from these mice was that these mutations interfered with the catabolism of sphingolipid and perturbed the endosomal/lysosomal progression. In all three of the mice models, the number of MVBs was significantly increased and mature lysosomes absent(Morales et al., 1999). This model demonstrated that MVBs were intermediate elements between endosomes and lysosomes and that both MVBs and lysosomes were transient structures (Morales et al., 1999).

Lysosomal Proteins

The proteome of the lysosome includes four classes of proteins. They are the soluble hydrolases, protective membrane proteins, membrane hydrolases and the activator proteins (Morales et al., 1999).

The soluble hydrolases comprise a large group of proteins that are synthesized as pre-proenzymes (Hasilik, 1992). The precursors undergo asparagine-linked glycosylation and carbohydrate processing which is completed in the Golgi apparatus. The glycosylation prepares the proteins for transport to the lysosomal compartment and may protect them from degradation within the lysosomal compartment (Bohley and Seglen, 1992). A large number of soluble hydrolases are peptidases. This group may be divided into endo and exo peptidases depending on their location of cleavage. The endopeptidases group is composed mainly of cysteine (Cathepsin B) (Mort and Buttle, 1997) and aspartic (Cathepsin D) (Bohley and Seglen, 1992) proteases, while the exopeptidase group is composed of cysteine and

serine proteases.

A different group of lysosomal proteins are the lysosomal protective membrane proteins. These are highly glycosylated integral membrane proteins. The best-studied protein of this group of proteins is the Lysosome Associated Membrane Protein-2 (LAMP-2). It consists of a short cytoplasmic tail, a trans-membrane domain and a large luminal domain (Pillay et al., 2002). LAMP-2 is found abundantly in the lysosomes and is considered a type I trans-membrane protein. LAMP-2 may participate in the aggregation of soluble lysosomal content.

A second type of integral membrane protein is the lysosomal acid phosphatase (LAP). This membrane hydrolase was crucial in the discovery of lysosomes by de Duve and is widely used as lysosomal marker. LAP is synthesized as a membrane bound precursor and transported to the lysosomes (Braun et al., 1989). In lysosomes, a soluble mature LAP is released by limited proteolysis (Gottschalk et al., 1989). In vitro, LAP is involved in the cleavage of various phosphomonoesters such as adenosine monophosphate and glucose 6-phosphate (Gieselmann et al., 1984). The *in vivo* function of LAP is not fully understood as LAP deficient mice had normal levels of phosphomonoesters (Suter et al., 2001) .

Activator proteins compose the final group of lysosomal proteins. These proteins assist various lysosomal enzymes in the catabolism of lipids. The group includes 5 activators, GM2 activator protein and saposins A to D. They function as detergents to facilitate the interaction of lipid substrates with their specific enzymes (Mahuran, 1998; O'Brien and Kishimoto, 1991). This will be discussed in greater details below.

Trafficking of Lysosomal Proteins

Targeting of newly synthesized lysosomal proteins can follow 2 different pathways depending on the physical nature of the protein. Trans-membrane proteins can interact directly with adaptor proteins to recruit clathrin and to traffic the protein to the lysosome. Thus, luminal proteins must bind trans-membrane receptors that then interact with adaptor proteins to traffic to the lysosomal compartment.

Integral Membrane Proteins

There are several lysosomal membrane proteins such as lysosomal integral membrane proteins (LIMPs), including limp-I and limp-II, lysosomal associated membrane proteins (LAMPs), including lamp-1 and lamp-2, as well as lysosomal membrane proteins with enzymatic activity such as lysosomal acid phosphatase (LAP) (Morales et al., 1999). Lysosomal membrane proteins can reach the lysosomal compartments via two distinct pathways: an indirect routing through the secretory pathway to the plasma membrane and endocytosis; LAP is an example of this mechanism (Braun et al., 1989) and a direct transport from the TGN to endosome and lysosome via clathrin-coated vesicles; this include limp-II (Barriocanal et al., 1986; Vega et al., 1991) lamp-1 and lamp-2 (Traub et al., 1996). These lysosomal membrane proteins are sorted in the Golgi apparatus from the secretory pathway by a signal found in their cytosolic tails. Two distinct classes of cytosolic signals able to mediate late endosome and lysosome targeting have been identified on lysosomal membrane proteins. One determinant, present in lamp-1 (Harter and Mellman, 1992; Hunziker et al., 1991), lamp-2 (Honing and Hunziker, 1995), limp-I and LAP (Peters et al., 1990), is based on a motif containing a critical tyrosine

residue. The second signal, found in limp-II (Sandoval et al., 1994), is related to the dileucine motif. These targeting motifs on the lysosomal proteins allow them to be recognized by an adaptor protein (AP-3) (Ohno et al., 1995). This adaptor protein then recruits the necessary components to induce the formation of clathrin coated vesicles (Robinson and Bonifacino, 2001).

Soluble Proteins

Soluble lysosomal proteins cannot interact directly with adaptor proteins. Proteins, such as cathepsins, must first bind to a trans-membrane receptor that in turn binds adaptor proteins (Hirst et al., 2001). Most soluble hydrolases are transported to the lysosomes by the mannose 6-phosphate (M6P-R) receptor. This receptor binds adaptor proteins that participate in the formation of clathrin-coated vesicles (Hirst et al., 2001). However, not all soluble proteins bind the M6P-R. Recently, a trans-membrane protein (sortilin) capable of trafficking to the lysosomal compartment has been identified. Sortilin (100 kDa) has been shown to bind adaptor proteins and traffic to the lysosomes (Nielsen et al., 2001). This protein is suspected to be an alternative sorting receptor, but no lysosomal proteins have been shown to bind to this protein.

Mannose-6-Phosphate Receptor (M6P-R)

The M6P-R plays a major role in the intracellular transport of newly synthesized soluble lysosomal enzymes. In the endoplasmic reticulum, the newly synthesized hydrolase acquires a preformed oligosaccharide from a lipid-linked intermediate (Kornfeld and Kornfeld, 1985) that is subsequently modified in the Golgi apparatus by the addition of phosphomannosyl residues by the UDP-N-

acetylglucosamine-1-phosphotransferase. This enzyme catalyzes the transfer of N-acetylglucosamine phosphate to mannose residues in the precursor's oligosaccharide chain (Hasilik et al., 1981; Kornfeld, 1986). N-acetylglucosamine residues are then removed by a phosphodiesterase (Varki and Kornfeld, 1980) and the exposed M6P residues recognized by the mannose of the M6P receptor (Kaplan et al., 1977). Subsequently, the lysosomal protein and the receptor are transported by clathrin-coated vesicle (Campbell and Rome, 1983; Geuze et al., 1985; Lemansky et al., 1987). These cargo vesicles fuse with endosomal membranes, where the receptors release the lysosomal enzymes due to the intra-luminal acidic pH of this compartment (von Figura and Hasilik, 1986).

However, some soluble lysosomal proteins such as the sphingolipid activator proteins are transported to the lysosomes in a M6P-independent manner (Rijnboutt et al., 1991). Fibroblasts from patients with mucolipidosis (I-Cell disease), caused by a mutation in the UDP- N-acetylglucosamine-1-phosphotransferase which prevents the formation of M6P residues, contain prosaposin in their lysosomes (Reitman and Kornfeld, 1981). Also, hepatocytes from these patients have near normal levels of several M6P-dependent soluble lysosomal hydrolases (Kornfeld, 1986; Scriver, 1995). Although the M6P-R pathway is an important trafficking mechanism to the lysosomes, it is clear that another mechanism also exists.

Sortilin

Sortilin is a 100 kDa type 1 trans-membrane protein expressed in most tissues with especially high expression in the brain, testis and skeletal muscle (Petersen et al., 1997). Sortilin has been classified on the basis of its primary sequence into a new

receptor family including the receptors SorLA or LR11 and SorCS, two recently identified type I receptors (Bu and Schwartz, 1998). This new family of receptors contains a luminal/extracellular region containing a cysteine-rich domain homologous to the yeast vacuolar sorting protein VPS10p and one trans-membrane region preceding a short intracellular domain that contains a signal for rapid internalization and trafficking (Marcusson et al., 1994).

A VPS domain forms the entire luminal domain of sortilin (Petersen et al, 1997). This VPS homology region is common to the yeast VPS10p, a vacuolar sorting receptor for carboxypeptidase Y (Marcusson et al., 1994). The VPS domain of sortilin, as well as those of SorLA/LR11 and SorCS, is preceded by a furin cleavage site (two for SorCS). It has been demonstrated that sortilin is synthesized as a precursor and converted to the mature protein by furin in the late Golgi compartments (Nielsen et al., 1999). The furin cleavage leads to the formation of a 44-amino acid propeptide that exhibits high affinity binding to the processed sortilin. Interestingly, receptor associated protein (RAP) and Neurotensin (NT) known ligands of sortilin, compete for the binding of propeptide indicating the prevention of propeptide cleavage may serve to prevent binding of RAP and NT (Mazella et al., 1998). Both RAP and NT are unable to bind sortilin in the absence of maturation by furin (Nielsen et al., 1999). Taken together, these observations show that the propeptide prevents recognition of prosortilin by its ligands and that cleavage and release of the propeptide is a necessary step for functional activity of the receptor. This mechanism of receptor activation is original since other receptors cleaved by furin, e.g. the insulin and the LRP receptors, are cleaved to generate the subunits that

form active receptors. In these cases, maturation does not involve exposure of binding sites to ligands since proreceptors are capable of ligand binding (Ullrich et al., 1986).

Finally, the cytosolic tail of sortilin closely resembles the cytosolic component of the M6P-R. Importantly, the cytosolic domain of sortilin contains motifs known to be involved in the trafficking from Golgi to endosome (Johnson and Kornfeld, 1992; Nielsen et al., 2001). In fact, chimeric constructs that contained the luminal domain of the M6P-R and the cytosolic component of sortilin, rescued the trafficking of M6P-R dependant proteins to the lysosomal compartment in M6P-R deficient cells (Nielsen et al., 2001). Functionally, no lysosomal ligands are known to bind to the luminal portion of sortilin. However, the cytosolic portion of sortilin was shown to bind the same adaptor protein, GGA, which the cytosolic portion of the M6P-R binds (Nielsen et al., 2001). Taken together, this suggests a novel role for sortilin, as a lysosomal sorting receptor.

Adaptor Proteins

The trafficking of proteins to various cellular locations depends on a group of proteins that have been called adaptor proteins. Two subgroups of adaptors proteins have been identified. The multimeric adaptor proteins were first identified several years ago. This group is composed of four different proteins that have been called AP-1, AP-2, AP-3 and AP-4. Each is composed of 4 polypeptide chains, the products of four different genes (Robinson and Bonifacino, 2001). Each is involved with a different trafficking destination. More recently, the monomeric adaptor proteins, named the Golgi localized, γ-ear containing, ARF binding protein (GGA),

were identified. This group of proteins is the product of a single gene and was discovered based on the similarities with the multimeric adaptors. This group of adaptor proteins is known to traffic between the trans-Golgi and the lysosomal compartment only (Dell'Angelica et al., 2000c; Hirst et al., 2001).

Multimeric Adaptors

The multimeric adaptors are a group of proteins that are composed of four polypeptide chains that are implicated in the trafficking of proteins to various cellular destinations (Robinson and Bonifacino, 2001). Each adaptor is a heterotetramer composed of a large β subunit, a medium size μ subunit, a small σ subunit and a second large subunit specific for each adaptor (γ for AP-1, α for AP-2, δ for AP-3 and ε for AP-4) (Kirchhausen, 1999). Although the adaptor subunits are tightly associated with each other, a number of techniques have been used to investigate the functions of the individual proteins. There is considerable evidence implicating the β-subunit in clathrin binding (Shih et al., 1995). The high degree of sequence conservation between β1 and β2 (Dell'Angelica et al., 1998), indicated by the similarity of their peptide maps even before the two proteins were cloned (Owen et al., 2000), made them likely candidates for this role. In an in vitro binding assay, purified β2-adaptin was shown to interact with clathrin with a stoichiometry of nearly one to one, and β2 was also found to compete with the entire AP-2 complex for binding to clathrin, suggesting that the β subunit contains the major clathrin binding site (Owen et al., 2000). More recently, experiments have been carried out using recombinant β1 and β2, and both proteins on their own were found to be capable not only of interacting with clathrin, but also of promoting clathrin assembly. In a

subsequent study, a clathrin-binding determinant on β2 was localized to the hinge region of the protein (Shih et al., 1995), and presumably the corresponding region of β1 mediates clathrin binding in the AP-1 complex. It has also been suggested that the β subunits may be involved in the recognition of receptor tails, based on a study in which the purified asialoglycoprotein receptor was found to bind to the β2 band on Western blots of clathrin-coated vesicles (Beltzer and Spiess, 1991) and one in which β2 was shown to bind a fusion protein containing the cytoplasmic tail of the EGF receptor (Nesterov et al., 1995). However, the most compelling evidence points to a role for the adaptor medium chains in receptor binding and sorting signal recognition. At least three distinct types of sorting signals have been identified, all of which can promote efficient endocytosis as well as influence intracellular trafficking: the YXXØ motif (where Ø is a bulky hydrophobic amino acid), the di-leucine motif, and the FXNPXY motif, of which the best characterized is the YXXØ motif. In 1995, Ohno et al. (Ohno et al., 1995) screened a yeast two-hybrid library using a triple repeat of such a motif (SDYQRL) from the cytoplasmic tail of TGN38 to search for interacting proteins. Out of over two million clones, two specifically bound to the SDYQRL motif, and both were found to encode the W2 subunit of the AP-2 complex. Alanine substitutions showed that the two residues essential for this interaction were the tyrosine and the leucine, and these are also the two residues that are essential for the efficient endocytosis of TGN38. Ohno et al. (Ohno et al., 1995) also showed that YQRL containing constructs bind to the W1 subunit of the AP-1 complex, although more weakly. In contrast, the YXXØ motif of the transferrin receptor, YTRF, only interacted with W2 in the two-hybrid system and not W1,

suggesting a mechanism for including the transferrin receptor (and presumably other proteins as well) in clathrin-coated vesicles budding from the plasma membrane, but not in clathrin-coated vesicles budding from the TGN (Ohno et al., 1995).

Information on the regulation of adaptor protein recruitment has come mainly from in vitro studies making use of either permeabilized cells or membrane fractions (Schmid and Smythe, 1991). These studies have shown that the binding of adaptors to their target membrane is not a simple event. The addition of GTPγS to permeabilized cells stimulates the recruitment of AP-1 onto TGN membranes, while addition of the fungal metabolite, brefeldin A (BFA), prevents AP-1 recruitment in vitro and causes AP-1 to redistribute to the cytoplasm when added to living cells (Robinson and Kreis, 1992). There is evidence that both GTPγS and BFA are acting via the ARF family of small GTP binding proteins. GTPγS causes ARF to become constitutively activated, while BFA inhibits the nucleotide exchange of ARF (Peyroche et al., 1996). More direct evidence for the involvement of ARF in AP-1 recruitment came from studies in which cytosol was depleted of ARF by gel filtration and AP-1 recruitment was found to be reduced, while full recruitment could be restored by the addition of recombinant ARF1 (Seaman et al., 1996). Thus, it appears that TGN membranes need to be 'primed' with ARF1 before AP-1 can bind, although since ARF1 also primes the membranes of the intermediate compartment and the Golgi stack prior to coatomer binding, it is unlikely that it contributes to the specificity of AP-1 binding. The priming of membranes by ARF1 may be due, at least in part, to its ability to activate phospholipase D (PLD) (Brown et al., 1993; Cockcroft et al., 1994). The addition of exogenous PLD stimulates the recruitment

of coatomer (a component of the COPI coat) onto membranes, while inhibitors of PLD block coatomer recruitment and also interfere with membrane traffic between the Golgi stack and the ER (Ktistakis et al., 1995). However, neither exogenous PLD nor PLD inhibitors have any effect on the recruitment of AP-1 adaptors onto the TGN, suggesting that ARF may stimulate coatomer recruitment and AP-1 recruitment in different ways. There are also important differences between the regulation of AP-1 recruitment and AP-2 recruitment. The association of AP-2 with the plasma membrane is not affected by BFA, either in vitro or in vivo, suggesting that ARF1 is not involved (Robinson and Kreis, 1992). However, GTPγS causes misrouting of AP-2 adaptors to an endosomal compartment (Seaman et al., 1993), and also inhibits the in vitro formation of clathrin-coated vesicles at the plasma membrane (Carter et al., 1993). Certain cationic amphiphilic drugs also cause AP-2 to bind to endosomes without affecting AP-1 recruitment (Wang et al., 1993). More recently, it has been shown that the addition of either constitutively active ARF1 or exogenous PLD also causes AP-2 adaptors to bind to the endosomal compartment. Neomycin, which inhibits PLD by binding to its cofactor PIP2, inhibits the binding of AP-2 not only to the endosomal compartment but also to the plasma membrane, suggesting that both events require PLD. These findings indicate that the putative AP-2 docking site is somehow activated by PLD, but that normally this activation only occurs at the plasma membrane (West et al., 1997). It is possible that the most divergent of the ARF isoforms, ARF6, may be involved in the normal recruitment of AP-2 since it has been localized to the plasma membrane (Cavenagh et al., 1996) and is BFA-insensitive (Peters et al., 1995). However, it is clear from these experiments

that AP-2 recruitment is regulated differently from AP-1 recruitment. There is also some evidence to suggest that phosphorylation of adaptor subunits may help to regulate not only the recruitment of adaptors onto membranes, but also their ability to co-assemble with clathrin (Wilde and Brodsky, 1996), although AP-1 recruitment onto membranes has been shown to be ATP-independent, at least in vitro (Simpson et al., 1996), suggesting that de novo phosphorylation is not required. In addition to the small GTP-binding proteins, heterotrimeric G proteins may also have a role in adaptor recruitment. The pre-incubation of cells with aluminum fluoride, which activates heterotrimeric G proteins, has been shown to prevent the BFA-induced redistribution of AP-1 from the TGN to the cytoplasm (Robinson and Kreis, 1992). Incubation with another activator of heterotrimeric G proteins, mastoparan, inhibits the AP-2-stimulated sequestration of ligand in assays for clathrin-coated vesicle formation at the plasma membrane (Carter et al., 1993). Certainly, the cell must have some means of sensing the amount of membrane in different compartments and thus ensuring that incoming and outgoing traffic are balanced, and at present GTP-binding proteins are the best candidates for proteins that might regulate vesicle budding.

AP-1

The AP-1 subunits are expressed in all mammalian tissues and cells. Disruption of the μ subunit of AP-1 is embryonic lethal in mice. The genetic analyses of AP-1 function all point to its role in protein sorting at the late-Golgi/TGN or endosomes, but it is still unclear what its precise function is (Meyer et al., 2000). In *S. cerevisiae*, AP-1 seems to be involved in late-Golgi sorting events involving clathrin, with the prevacuolar compartment or vacuole as the likely destinations for

the proteins sorted (Boehm and Bonifacino, 2001). This function, however, is highly redundant with that of the GGAs, and is dispensable for viability. In *C. elegans*, the available evidence suggests that AP-1 directs protein sorting to dendritic vesicles destined for a specific domain of the neuronal plasma membrane (Boehm and Bonifacino, 2001). In mammals, the AP-1A isoform has been implicated in the retrieval of mannose 6-phosphate receptors from the endosomal system to the TGN, and the AP-1B isoform in the sorting of integral membrane proteins from the TGN or endosomes to the basolateral plasma membrane domain of polarized epithelial cells (Meyer et al., 2000). Unlike in *S. cerevisiae*, all of these functions appear to be essential for the development and viability of *C. elegans* and mice (Boehm and Bonifacino, 2001).

AP-2

Unlike the other multimeric adaptors, AP-2 does not localize to the Golgi apparatus. The role of AP-2 in human cells in culture was analyzed using a dominant-negative approach. The μ2 subunit of AP-2 had been previously shown to interact with tyrosine-based sorting motifs of the YXXØ-type (Ohno et al., 1995). Mutation of D^{176} to A (Aguilar et al., 1997) or W^{421} to A (Nesterov et al., 1995) in μ2 was found to block this interaction. When μ2-D^{176}A-W^{421}A was expressed in HeLa cells under the control of a tetracycline-inducible promoter, the mutant subunit was found to replace the endogenous μ2 subunit in the AP-2 complex. Endocytosis of the transferrin receptor was severely impaired in these cells (Nesterov et al., 1995), thus demonstrating that AP-2 mediates rapid receptor endocytosis in human cells. The genetic analyses of AP-2 function in various organisms again illustrate the dichotomy

between the requirements in yeast and higher eukaryotes. In *S. cerevisiae*, AP-2 is not essential for endocytosis or cell viability, nor does it seem to interact physically or genetically with clathrin (Boehm and Bonifacino, 2001). In higher eukaryotes, on the other hand, AP-2 plays a critical role in clathrin-mediated endocytosis in different cell types and is required for the development of multicellular organisms. These findings from genetic analyses are in agreement with the role of AP-2 in endocytosis previously suggested by extensive biochemical and morphological studies of mammalian cells (Boehm and Bonifacino, 2001).

AP-3

AP-3 is involved in the transport of lysosomal integral membrane proteins (Dell'Angelica et al., 1999). AP-3 is expressed in various species, from yeast to mammals. AP-3 functional studies were greatly facilitated by the naturally occurring mice and human mutation (Dell'Angelica et al., 2000a). Interestingly, although mutation to AP-1 subunits is embryonic lethal, AP-3 mutations are not (Boehm and Bonifacino, 2001). The phenotypes of pearl and mocha, which have mutations in their AP-3 complex, mice are reminiscent of the human genetic disorder, Hermansky-Pudlak syndrome. Like the AP-3 mutant mice, HPS patients exhibit hypopigmentation of the eyes and skin, prolonged bleeding and lysosome abnormalities (Dell'Angelica et al., 2000a). Indeed, two human patients suffering from a particular form of this disease, termed HPS type 2 (Shotelersuk et al., 2000), have been shown to carry mutations in the gene encoding the β3A subunit of AP-3 (Dell'Angelica et al., 1999). The mutant genes were transcribed and the corresponding mRNAs translated, but the resulting protein products underwent rapid

proteasomal degradation. The remaining AP-3 subunits were also degraded, resulting in very low levels of assembled AP-3 in the patient cells.

As in the pearl and mocha cells, lysosomal membrane proteins such as CD63 and lamp-1 were misrouted via the plasma membrane in HPS-2 cells (Dell'Angelica et al., 1999). Misrouting of the lysosomal trans-membrane proteins limp-II and lamp-1 could also be observed upon inhibition of the expression of μ3A in NRK (rat) and HeLa (human) cells, respectively, using an antisense RNA approach.

The results of the genetic analyses of AP-3 function in various organisms are all consistent with a role in transport to lysosome-related organelles, including the yeast vacuole, fly pigment granules and mammalian melanosomes, platelet dense bodies and some lysosomes (Robinson and Bonifacino, 2001). In yeast, the role of AP-3 is limited to sorting of some cargo molecules such as ALP and Vam3p (Cowles et al., 1997). In mammals, AP-3 appears to participate in the sorting of a larger set of proteins, including lysosomal membrane proteins and some melanosomal proteins (Kantheti et al., 1998). The impact of AP-3 deficiency on the sorting of these proteins is, however, only partial, suggesting the existence of alternative pathways for sorting of these proteins to lysosome-related organelles. The possibility remains, however, that there could be a stricter requirement for AP-3 in the sorting of a smaller set of proteins. The tissue-specific manifestations of AP-3 deficiency in higher eukaryotes could thus reflect the more severe missorting of specialized proteins. The AP-3B isoform has been implicated in the generation of a subset of synaptic-like microvesicles and the budding of synaptic vesicles from PC12 donor membranes (Faundez et al., 1998).

An important issue that remains to be elucidated is the intracellular localization of AP-3. The studies done so far indicate that it is localized to the TGN, endosomes, or both, but it is unclear at which of these compartments lysosomal, melanosomal and neurotransmitter/synaptic vesicles proteins are sorted. The requirement for clathrin for AP-3 function in higher eukaryotes is also unclear. Finally, the subset of synaptic vesicles that are generated in an AP-3-dependent fashion and the specific proteins that require AP-3 for sorting into synaptic vesicles remain to be identified.

AP-4

This is the most novel of the multimeric adaptor proteins identified only recently. This adaptor protein is localized in all tissues and cells examined and localizes to the trans-Golgi network. Recent work has implicated this adaptor protein in the trafficking of proteins to the basolateral compartment (Simmen et al., 2002). Although little is known about this adaptor complex, functional studies have demonstrated that mutant AP-4 subunits prevent the proper association of the complex and cause the misrouting of a subset of basolateral-targeted proteins.

Golgi localized, γ-ear containing, ARF binding protein (GGA)

The GGAs, a family of three similar proteins, are the most recently discovered adaptor proteins. The GGAs are monomeric adaptor proteins composed of four domains: a VHS domain that binds to the tail of the sorting receptor via a specific protein motif, a GAT domain which interacts with ARF, which is necessary for the recruitment of the GGAs to the Golgi membrane and the HINGE and EAR domains that bind clathrin and accessory proteins to form clathrin coated vesicles

(Hirst et al., 2000). These adaptors were discovered using several approaches including database searches for novel proteins that had adaptor-like domains. These proteins are highly conserved and have been identified in species such as yeast, drosophila, mice and humans. The GGAs have been localized to several cell types where endogenous GGA1, GGA2 and GGA3 localize predominantly to the trans-Golgi region in NRK, HeLa, Cos7 and human embryonic skin cells. The VHS domain of GGA2 interacts with a dileucine-sorting motif present in the cytoplasmic tail of sortilin (Nielsen et al., 2001; Takatsu et al., 2001). Similarly, the M6P-R interacts through an acidic-cluster-dileucine motif with the VHS domain of GGA proteins (Puertollano et al., 2001a; Takatsu et al., 2001; Zhu et al., 2001). Many other trans-membrane proteins lacking such a motif do not interact with GGA proteins, which indicate their specificity for a subset of transported proteins. The hinge domain of mammalian (Puertollano et al., 2001b) and yeast (Costaguta et al., 2001) GGA proteins interacts directly with clathrin in vitro. The presence of the ear domain strengthens the interaction, while the ear domains of certain GGA proteins can interact with clathrin on their own. Although direct interaction in vivo has not been shown, indirect evidence is strongly in favor of this conclusion. In mammalian cells, over-expression of GGA3 recruits clathrin to the TGN. A truncated GGA3 construct lacking the clathrin-binding hinge and ear domains causes M6PR to compact at the TGN rather than be packaged into vesicles, which suggests that the GGA-clathrin interaction is required for vesicle formation (Puertollano et al., 2001b). The ear domain of human GGA proteins interacts with γ-synergin (Takatsu et al., 2001), a Golgi-localized protein partner of γ-adaptin that has an unknown function (Page et

al., 1999). γ-synergin has no homolog in yeast, and the ear domains of yeast and human GGA proteins are not very well conserved; hence the role of the ear domain in yeast is even less clear. However, a binding partner shared by the mammalian GGA ear and γ-adaptin ear shows that these two domains indeed share similar functions (Page et al., 1999).

Sphingolipid Activator Proteins

The degradation of glycosphingolipids within the lysosomes is dependant on the activity of several hydrolases. However, glycosphingolipids with short oligosaccharide chains require a group of proteins, known as the sphingolipid activator proteins to be degraded by their respective hydrolases. Five sphingolipid activator proteins have been identified to date, the G_{M2} activator protein (G_{M2}AP) and the four Saposins (A, B, C and D). The four saposins are derived from the partial proteolysis of a common precursor known as prosaposin. Both prosaposin and G_{M2}AP are the products of separate genes. Deficiency of the activator proteins cause several variant forms Metachromatic Leukodystrophy, Gaucher's disease and G_{M2} gangliosidosis (Sandhoff, 1977).

Functions of G_{M2} activator proteins and its related diseases

The enzymatic degradation of G_{M2} requires β-hexosaminidase A, a water-soluble enzyme that acts on substrates of the membrane surface (Mahuran, 1991). Hexosaminidase A is a heterodimer encoded by two different genes, the HEXA gene (Nakai et al., 1991) and the HEXB gene (Bikker et al., 1988) encoding the α subunit and β subunit of β-hexosaminidase A respectively. Two other Hex isozymes, formed by αα subunit (Hex S) and ββ subunit (Hex B) are also present. Mutations of the

HEXA gene are associated with deficient activity of Hex A and S but normal level of Hex B, causing a B-variant of Tay-Sachs disease. Mutation of Hex B is associated with deficient activity of Hex A and B, resulting in the o-variant of Sandhoff's disease (see figure A).

These enzymes are responsible for the degradation of specific glycosphingolipids. However, glycosphingolipid substrates with short oligosaccharide chains cannot reach the active site of water-soluble β-hexosaminidase A and cannot be degraded. $G_{M2}AP$ binds GM_2 ganglioside and other related short gangliosides and forms water-soluble complexes (mostly in a 1:1 molar ratio). $G_{M2}AP$ is considered a biological detergent that has been proposed to function in one of two mechanisms. The 'liftase' hypothesis consists of $G_{M2}AP$ recognizing G_{M2} within the membrane, binding to and lifting the lipid out of the bilayer and by presenting it to the water-soluble β-hexosaminidase A. The second model proposes that the activator binds lipids already removed from the lipid bilayer, forming an activator-lipid complex that can be recognized by the water-soluble β-hexosaminidase A. The enzymatic reaction takes place in fluid-phase. In addition, formation of the ternary complex presumably involves a protein-protein interaction between the GM_2 activator and β-hexosaminidase A (Kytzia et al., 1983). Mutations in the gene encoding the $G_{M2}AP$ activator protein (Heng et al., 1993) give rise to an AB-variant of Tay-Sachs (Mahuran et al., 1990) characterized by normal levels of Hex A and B enzymes, demonstrating the importance of this protein.

Biosynthesis and Intracellular Processing of Prosaposin

Human prosaposin is a 524 amino acid glycoprotein containing a 16-residue

signal peptide sequence and five potential N-linked glycosylation sites (O'Brien et al., 1988). The N-terminal signal sequence directs prosaposin into the lumen of the endoplasmic reticulum (ER), where it receives high mannose, asparagine-linked oligosaccharides. Prosaposin is then transported to the Golgi apparatus, where the oligosaccharide chains are trimmed and remodeled like most glycoproteins (Kornfeld and Kornfeld, 1985). Within the Golgi apparatus, prosaposin is sorted to different destinations. Prosaposin was first identified as four similar polypeptide domains placed in tandem from analysis of a cDNA encoding sulfated glycoprotein-1 (SGP-1), the rat homologue of human prosaposin. One of the domains of SGP-1 was identified as homologous with human Saposin B. These results suggested that partial proteolytic processing of prosaposin/SGP-1 could generate the saposin proteins. However, Saposin A and D had not been recognized until O'Brien et al (O'Brien et al., 1988) analyzed the full sequence of the precursor of human Saposin C and found that it also encoded both Saposin B and C and two additional putative Saposins, A and D. Immunoprecipitation of whole cell lysates using anti-saposin B antibody demonstrated that the precursor of Saposin B was first synthesized as a 65 kDa glycoprotein and then converted to a 70 kDa polypeptide by post-translational glycosylation. The 65 kDa precursor was rapidly processed into mature 8-13 kDa saposin via 52 kDa and 35 kDa intermediates. The 70 kDa protein was the only form found in cultured medium. Pulse-chase labeling experiments of Sertoli cells in culture (Igdoura et al., 1996) confirmed the same biosynthetic relationship between the 65 and 70 kDa forms of rat prosaposin in vivo.

Structure and Functions of Saposins

All saposins contain about 80 amino acids and each has one N-glycosylation site, except human saposin A that contains two N-glycosylation sites. Their structures are similar to each other since they contain six identically placed cysteine residues and two conserved proline residues. It has been noted that saposins have a remarkable similarity with other proteins, called SAPLIP (saposin-like proteins), which have a cysteine pattern identical to saposins, a similar size and stability under extreme conditions (Munford et al., 1995). These proteins include surfactant B, NK-lysin, and plant aspartic proteinases (Liepinsh et al., 1997; Ponting and Russell, 1995; Stenger et al., 1998; Vaccaro et al., 1999).

Based on helical wheels with appropriate positioning of proline residues and glycosylation sites and the localization of three potential disulfide bonds formed by the 6 cysteine residues, a hypothetical secondary structure of saposins were predicted (O'Brien and Kishimoto, 1991). This suggested a similar structure between Saposin A, C and D, but a different structure for Saposin B because of the presence of a large β-sheet region and a lengthy C-terminal helical region not present in saposin A, C, or D. However, they all form three internal disulfide bridges in the hydrophobic interior, which may explain their stability to heat (Morimoto et al., 1988; O'Brien et al., 1988).

The similar protein sequences and predicted structures of saposins would indicate a common structure-function rationale. However, the functional properties of saposin B were thought to be different from the other saposins because Saposin B was shown to interact with lipid substrates forming a lipid/saposin B complex while the other saposins interact directly with enzymes.

Saposins

Saposin B (previously called sphingolipid activator protein) was the first activator protein discovered. Saposin B activates the hydrolysis of sulfatide by arylsulfatase A, the hydrolysis of G_{M1} ganglioside by acid β-galactosidase, and globotriaosylceramide by α-galactosidase (see figure A) (Inui and Wenger, 1984; Li et al., 1988). Human hereditary deficiency of Saposin B results in tissue accumulation of cerebroside sulfate and other glycolipids and a clinical phenotype of metachromatic leukodystrophy (Inui and Wenger, 1984; Stevens et al., 1981), a disease characterized by progressive neurological degeneration. Saposin B was also shown to be the activator of degradation of additional glycolipids and glycerolipids (Li et al., 1988). Saposin B seems to act as a detergent-like protein solubilizing multiple lipid substrates for enzyme hydrolysis. Many other lipids, including G_{M2} ganglioside and sphingomyelin also bind to saposin B, although saposin B does not have stimulatory effects on the lysosomal hydrolysis of these lipids (Inui and Wenger, 1984).

In vitro saposin B-lipids binding assay followed by enzymatic hydrolysis studies (Vogel et al., 1991) demonstrated that saposin B activated more efficiently the hydrolysis of sulfatide by arysulfatase A than globotriaosylceramide by α-galactosidase A, or GM_1 ganglioside by β-galactosidase A. Furthermore, the molar ratios of lipids, including GD_1a, GM_1, GM_2, GM_3 gangaliosides and sulfatide were found to be 1:2 (Vogel et al., 1991), which suggested that Saposin B may exist as a dimer during its association with lipids. However, this has not been confirmed. In summary, Saposin B acts on a broad range of glycolipids within an acidic

enviroment, to stimulate their specific hydrolysis within lysosomes.

Saposin C, previously called sphingolipid activator protein-2, was identified (Ho and O'Brien, 1971) in 1971 and shown to activate the hydrolysis of glucosylceramide by β-glucosidase (Morimoto et al., 1989). The activation of the hydrolysis of galactocerebroside by β-galactosidase by saposin C has also been reported (see figure A) (Glew et al., 1988; Radin, 1984). Deficiency of Saposin C has been linked to a variant form of Gaucher's disease, a form of splenohepatomegaly and neurological defects (Christomanou et al., 1986).

Unlike Saposin B, Saposin C does not appear to interact with substrate lipids such as glucocerebroside but to bind and activate the enzyme glucocerebrosidase. This was demonstrated when sepharose-linked Saposin C used in an affinity column for glucocerebroside, did not form complexes with this lipid (Peters et al., 1977). At least two modes of action were proposed for this saposin. 1) The activation effect of Saposin C might derive from an induction in the conformational change of the enzyme for optimal catalysis (Fabbro and Grabowski, 1991; Qi et al., 1994). 2) Saposin C most likely reconstitutes the enzyme activity by favouring the glucosylceramidase localization on lipid surfaces of appropriate composition (Vaccaro et al., 1995; Vaccaro et al., 1993).

Saposin A can stimulate the enzymatic hydrolysis of 4-methylumbelliferyl-β-glucoside, glucocerebroside, and galactocerebroside in vitro (see figure A) (Morimoto et al., 1989). Stimulation of acid β-glucosidase by Saposin A is similar to that by Saposin C but the degree of stimulation is weaker. The binding site of saposin A and C were shown to be the same (Morimoto et al., 1990); hence it is thought that

these proteins have similar functions.

Saposin D is the least understood of the saposins. It has been reported to be involved in sphingomyelin and ceramide hydrolysis (Azuma et al., 1998; Morimoto et al., 1988), but no detailed information on its mechanism of action is available. However, the addition of saposin D to the culture medium of fibroblasts from patients with prosaposin deficiency lead to a decrease in the accumulation of ceramide (Klein et al., 1994), thus the function of saposin D seems to be related to ceramide degradation rather than sphingomyelin degradation. It was also found that Saposin D, but not the other three saposins, stimulates the degradation of ceramide by a partially purified preparation of ceramidase and that the saposin poorly interacts with ceramide at acidic pH values (Azuma et al., 1994). Recently, saposin D has been demonstrated to associate with cell membranes and was highly dependent on the composition of the bilayer (Tatti et al., 1999). The presence of acidic phopholipids such as phophotidylserine or phosphatidic acid greatly favoured the interaction of saposin D at low pH (Ciaffoni et al., 2001).

Distribution and Possible Functions of Prosaposin

Prosaposin also exists as an unprocessed 70 kDa secretory protein in many tissues and body fluids (O'Brien et al., 1988). The highest concentration of prosaposin has been reported in testis, seminal plasma, cerebral gray matter, cerebrospinal fluid and human milk, and lower concentration in liver, spleen, and other organs (Morales et al., 1996). Interestingly, it appears that the secreted from of the protein may act a neurotrophic factor (O'Brien et al., 1994; Qi et al., 1994). The active neurotrophic region is localized to a 12 amino acid residue at the NH_2-terminal

end of Saposin C (LIDNNKTEKEIL) (O'Brien et al., 1995). Several synthetic peptides derived from this region are equally as bioactive as prosaposin and are called prosaptides (Misasi et al., 1996; Qi et al., 1996). Nanomolar concentrations of this prosaptide as well as prosaposin can stimulate neurite outgrowth and choline acetyltransferase activity in neuroblastoma cells (O'Brien et al., 1994). In addition, prosaposin has been shown to protect hippocampal neurons from ischemic damage and to prevent apoptosis of cerebellar granule neurons in culture submitted to toxicity (Campana et al., 1998; Sano et al., 1994). Very interestingly, the signaling pathways involved in Schwann cell survival by prosaposin and prosaptide have recently been identified through the PI3K/Akt pathway, although no cell surface receptor has yet been identified (Campana et al., 1999). Prosaposin has also been found to activate the MAPK pathway by a G-protein-dependent mechanism essential for enhanced sulfatide synthesis by Schwann cells acting as a myelinotrophic factor (Campana et al., 2000).

Prosaposin has been found in many body fluids, however, little is known concerning its physiological function. Rat prosaposin is rich in seminiferous fluids and may be associated with the head and tails of spermatozoa once it is secreted into the lumen (Griswold et al., 1986). These may suggest that prosaposin may be involved in the release and/or maturation of spermatozoa during spermatogenesis. It has also been suggested that prosaposin may be involved in the modification of the sperm membrane by means of glycolipid transport. Since glycolipids were shown to mediate heterologous cell contacts, including the preferential adhesion of different cell types (Marchase, 1977), it is possible that glycolipid modification of Sertoli cell

and /or germinal cell membranes play a role in certain processes such as the induction of sperm maturation. Nevertheless, further investigation is required to clarify the functions of secreted prosaposin in the male reproductive system (Collard et al., 1988).

Prosaposin Related Diseases

The first demonstration of a role for saposins in human disease came from the discovery of a variant form of metachromatic leukodystrophy with normal arylsulfatase A activity and deficiency of saposin B (Stevens et al., 1981). Patients with this disease present neurological deterioration and demyelination, severe progressive mental retardation, and tissue accumulation of sulfatide in brain and myelinated nerves. However, unlike the classical form of metachromatic leukodystrophy, in which arysulfatase A is deficient, patients who lack saposin B have normal or nearly normal activities of arysulfatase A (Holtschmidt et al., 1991; Kretz et al., 1990). Reloading of saposin B in cultured fibroblasts from patients with saposin B deficiency corrects the impaired degradation (Stevens et al., 1981). However, the fact that saposin B also activates the hydrolysis of other lipids by different enzymes is reflected by the composition of the storage material, which in addition to sulfatide, also contains globotriaosylceramide and GM3 ganglioside in saposin B deficiencies (see figure A) (Li et al., 1985).

Point mutations and amino acid insertions have been reported from three patients with saposin B deficiency, an inherited autosomal recessive disease. One is due to a single C-T transition in the 23rd codon of saposin B domain resulting in a threonine to isoleucine amino acid substitution (Kretz et al., 1990). A second one is

a G to C mutation leading to a substitution of a cysteine residue for a serine residue within the saposin B domain. The last point mutation resulted from an insertion of 33 nucleotides in the last third of the saposin B domain. In the first case, a polar amino acid (threonine) is replaced with a nonpolar amino acid (isoleucine) that in turn appears to eliminate the glycosylation signal and to increase its proteolytical degradation owing to exposure of a proteolytical site (arginine) two residues to the amino-terminal side of the glycosylation site. The same proteolytical hydrolases that cleaves prosaposin at dibasic residues at the saposin boundaries could also cleave the mutant protein. The second point mutation destroys the disulfide bonds and the correct three-dimensional structure. The 33 nucleotide insertion occurs in the last one-third of the saposin B domain, changing its hydropathy profile significantly and perhaps leading to instability (O'Brien and Kishimoto, 1991).

A deficiency in saposin C was first demonstrated in a variant form of Gaucher's disease (Christomanou et al., 1986). A 200-fold elevation of glucocerebroside in spleen but normal glucosylceramide β-glucosidase activity was found in a Gaucher's disease patient (O'Brien and Kishimoto, 1991). Two point mutations in the saposin C coding region, G1154 to T (Schnabel et al., 1991) and T1153 to G (Rafi et al., 1993), changing the cysteine residues to phenylalanine and glycine respectively, lead to an atypical variant of Gaucher's disease (see figure A), thus, indicating the importance of the cysteine residues for the stability of mature saposins.

Another type of mutation resulting in total prosaposin deficiency was reported in two siblings of fourth cousin parents (Harzer et al., 1989; Schnabel et al.,

1992). Biochemical analysis demonstrated the accumulation of different sphingolipids including glucosylceramide, galactosylceramide, and ceramide in patient's tissues. Sequence analysis demonstrated an A-T transversion in the initiation codon resulting in the absence of prosaposin synthesis. Clinically, these patients presented hyperkinesia, respiratory insufficiency, and hepatosplenomegaly and died by 16 weeks. Saposin D was implicated in the degradation of sphingomyelin (Morimoto et al., 1988). Interestingly, sphingomyelin levels and sphingomyelinase activity were normal in these patients indicating that saposins are not directly involved in the degradation of sphingomyelin. Thus, the significance of saposin D as an activator protein is still in doubt. Recent evidence has implicated the saposin D domain in the trafficking of prosaposin to the lysosomes (Zhao and Morales, 2000). The implication from this work suggested that the D domain along with the adjacent carboxy-terminal region bound to sphingomyelin and a sorting receptor to traffic prosaposin (Zhao and Morales, 2000). This could explain why no mutation in the D domain has ever been found. If it were implicated in the trafficking of the protein, the consequences of a mutation in the D domain would be probably lethal.

Sphingolipids

Sphingolipids differ from glycerolipids in that they are based on a long chain sphingosine backbone rather than a glycerol backbone. Sphingolipids have been implicated in a number of cellular functions including cell-signaling pathways that regulate calcium homeostasis, cell cycle progression and apoptosis (Merrill et al., 1997). The synthesis of sphingolipids occurs in the endoplasmic reticulum (ER) and the Golgi apparatus. In an initial synthesis step, L-serine is converted into

dihydroceramide on the cytosolic side of the ER (see figure B) (van Echten and Sandhoff, 1993). Typically, free sphingoid bases are present in very low concentrations because *de novo* synthesized bases are rapidly converted to dihydroceramide (Merrill et al., 1997). Dihydroceramide is then converted to ceramide, which is then modified in one of two ways.

The addition of phosphorylcholine to ceramide forms sphingomyelin (see figure B), an event that is catalyzed by sphingomyelin synthase (Ullman and Radin, 1974). Sphingomyelin has been implicated in cell signaling. It would seem that sphingomyelin hydrolysis is important in monocytic differentiation, and other cell signaling cascades (Okazaki et al., 1989; Okazaki et al., 1990). The effector molecule of sphingomyelin hydrolysis is ceramide, which functions as a signaling molecule involved in programmed cell death via TNF-α or Fas ligand which, when binding to their respective receptors, activates sphingomyelinase to catalyze sphingomyelin degradation (Cifone et al., 1994). The regulation of sphingomyelin is therefore closely regulated in the cell (Luberto and Hannun, 1998). Sphingomyelin has also been localized to lipid raft microdomains and seems to provide the lipid scaffold that the microdomain is built upon (Brown and London, 1998)(Simons and Ikonen, 1997). These microdomains have been implicated in the sorting of proteins at the trans-Golgi network (Simons and Ikonen, 1997; Simons and van Meer, 1988).

On the other hand, the stepwise addition of sugar residues forms glycosphingolipids (see figure B) (Coste et al., 1986). Glycosphingolipids are components of the plasma membrane of eukaryotic cells (Neuberger and Deenen, 1985). Glycosphingolipids are composed of a ceramide moiety that acts as a

membrane anchor and a hydrophilic extracellular oligosaccharide chain composed of various sugar residues. The varying composition of those residues leads to the wide range of naturally occurring glycosphingolipids. Glycosphingolipids participate in a wide range of cellular function such as cell adhesion, cell growth, differentiation as well as interacting with membrane bound receptor (Karlsson, 1989). Recently, it was shown that glycosphingolipids participate in the trafficking of proteins to the melanosome, an organelle that belongs to the family of lysosome related organelles (Sprong et al., 2001).

The inhibition of sphingolipid synthesis has been studied for many years. Several compounds are known to inhibit the synthesis of sphingolipids. The more common compounds are fumonisin B_1, PDMP and D609. Fumonisin B_1 inhibits the formation of sphingosine by acting as a competitor in the formation of dihydroceramide from sphinganine (see figure B) (Wang et al., 1991). This compound comes from the mold *Fusarium moniliforme* and has been shown to cause neuronal degeneration, liver and renal toxicity and cancer (Wang et al., 1991). This compound has the effect of preventing the synthesis of all down stream sphingolipid components including the glycosphingolipids and sphingomyelin (Merrill et al., 1993). Recent studies have shown that fumonisin B_1 can induce apoptosis (Lim et al., 1996), decrease axonal growth (Harel and Futerman, 1993), and interfere with the transport of glycosylphosphatidylinositol-anchored proteins (Horvath et al., 1994). PDMP on the other hand specifically inhibits the glycosphingolipids (see figure B). This compound inhibits glucosylceramide synthase, the initial branch point for glycosphingolipids (Inokuchi et al., 2000) and causes the accumulation of ceramide

and sphingosine (Merrill et al., 1997). D609 is a specific inhibitor of sphingomyelin synthase (see figure B); hence the production of sphingomyelin is blocked in cells treated with this compound. The addition of 50 – 200 μg/ml significantly reduced the amount of sphingomyelin founds in the cell (Luberto and Hannun, 1998).

In conclusion, this review has shown that the trafficking of prosaposin to the lysosomal compartment requires further investigation. The objectives of the present investigation are to define the role of the D functional domain and the carboxy-terminal region of prosaposin and to determine whether sphingomyelin plays a role in the normal transport of this molecule. The final objective of this study is to elucidate the mechanism of transport of prosaposin to the lysosome.

Fig. A The degradation of glycosphingolipids. This diagram demonstrates the step-wise degradation of glycosphingolipids and the specific actions of enzymes and activator proteins. The site of blockage caused by a mutant enzyme or activator is shown along with the associated disease phenotype caused by the blockage.

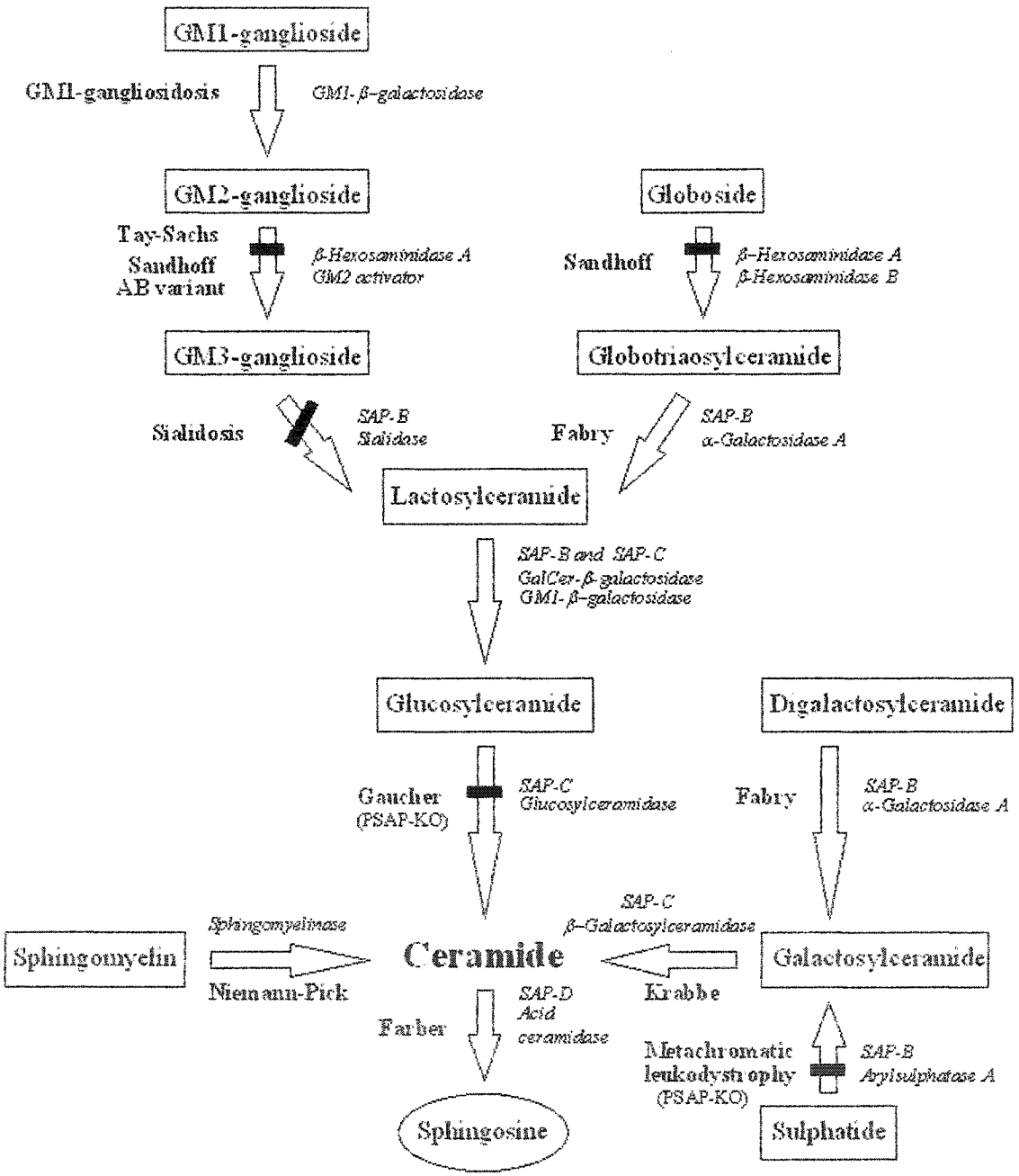
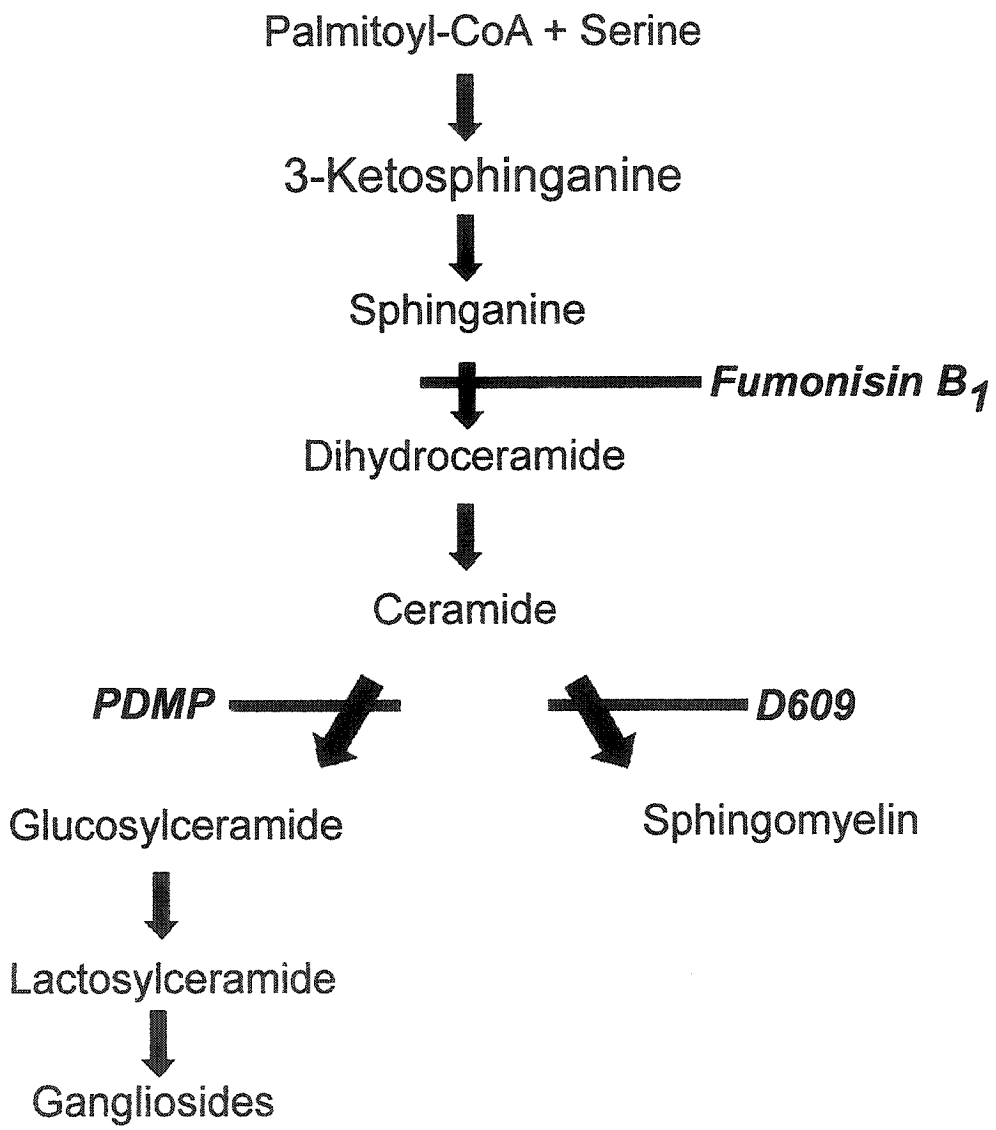

GM1-ganglioside
GM1-gangliosidosis
GM1-β-galactosidase
GM2-ganglioside
Globoside
Tay-Sachs
Sandhoff
AB variant
β-Hexosaminidase A
GM2 activator
Sandhoff
β-Hexosaminidase A
β-Hexosaminidase B
GM3-ganglioside
Globotriaosylceramide
Sialidosis
SAP-B
Sialidase
Fabry
SAP-B
α-Galactosidase A
Lactosylceramide
SAP-B and SAP-C
GalCer-β-galactosidase
GM1-β-galactosidase
Glucosylceramide
Digalactosylceramide
Gaucher
(PSAP-KO)
SAP-C
Glucosylceramidase
Fabry
SAP-B
α-Galactosidase A
SAP-C
β-Galactosylceramidase
Sphingomyelinase
Sphingomyelin
Ceramide
Galactosylceramide
Niemann-Pick
Krabbe
Farber
SAP-D
Acid
ceramidase
Metachromatic
leukodystrophy
(PSAP-KO)
SAP-B
Arylsulphatase A
Sphingosine
Sulphatide

Fig. B Sphingolipid synthesis pathway and site of inhibitor action. The formation of sphingolipids is outlined and the site of inhibitors shown at their specific sites of action.


Palmitoyl-CoA + Serine
3-Ketosphinganine
Sphinganine
Fumonisin B1
Dihydroceramide
Ceramide
PDMP
D609
Glucosylceramide
Sphingomyelin
Lactosylceramide
Gangliosides

MATERIALS AND METHODS

Construction of expression vector

Cultured cells do not normally synthesize large amounts of prosaposin. In view of this we used an expression vector to establish a stably transfected CHO cell line that over-expressed human prosaposin. The expression vector P91023(B) (Murray et al., 1983) was kindly provided by Dr. R. J. Kaufman (Genetic Institute, Cambridge). A prosaposin cDNA containing a 9 bp insertion of exon 8 (Morales et al., 1998; Zhao et al., 1998b), was cloned into the EcoRI site of the vector.

Transfection of CHO cells and selection of stably transformed cells

CHO cells (1-4 x 10^6 cells) were seeded on 100 mm dishes 16 h prior to the addition of DNA. Transfection was conducted using calcium phosphate with 15 μg of DNA. After 72 h in culture, the cells were trypsinized and transferred into 100 mm dishes and selected in a minimum essential medium devoid of nucleosides with 10% dialyzed fetal serum. The resistant colonies were put into culture medium containing 0.02 μM methotrexate. The concentration of methotrexate was progressively increased up to a level of 1 μM. The selected cell lines were analysed for expression of prosaposin mRNA and its translation product.

Northern blotting

For Northern blot analysis, the mRNA from transformed CHO cells grown in T-150 culture flasks were purified by CsCl gradient centrifugation and resolved by agarose gel electrophoresis (Maniatis et al., 1982). The prosaposin cDNA was labeled with the DIG luminescent detection kit from Boehringer Mannheim (Laval,

Quebec, Canada), and probed according to the manufacturer specifications.

Immunoprecipitation of prosaposin

Subconfluent transformed CHO cells stably expressing human prosaposin in T-75 flasks were incubated for 1 h in methionine-free medium and labeled for 1 h with ^{35}S-methionine at 0.2 mCi/flask. The cells were washed twice with phosphate-buffered saline and solubilized in 1 ml of 50 mM Tris-HCl (pH 8.0) containing 0.15 M NaCl, 0.5% NP-40 and 10 µg/ml PMSF. The cell extract was then precleared by incubation with 50 µl preimmune rabbit serum for 1 h and 500 µl of 10% S. aureus polysaccharides were added. After centrifugation, the supernatant was incubated with an antiserum against prosaposin overnight at 4°C with shaking. The immune complex was precipitated by the addition of 50 µl of Staphylococcus aureus suspension. The antigen-antibody complex was denatured by boiling in the SDS-PAGE loading buffer for 5 min and was analyzed by gel electrophoresis using a 10% gel (Laemmli, 1970). The gels were subsequently impregnated with Amplify (Amersham) and the autoradiographs of dried gels were obtained on an X-ray film at -80°C with two intensifying screens.

Confocal microscopy

Cell culture I: Chinese ovary (CHO) cells were cultured in minimum essential medium (Gibco, Montreal, Quebec) supplemented with 10% fetal calf serum and antibiotics. Chinese hamster Ovary (CHO) cells transfected with a stable expression vector, P91023 (B), containing a human prosaposin cDNA (Lefrancois et al., 1999) were incubated in Coon's F12 medium (Sigma, St. Louis, MO) supplemented with 10% FBS, antibiotics, and 0.1% methotrexate (Sigma, St. Louis, MO) for 72 hours. The cells were

then trypsinized and resuspended in methotrexate supplemented media at a concentration of 2 X 10^4 cells/ml. 2.5 ml of the cell rich medium was added to six wells, containing three coverslips each. 6 plates were prepared, and incubated overnight at 37°C.

Cell culture II: COS-7 cells were cultured in DMEM (Sigma, St. Louis, MO) supplemented with 10% FBS and antibiotics. The cells were harvested then plated (5 X 10^5/100ml) in NuSerum supplemented medium overnight in preparation for a transient transfection. The cells were transfected with a wild-type prosaposin construct or an Albumin/Sap-D/COOH (Alb-D-COOH) construct as described by Zhao and Morales (Zhao and Morales, 2000). The NuSerum incubated cells were tranfected using a DEAE-dextran/chloroquine phosphate protocol and then plated using DMEM supplemented with 10% FBS and antibiotics overnight.

Cell culture III: Normal rat kidney (NRK) cells were provided by Dr. D. Laird (Department of Anatomy and Cell Biology, University of Western Ontario, Canada) and grown in F12 medium supplemented with 10% fetal calf serum. (Gibco, Montreal, Quebec).

Inhibitors: The medium was removed from the dishes and the cells were washed with PBS for one minute. The CHO and COS-7 cells received FB_1 (25 μg/ml), PDMP (25 μg/ml), or D609 (100 μg /ml) supplemented medium (Biomol Research laboratories, Inc., PA). Fresh medium was added to other plates to serve as negative controls. The plates were then incubated for 24 hours at 37°C.

Exogenous lipids: CHO and COS-7 cells received one of the following treatments: ceramide, dihydroceramide, or sphingomyelin (Biomol Research Laboratories, Inc., PA) at a final concentration of 5 μM. One set of plates received

no lipids. The plates were then incubated for 24 hours at 37°C. In the case of the CHO cells, separate plates were also incubated for 12 and 48 hours. Incubation at 48 hours was of interest because the turnover time of sphingomyelin in vitro at 37°C is 25-26 hours.

Immunofluorescent staining: The cells were washed 3 times in PBS, and in the case of some of the COS-7 cells, also incubated in 60 nM LysoTracker (Molecular Probes Inc. Eugene, OR) for 30 min. LysoTracker labels the acidic compartments of the cell, which includes endosomes, lysosomes and the trans-Golgi network. The cells were then fixed on coverslips with 3.8% paraformaldehyde (Sigma, St. Louis, MO) for 30 minutes at room temperature and then rinsed twice with PBS and treated with 0.5% triton X-100 (Boehringer Mannheim, Indianapolis, IN) for 30 minutes at room temperature. The cells were blocked with 100 µl of 10 % goat serum for 1 hour, followed by 100 µl of primary antibody diluted 1:200 in PBS overnight at 4°C. The cells were then washed in 0.05% tween-20 (Sigma, St. Louis, MO) three times for 5 minutes each. The appropriate FITC or Alexa conjugated secondary antibody (Sigma, St. Louis, MO) was diluted 1:200 and 100 µl of the antibody solution was placed on each coverslip for 1 hour at room temperature. The cells were then washed with 0.05% tween-20 three times for 5 minutes each, followed by a rinse with distilled water. The coverslips were mounted face down on microscope slides with 90% Mowiol (Calbiochem, La Jolla, CA) in PBS to be viewed on a Zeiss 410 confocal microscope (Carl Zeiss, Germany). The slides were stored in a light proof black box.

Electron microscopy

Preparation of culture cells for electron microscopy

Culture cells were detached from the culture dishes with 0.1% (w/v) trypsin in HBSS (Hank's Balanced Salt Solution). The cells were pelleted at 1000 Xg during 7 min and the supernatant discarded. The cell pellets were carefully detached from the centrifuge tubes with a needle and fixed in 4% paraformaldehyde and 0.5% glutaraldehyde in 0.05 M phosphate buffer. The cell pellets were dehydrated in ethanol and embedded in Lowicryl K11M.

Tissue culture of rat Sertoli cells

Three mice were anesthetized using Somnotol (MTC Pharmaceutical Inc.) and their testis removed under sterile conditions. The tunica albuginea was removed and the seminiferous tubules cut into 2 mm pieces and placed in 10 ml of DMEM supplemented with 10% FBS and antibiotics overnight at 37°C.

Cell culture

COS-7 cells were plated on 100 mm plates and transfected as described above. Some plates did not receive inhibitors or exogenous lipids and were used for the dominant negative experiment.

Inhibitors

10 ml media supplemented with FB_1, PDMP or D609, were added to the plates at the same concentration as above and incubated for 24 hours at 37°C. 10 ml of fresh medium was added to a control plate of seminiferous tubules.

Exogenous lipids

Cultured seminiferous tubules treated or not with the sphingolipid inhibitors

received either ceramide or sphingomyelin at a concentration of 5 μM. One set of plates received no lipids.

Preparation of Lowicryl sections

Pieces of seminiferous tubules or COS-7 cells were fixed for 1 hour using 5% paraformaldehyde and 0.5% glutaraldehyde in 1M sodium buffer. The cells were then placed in 1% agarose for structural support and dehydrated in ascending concentrations of ethanol (30, 50, 70, 90 and 100%) for 1 hour each and then in 1:1 ethanol/Lowicryl for 1 hour and then 2:1 Lowicryl/ethanol overnight. The tissue was then placed in pure Lowicryl for 4 hours before being embedded within gelatin caplets. Ultrathin sections were cut and placed on formvar coated nickel grids as described previously by Sylvester et al. (1989) (Sylvester et al., 1989) and Hermo et al. (1991) (Hermo et al., 1991).

Electron microscopy immunocytochemistry

40 μl-drops of 10% goat serum were placed on a rubber mat within a petri dish and the grids were incubated on the drops tissue side down for 15 minutes. The grids were then placed on drops of anti-prosaposin antibody diluted 1:20 in TBS, at a concentration of 50 μg/μl (seminiferous tubules), or anti-myc (1:10) antibodies (COS-7 cells) and incubated for 1 hour. Four washes of five minutes each with 0.1% tween-20 were followed by a second 15-minute incubation with goat serum. The grids were then incubated on secondary anti-rabbit antibody conjugated to 15 nm gold diluted 1:100, at a concentration of 0.185 μg/μl (seminiferous tubules) or anti-rabbit conjugated to 15 nm gold and anti-mouse conjugated to 10 nm gold (COS-7 cells). Four 5-minute washes with 0.1% tween-20 in TBS and two washes in distilled

water was followed by counter staining with uranyl acetate for 2 minutes and lead citrate for 30 seconds. The grids were then viewed on a Philips 400 electron microscope. The specifity of the anti-prosaposin antibody was tested on immunoblots of rat lysosomal proteins (Igdoura and Morales, 1995). Anti-cathepsin A and L were used as a specific marker for lysosomes. The specificity of these antibodies was tested immunocytochemically (Igdoura et al., 1995; Satake et al., 1994)

Preparation of Epon sections

The COS-7 cells were trypsinized, pelleted and fixed for 1 hr with 2.5% glutaraldehyde in 0.1M phosphate buffer. After embedding in 1% agarose, the cells were post-fixed with osmium-ferrocyanide. Increasing concentrations of ethanol were used for subsequent dehydration. The cells were then embedded in Epon. Semithin sections were cut and mounted on 200 mesh copper grids. Staining of the grids was done with uranyl acetate for 5 min, followed by lead citrate for 2 min. The grids were viewed on a Philips 400 electron microscope.

Statistical analysis

Quantitative analysis was performed to determine the colloidal gold density of prosaposin labeling in lysosomes. Lysosomes were selected in a manner that included the following criteria; lysosomes had to be spherical, range from 0.2 μm to 0.4 μm in diameter, and have moderate electron density (Igdoura et al., 1996). Thirty lysosomes per condition were examined from 3 different grids that came from three mice. The 15 nm gold particles in each lysosome were counted and the area of each lysosome was determined using a MOP-3 instrument (Carl Zeiss, Germany). The average lysosomal density was measured by dividing the number of gold particles by

the lysosomal area. The mean density and the standard deviation from the mean were calculated for each group, and the results were analyzed statistically using a student t-test.

Plasmids and antibodies

The mutant GGA3-GFP construct is from Dr. Juan Bonifacino's lab (CBMB, NIH, Bethesda, MD). The mutant AP-3 cells are from Dr. William Gahl's lab (NICHD, NIH, Bethesda, MD). The full-length sortilin construct was from Dr. Claus Petersen (University of Aarhus, Denmark). The truncated form of sortilin was generated in our lab. It included the luminal and trans-membrane regions without the cytosolic portion sub cloned into the pcDNA3.1B expression vector. The anti-cathepsin B antibody was a generous gift from Dr. John S. Mort (Shriner's Hospital, Montreal, QC). The anti-$G_{M2}AP$ antibody and plasmid were a generous gift from Dr. Don Mahuran (Hospital for Sick Children, Toronto, ON). The anti-prosaposin antibody was generated in our laboratory against the N-terminus and the functional domains A-B of prosaposin. The characterization and specificity of the prosaposin antibody was discussed in a previous paper (Zhao and Morales, 2000). The anti-myc antibody was purchased from Invitrogen (Mississauga, ON).

SDS-PAGE

Immunoblotting

Culture media from confluent transformed CHO cells (6 ml) treated or not with fumonisin B_1 were collected and concentrated. Previously 300 µl of 1% non-idet, 1 mg of aprotinin B and 2 mg of trypsin inhibitor in 3 ml of buffer phosphate was added to the media. The concentrated media were denatured in 2x sample buffer

(0.625 M Tris/HCl pH 6.8, 20% glycerol, 50 mM dithiothreitol, 3% SDS and 0.001 bromophenol blue) by heating at 100°C, and then electrophoresed on SDS gradient polyacrylamide gel (8-18%) (Laemmli, 1970). Proteins were transferred from the unfixed gel onto a nitrocellulose sheet. The nitrocellulose was blocked with 20% goat serum in TBS, incubated with the anti-prosaposin antibody (1:100), and then with alkaline phosphatase conjugated goat anti-rabbit IgG (1:1000). The blot was then developed using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates. The quantitative interpretation of the results was carried out by assessing the relative density of bands by densitometric scanning of each band. In all cases three scans were made of each band and averaged to obtain accurate results. A Zeineh Soft Lazer Scanning Densitometer (Biomed Instruments Inc., California, USA) was used for the measurements.

Co-immunoprecipitation assays

In order to test the possible protein/protein interactions of sortilin with prosaposin, $G_{M2}AP$ or cathepsin B, the proteins were in vitro translated using Promega's TnT Coupled Reticulocyte Lysate System (Promega, Madison, WI). Prosaposin, G_{M2} Activator protein, sortilin and truncated sortilin plasmids were incubated with the rabbit reticulocyte lysate from the kit along with RNA polymerase and a complete amino acid mixture. Proteins were labeled using the Transcend Non-Radioactive Translation Detection System (Promega, Madison, WI) for visualization that incorporates biotinylated lysine residues into the polypeptide chain. The proteins were then incubated together and then incubated with the appropriate antibody, anti-prosaposin, anti-$G_{M2}AP$ or anti-cathepsin B. The complex was pulled down using

protein A covered sepharose beads and washed using MatchMaker Co-IP Kit solutions three times. (Clontech, Palo Alto, CA). The proteins were then suspended in reducing sample buffer and boiled at 100°C for 5 min. The samples were subsequently run on 12.5% acrylamide gel, transferred to nitrocellulose paper, and visualized with a strepavidin molecule conjugates to horseradish peroxidase (Promega, Madison, WI). The strepavidin was applied to the nitrocellulose for 1 hour diluted to 1:10000. The blot was developed using a chemiluminescent substrate and exposed to radiographic film for 10 minutes.

RESULTS

I. Effect of sphingolipid depletion on the targeting of prosaposin to lysosomes

A recent study demonstrated that prosaposin reaches the lysosomes in a mannose 6-phosphate receptor independent manner (Igdoura et al., 1996). The same study demonstrated that this protein is tightly bound to the inner leaflet of the Golgi membrane. To assess if a protein/lipid interaction influenced the targeting of prosaposin to the lysosomes, both glycosphingolipids and sphingomyelin were depleted in CHO, CHO transfected with a human prosaposin cDNA and NRK cells using Fumonisin B1 (GSL and Sm), PDMP (GSL) and D609 (Sm). The reason for studying these membrane lipids in the trafficking process is the well-documented interaction between prosaposin and sphingolipids (Vaccaro et al., 1999).

Lysosomal Markers

Anti-cathepsin L antibody is a specific marker for the lysosomal compartment and was used to identify lysosomes in Lowicryl embedded cells (Fig. 1a). The antibody labelled membrane bound structures of more than 0.2 μm in diameter showing various degrees of electron density. These structures were considered lysosomes. Electron-lucent vesicles less than 0.1 μm in diameter found around or near the Golgi apparatus were unlabelled and were identified as secretory vesicles.

Effect of Fumonisin B_1 in NRK and CHO cells

Quantitative analysis of electron micrographs taken from NRK cells reacted with anti-prosaposin antibody demonstrated a decrease in density of colloidal gold

labelling of 59 % in the lysosomes of fumonisin B_1 treated cells as compared to the non-treated NRK cells (Figs. 1b, 1c and 2). Chinese hamster ovary (CHO) cells showed a low level of expression of prosaposin. Fumonisin B_1 treatment reduced the density of gold labelling with anti-prosaposin antibody by 85 % compared to the non-treated cells (Figs. 2, 3).

Selection of Stably Transformed CHO Cells

A major problem during the course of this investigation was the low level of expression of prosaposin in culture cells. Thus, in order to better define the role of GSLs in the transport of prosaposin, CHO cells were transformed with an expression vector containing a human prosaposin cDNA, followed by the dihydrofolate reductase sequence.

Northern blot analysis of transfected clones selected with 0.065 μm methotrexate demonstrated that two of them, designated clone 66 and 62, exhibit the presence of bicistronic mRNAs containing both endogenous prosaposin (3.15 kb) and prosaposin/dihydrofolate reductase sequence (3.50 kb) respectively (Fig. 4).

Since clone 66 produced larger quantities of prosaposin mRNA, it was tested for the production of prosaposin protein. Figure 5 shows two SDS polyacrylamide gel electrophoresis of immunoprecipitated prosaposin from stably transformed CHO cells (clone 66). Autoradiography revealed two labeled proteins corresponding to apparent molecular masses of 65 kDa found in cell lysates and 70 kDa found in culture media. The intensity of the labeled band of clone 66 was 4 to 7 folds higher than that of the control CHO cell line transfected with the same vector without the prosaposin cDNA (clone 74).

Immununogold labeling with anti-prosaposin antibody of CHO cells transfected with the expression vector carrying the human prosaposin cDNA (clone 66) showed intense labeling of the lysosomes (Fig. 6a, 6b). When the numbers of colloidal gold particles per μm^2 of lysosomes was compared between clones 66 and clone 74 CHO cells (control), a 5-fold increase was found in the transformed cells (Fig. 6c). Sections of control CHO cells and of clone 66 CHO cells incubated with normal rabbit serum did not show any reactivity in the lysosomes (data not shown).

Effect of Fumonisin B_1 on stably transfected CHO cells (clone 66)

Overexpression of prosaposin caused a heavy labelling of the lysosomes of CHO cells (clone 66) with anti-prosaposin antibody. Fumonisin B_1 treatment decreased the density of immunogold labelling by 55 % in the lysosomes of these cells compared to the non-treated cells (Fig. 2, 7a, 7b).

On the other hand, the gold particle density increased by 3 folds in the small vesicles (less than 0.1 μm in diameter) found around the nucleus or near the Golgi apparatus in fumonisin B_1 treated cells compared to the non-treated cells (Fig. 8a, 8b, 8c). An increase in labelling of these vesicles was also found in the other cell lines treated with fumonisin B_1 (data not shown).

Immunoblotting with anti-prosaposin antibody revealed that culture media from clone 66 CHO cells contained a major 70 kDa band and that this band was approximately 3 fold stronger in fumonisin B_1 treated cells (Fig. 9).

Immunogold Labeling Studies with Anti-Cathepsin A Antibody

Cathepsin A is a lysosomal hydrolase known to be targeted to the lysosomes via the mannose 6-phosphate receptor. In order to determine if the mannose 6-

phosphate receptor pathway was affected by the fumonisin B_1 treatment an anti-cathepsin A antibody was used. Quantitative immunoelectron microscopy using anti-cathepsin A antibody with NRK, CHO, and clone 66 CHO cells treated or not with fumonisin B_1 showed no significant differences ($p \leq 0.05$) in the immunogold labeling density in the lysosomes (Fig. 10).

Confocal Microscopy of Clone 66 Cells Treated with FB_1, PDMP and D609

Control untreated cells (clone 66) showed an intense reaction with anti-prosaposin antibody in the perinuclear Golgi region. A granular reaction was also observed on the periphery of this region that appears to correspond to immunostained lysosomes. Fumonisin B_1 treatment produced a loss of granular staining and a slight decrease in Golgi fluorescence. PDMP treated cells displayed a pattern similar to the untreated control cells. Finally, the D609 treated cells did not display any immunoreactivity (Fig. 11)

II. Role of sphingomyelin in the transport of prosaposin to the lysosomes

Sphingomyelin was shown to conditionally interact with the D domain of prosaposin prior to its transport to the lysosomes. However, the evidence for the role of sphingomyelin in this process was based on the use of sphingolipid inhibitors. Thus, one of the objectives of our investigation was to examine the direct effect of sphingomyelin and sphingomyelin precursors in the transport of prosaposin to the lysosomes using sphingomyelin depleted cells.

Treatment of sphingolipid depleted cells with exogenous sphingolipids – confocal microscopy

Control Cells: In order to determine if the incubation of CHO cells with

sphingomyelin, dihydroceramide and ceramide alters or not the distribution of prosaposin to the lysosomes, CHO cells were incubated with these lipids for 12, 24 and 48 hours and stained with α-prosaposin antibody. The immunostaining yielded a perinuclear Golgi-like staining and a granular reaction typical of lysosomes comparable to CHO cells which did not receive the lipid supplementation (Lefrancois et al., 1999) (Figs.12A-D, 13A-D, 14A-D).

Effect of dihydroceramide, ceramide and sphingomyelin in CHO cells treated with Fumonisin B_1: Treatment of cells with the sphingolipid inhibitor Fumonisin B_1 showed a decrease in immunostaining with α-prosaposin antibody (Figs.12E, 13E, 14E). Incubation of FB_1 treated cells with ceramide, dihydroceramide, or sphingomyelin for 12, 24 and 48 hours increased the immunostaining pattern similar to that of the control cells (Figs.12F-H, 13F-H, 14F-H). FB_1 blocks the synthesis of dihydroceramide but does not interfere with the conversion of exogenous dihydroceramide or ceramide to sphingomyelin. Hence the addition of sphingomyelin or the restoration of the sphingomyelin pathway restituted the cytoplasmic staining in CHO cells.

Effect of dihydroceramide, ceramide and sphingomyelin in CHO cells treated with D609: Compared with non-treated cells, D609 significantly decreased the cytoplasmic immunostaining with α-prosaposin antibody (Figs.12M, 13M, 14M). The addition of ceramide or dihydroceramide for 24 hours did not modify this weak staining (Figs.13N-O), while the addition of exogenous sphingomyelin for 24 hours (Fig. 13P) increased the staining to a level similar to that of the control cells (Fig.13A). D609 inhibits the conversion of ceramide to sphingomyelin. Therefore,

exogenous ceramide and dihydroceramide cannot restore the sphingomyelin pathway due to the distal action of this inhibitor. When the cells were incubated with ceramide, dihydroceramide, and sphingomyelin for 12 hours (Figs.12N-P), the immunostaining pattern of α-prosaposin was weak, indicating that exogenous sphingomyelin did not have sufficient time to function. When the cells were treated with D609, followed by 48-hour incubation with ceramide, dihydroceramide, and sphingomyelin, no labeling was detected with α-prosaposin antibody (Figs.14N-P). Incubation at 48 hours was of interest because the turnover time of sphingomyelin in vitro at 37°C is 25-26 hours. Hence, at 48 hours exogenous sphingomyelin should be degraded explaining the lack of immunostaining found in these cells. The cells incubated with sphingolipid precursors, including sphingomyelin, for 48 hours after FB_1 treatment were labeled (Figs.14F-H). Although sphingomyelin may be broken down in these cells they are capable of synthesizing sphingomyelin by using ceramide from catabolized sphingolipids due to the location of the FB_1 blockage (Table 1).

Treatment of the CHO cells with PDMP: Incubation of CHO cells with PDMP that selectively blocks the production of glycosphingolipids but not of sphingomyelin (Lefrancois et al., 1999) (Table 1), yielded a similar immunostaining to the control cells (Figs.12I-L, 13I-l, 14I-L). This suggests that the transport of prosaposin to the lysosomes did not require the presence of glycosphingolipids. The addition of ceramide, dihydroceramide or sphingomyelin for 12, 24 and 48 hours did not change the immunostaining of the cells reacted with anti-prosaposin antibody.

Cathepsin B staining of CHO cells: The inhibitors and the exogenous sphingolipids were also added to CHO cells labeled with α-cathepsin B antibody (Figs.15A-L).

Cathepsin B is a soluble lysosomal protein that is transported from the *trans*-Golgi to the lysosomes by the M6P receptor (Mort and Buttle, 1997). As expected, inhibition of lipid synthesis with the inhibitors FB_1 or D609 yielded a labeling pattern similar to that of untreated control cells labeled with anti-cathepsin B antibody. The addition of exogenous ceramide, dihydroceramide, or sphingomyelin alone, for 24 hours or in conjunction with any of the inhibitors did not change the immunostaining of cells labeled with α-cathepsin B antibody. This indicated that the treatment with the inhibitors and the addition of exogenous lipids did not affect the protein synthetic machinery or other cell biological functions, such as M6P lysosomal targeting.

Treatment of sphingolipid depleted seminiferous tubules with exogenous sphingolipids – electron microscopy

In order to examine the transport and distribution of endogenous prosaposin, we used isolated seminiferous tubules from mouse testes. In this model system two cell types are present, the germinal cells and the Sertoli cells. Sertoli cells are the somatic components of the seminiferous tubules, which have lysosomes containing prosaposin (Igdoura and Morales, 1995). Immunogold labeling of seminiferous tubules with the α-prosaposin antibody labeled specifically the lysosomes of Sertoli cells (Fig.16). The quantitative analysis of immunogold labeling of the lysosomes of Sertoli cells from seminiferous tubules incubated in culture medium alone yielded an average density of 14.57 gold particles/μm^2 (Fig.17). Sertoli cell lysosomes from seminiferous tubules supplemented with ceramide yielded a density of 16.88 gold particles/μm^2 while the tissue treated with sphingomyelin produced a density of 15.87 gold particles/μm^2. These results were not statistically significantly different when

compared to untreated control seminiferous tubules (Figs.16, 17).

Treatment of Seminiferous Tubules with Fumonisin B_1: Tissue treated with 25 μg/ml of FB_1 displayed a reduction of lysosomal immunogold labeling compared to control tissue. Still, few grains were localized in the lysosomes and background was minimal. Statistical analysis supported these observations with the average density decreased to 3.79 gold particles/μm^2. This represented a reduction of 74% in comparison to the untreated control cells. This reduction was shown to be statistically significant using a student t-test. Seminiferous tubules treated with FB_1 and then supplemented with ceramide produced a gold density value of 16.70 gold particles/μm^2 while the sphingomyelin-supplemented tissue yielded a value of 17.63 gold particles/μm^2 in the lysosomes of Sertoli cells. These values were comparable to the density of untreated control Sertoli cells and were not statistically different from the untreated control values, suggesting that these lipids restore the lysosomal targeting of prosaposin (Figs. 16,17).

Treatment of Seminiferous Tubules with PDMP: As expected from the confocal microscopy data, tissue treated with 25 μg/ml of PDMP did not show a decrease in gold particle labeling in the lysosomes of Sertoli cells. The gold particle density in the PDMP treated tissue was 14.39 gold particles/μm^2. This was not statistically different from the untreated control tissue (Figs. 16,17).

Treatment of Seminiferous Tubules with D609: Tissue treated with 100 μg/ml of D609 exhibited a marked decrease in lysosomal labeling compared to control Sertoli cells. Quantitative studies supported the qualitative analysis, registering the average density of immunogold grains to be 3.16 gold particles/μm^2. This represented a

reduction of 78% in comparison to untreated control Sertoli cells and was shown to be statistically significant using a student t-test. The mouse testis treated with D609 and then supplemented with ceramide did not show a restoration of labeling. The density of grains in this sample was 2.38 gold particles/μm^2, a reduction in labeling of over 80 %. This reduction was also shown to be statistically significant. The sphingomyelin supplemented tissue however had a comparable level of density compared to untreated control tissue of 17.70 gold particles/μm^2 suggesting that sphingomyelin is involved in the transport of prosaposin to the lysosomes of Sertoli cells (Figs. 16,17).

III. Role of functional domain D and the Carboxy-terminus in the targeting of prosaposin to the lysosomes

The objective of this investigation was to assess the role of the functional domain D and the C-terminus of prosaposin in the transport of this molecule to the lysosomes. Thus, a chimeric construct formed by these 2 prosaposin domains along with albumin was transfected into COS-7 cells as a dominant negative competitor to determine if it uses the same lysosomal pathway as prosaposin. Additionally, the targeting of this construct to the lysosomes was assessed in cells treated with the sphingolipid synthesis inhibitors supplemented or not with sphingomyelin.

Wild Type Construct Transfected COS-7 cells

Cultured cells transfected with a prosaposin wild type construct or with the albumin-prosaposin chimeric construct were incubated with sphingolipid inhibitors, supplemented with sphingomyelin and immunostained with a myc-tag antibody. As expected, cells transfected with the full-length prosaposin cDNA, linked to a myc-

tag, showed a strong immunoreaction to the α-myc antibody (Figs.18A-C). The antibody yielded an intense perinuclear reaction as well as a cytoplasmic punctate staining. LysoTracker (red fluorescence), which is known to stain acidic compartments (*trans*-Golgi, endosomes and lysosomes), also produced a Golgi-like intense reaction in the perinuclear region and a punctate staining in the cytoplasm of the transfected cells. Several overlapping images, appearing as yellow fluorescence, could be seen, demonstrating that recombinant prosaposin and the LysoTracker dye shared the same compartment. Cells treated with D609 lost the green prosaposin staining (Fig.18D). The LysoTracker staining was decreased but still present in punctate structures (Fig.18E). No overlapping was found under this experimental condition (Fig.18F). When these cells were supplemented with exogenous sphingomyelin, both the green prosaposin staining in the lysosomal compartment and the red LysoTracker staining of the Golgi and lysosomal compartment returned (Figs.18G-I). Several overlapping structures could be seen suggesting the return of prosaposin into the lysosomal compartment substantiating the hypothesis that sphingomyelin is required for the transport of prosaposin to the lysosomes.

COS-7 Cells Transfected with the Albumin-Prosaposin Chimeric Construct

Cells transfected with this construct designated Albumin/SAP-D/COOH/myc-tag, yielded a strong immunostaining in the perinuclear region and in cytoplasmic punctate structures (Figs.19A-I). Thus, albumin linked to the D domain plus the COOH-terminal region of prosaposin appeared to be redirected to the lysosomes. Based on this result, it was hypothesized that the COOH-terminal region was required to interact with a trafficking protein after a conditional interaction of the

prosaposin D domain with sphingomyelin present in the Golgi membrane. Our results showed that Albumin/SAP-D/COOH/myc-tag transfected cells treated with D609 lost all prosaposin staining in their lysosomes. No overlap between the immunostaining and LysoTracker was ever found in these cells. Only the red staining of the LysoTracker was found in the Golgi and in the endosomal/lysosomal compartment. Cells treated with D609 and supplemented with sphingomyelin showed a green staining pattern similar to untreated control cells. Cytoplasmic green punctate structures as well as intense Golgi perinuclear reactions were seen in the transfected cells. LysoTracker frequently overlapped with the green immunostaining demonstrating that the Albumin/SAP-D/COOH/myc-tag construct reached the lysosomal compartment. This result provided additional evidence that sphingomyelin is required for prosaposin transport to the lysosomes.

Effect of Dominant Negative Competitors on Endogenous Prosaposin

The objective of this study was to examine the effect of a dominant negative competitor on the expression of prosaposin and on the morphological phenotype of the lysosomal compartment of transfected cells. The dominant negative and control constructs used for transfection were synthesized by linking the D functional domain of prosaposin to the secretory protein albumin. The carboxy-terminal domain of prosaposin was only added to the dominant negative constructs. The plasmid used for transfection contained a myc-tag, which allowed for the detection of the chimeric constructs with a α-myc antibody. The α-myc antibody was visualized with a secondary antibody conjugated to 10 nm colloidal gold particles. Endogenous prosaposin was detected with a primary α-prosaposin antibody, and a secondary

antibody conjugated to 15 nm gold particles.

Plasmid only: As a negative experimental control, COS-7 cells were transfected with the plasmid only. The immunogold labeling of these cells mimicked that of the Alb-D construct cells, with high number of endogenous protein gold particles (15 nm) localized to the lysosomes. Small gold particles (10 nm) were absent from the lysosomes (Fig.20A).

Alb-D Construct: In COS-7 cells transfected with the Alb-D construct, 10 nm gold particles representing the constructs were absent from the lysosomes. Instead, these cells showed a strong reaction with 15 nm gold particles representing endogenous prosaposin labeling (Fig. 20B).

Alb-D-COOH Construct: Immunogold labeling in the lysosomes of cells transfected with the Alb-D-COOH construct showed an overwhelming majority of 10 nm construct-associated gold particles, and weak labeling of endogenous-associated 15 nm particles (Fig. 20C). This observation was further confirmed by a quantitative analysis that demonstrated a statistically significant decrease of endogenous prosaposin immunogold labeling and an increase in the immunogold labeling of the chimeric construct (Fig. 20D)

Morphological Phenotype of Dominant Negative Competitors

The purpose of this experiment was to examine the morphological effects on the lysosomes of COS-7 cells transfected with the dominant negative competitor. The rationale was that the Alb-D-COOH construct competes out the endogenous prosaposin, it should act as a dominant negative competitor that inhibits the transport of prosaposin to the lysosomes. Prosaposin deficient lysosomes should exhibit

accumulation of undigested membranes due to the inability of these organelles to digest sphingolipids (Morales et al., 2001).

Plasmid only: As a negative experimental control, some COS-7 cells were transfected with the plasmid only. The morphology of multivesicular bodies and mature lysosomes in these cells were similar to that of the Alb-D construct cells and wild-type cells (Fig.21A, B).

Alb-D Construct: The multivesicular bodies and mature lysosomes of COS-7 cells transfected with Alb-D constructs were similar to the wild-type morphology (Figs.21C).

Alb-D-COOH Construct: In cells transfected with Alb-D-COOH, lysosomal morphology was compromised. Specifically, no electron-dense mature lysosomes were observed. In the perinuclear region of these cells there was an accumulation of abnormal multivesicular bodies, which contain large quantities of undegraded membrane (Figs.22A, B).

IV. Role of sortilin in the targeting of prosaposin and $G_{M2}AP$ to the lysosomes

Recently, a novel trans-membrane Golgi protein known as sortilin was shown to bind the GGAs and traffic to the lysosomal compartment. In fact, chimeric proteins that had the sortilin cytosolic domain fused to the luminal domain of the M6P-R could restore the trafficking of M6P-R dependant proteins in cells that lacked the M6P-R. Hence, we tested whether or not sortilin is the sorting receptor for prosaposin and $G_{M2}AP$.

Expression and localization of truncated sortilin

In vitro translated full-length or truncated (Δcytosolic) sortilin (Fig. 23A)

were run on a 12.5% acrylamide gel and transferred to nitrocellulose paper. The membrane was blotted for sortilin using an anti-sortilin monoclonal antibody. The full-length sortilin produced a band at approximately 95 kDa while the truncated sortilin was slightly lighter (Fig 23B). COS-7 cells were then transiently transfected with the truncated construct to examine its intracellular localization. The cells were stained for the construct via a myc antibody and for a Golgi marker, anti-Golgin. The construct was retained in the Golgi apparatus and localized to the same compartment as the Golgi marker (Fig 23C).

Truncated sortilin abolishes the transport of prosaposin and $G_{M2}AP$

In this experiment COS-7 cells were transfected with a dominant negative sortilin construct lacking the cytoplasmic region implicated in the binding of GGAs (Nielsen et al., 2001). The construct was linked to a myc-tag to identify transfected from non-transfected cells and to distinguish truncated from endogenous sortilin. The cells were immunostained with the anti-prosaposin (Fig. 24A-D), anti-$G_{M2}AP$ (Fig. 25A-D) or anti-cathepsin B (Fig. 26A-D) antibody followed by incubation with a secondary antibody conjugated to Alexa 488 (green fluorescence). To detect the construct, the same culture cells were immunostained with an anti-myc antibody followed by a secondary antibody conjugated to Alexa 568 (red fluorescence). Non-transfected cells (Fig. 24A, 25A) showed the characteristic anti-prosaposin and anti-$G_{M2}AP$ immunostaining in the perinuclear region as well as in cytoplasmic punctate structures. The transfected cells (Fig. 24B, 24D, 25B, 25D) displayed a perinuclear immunostaining with the anti-prosaposin, anti-$G_{M2}AP$ and the anti-myc antibodies. However, no punctate structures were seen in transfected cells suggesting that over-

expression of truncated sortilin competed for the binding of prosaposin and abolished its transport to the lysosomal compartment. The immunostaining pattern of the anti-cathepsin B antibody was not different in the transfected or non-transfected cells (Fig. 26A-D)

Co-immunoprecipitation shows association of sortilin and SAPs

Full-length sortilin, truncated sortilin, prosaposin and $G_{M2}AP$ were *in vitro* translated and used in a co-immunoprecipitation assay. The binding of sortilin and truncated sortilin to prosaposin or $G_{M2}AP$ was tested using an anti-prosaposin or anti-$G_{M2}AP$ antibody and protein A conjugated sepharose beads. Prosaposin was able to pull down full-length (Fig. 27, Lane 1) and truncated sortilin (95 kDa band) (Fig. 27, Lane 2). This demonstrates that the luminal domain of sortilin binds prosaposin, and hence supports the functional studies in COS-7 cells transfected with the dominant negative form of sortilin. Similarly, $G_{M2}AP$ was able to pull down full-length (Fig. 27, Lane 3) and truncated sortilin (Fig. 27, Lane 4) demonstrating that sortilin interacts with both sphingolipid activator proteins. On the other hand, cathepsin B was not pulled down either by truncated or full-length sortilin (Fig. 27, Lane 5 and 6).

Dominant negative GGA abolishes the transport of SAPs

To substantiate our experimental evidence showing that prosaposin and $G_{M2}AP$ use sortilin to traffic to the lysosomes, COS-7 cells transfected with the dominant negative GGA construct were immunostained with anti-prosaposin (Fig. 28A), anti-$G_{M2}AP$ (Fig. 28B), anti-cathepsin B (Fig. 28C) or anti-LAMP-2 antibodies (Fig. 28D). Since the GGA construct was linked to the green fluorescence

protein (GFP), transfected cells were recognized from non-transfected cells by their green fluorescence. While transfected cells did not exhibit immunostaining for anti-cathepsin B, anti-prosaposin, or anti-$G_{M2}AP$ antibodies, the anti-LAMP-2 antibody produced a strong granular and perinuclear immunostaining. Non-transfected COS-7 cells were immunostained by the four antibodies.

AP-3 mutant does not affect the transport of SAPs

Human fibroblasts carrying a mutation in the μ subunit of AP-3, were immunostained with anti-prosaposin antibody (Fig. 29A), anti-$G_{M2}AP$ (Fig. 29B), anti-cathepsin B antibody (Fig. 29C), or anti-LAMP-2 antibody (Fig. 29D). While anti-cathepsin B, anti-prosaposin and anti-$G_{M2}AP$ antibodies yielded a perinuclear Golgi-like reaction and a cytoplasmic punctate reaction typical of lysosomes; anti-LAMP-2 antibody did not generate immunostaining. As expected, anti-LAMP-2 antibody immunostained punctate structures in wild-type fibroblasts (Fig. 29D, inset).

Fig. 1 (A) CHO cell (clone 66) cell labelled with anti-Cathepsin L antibody. The labelling is seen over a large membrane bound structure (L) of approximately 0.3 μm in diameter. x40,000. (B) NRK cell incubated in control media without fumonisin B_1 for 48 hours. Note lysosomes (L) moderately labelled with anti-prosaposin antibody. x35,000 (C) NRK cell incubated in media containing fumonisin B_1 for 48 hours. Notice lysosome (L) weakly labelled with anti-prosaposin antibody. x35,000.

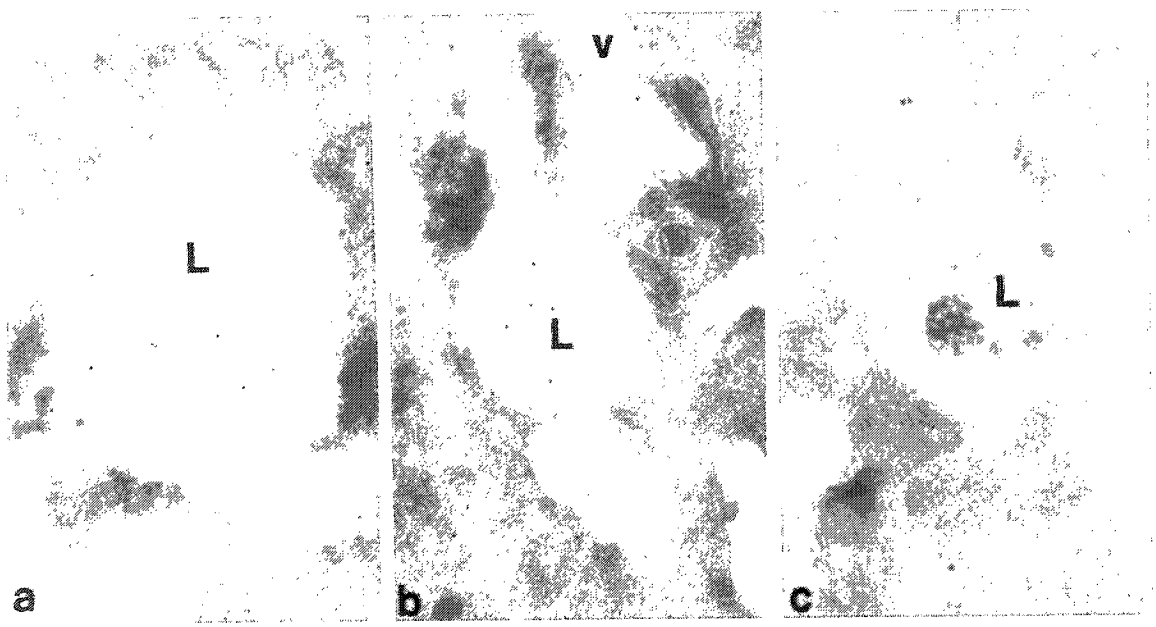

L
a
v
L
b
L
c

Fig. 2 (A) CHO cell grown in control media. Note lysosome (L) moderately labelled with anti-prosaposin antibody. x44,250. (B) CHO cell treated for 48 hours with fumonisin B_1 and labelled with anti-prosaposin antibody. Notice lysosome (L) unlabelled. x34,000.

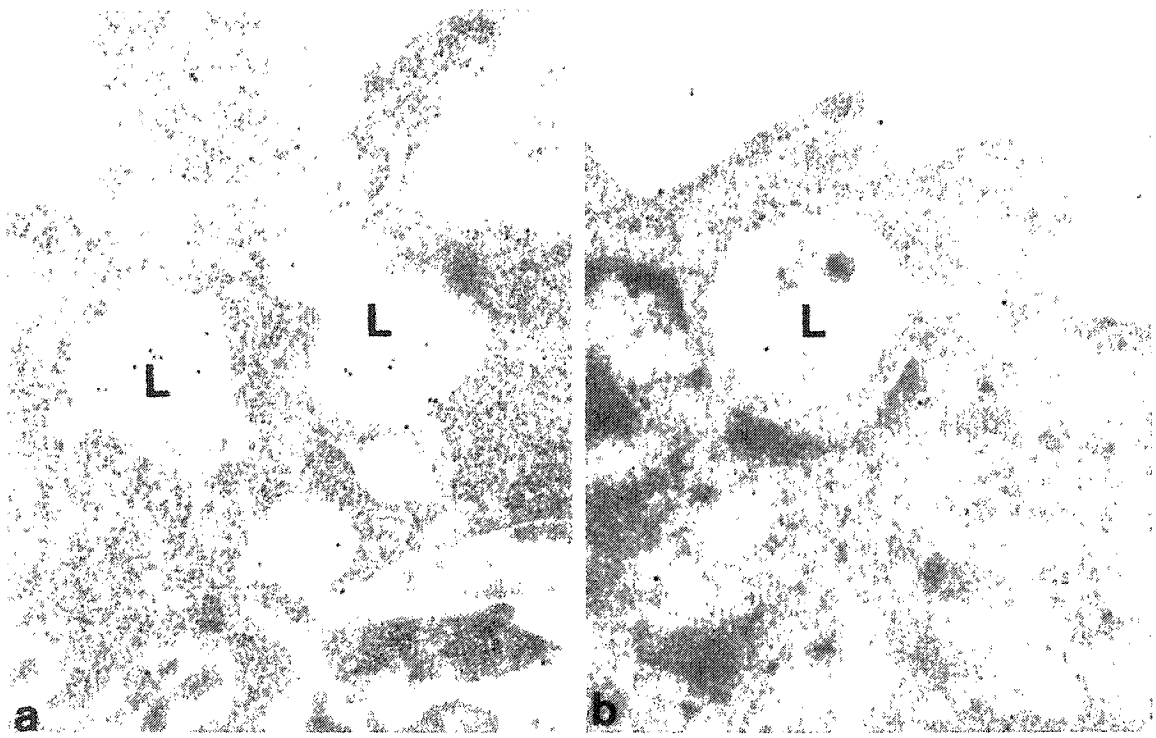

L
L
a
L
b

Fig. 3 Bar graph showing the number of colloidal gold particles per μm^2 of lysosome of three cell lines treated (white bars) or not (black bars) with fumonisin B_1. NRK and CHO cells were immunoreacted with anti-prosaposin antibody. Error bars represent standard deviation of the mean.

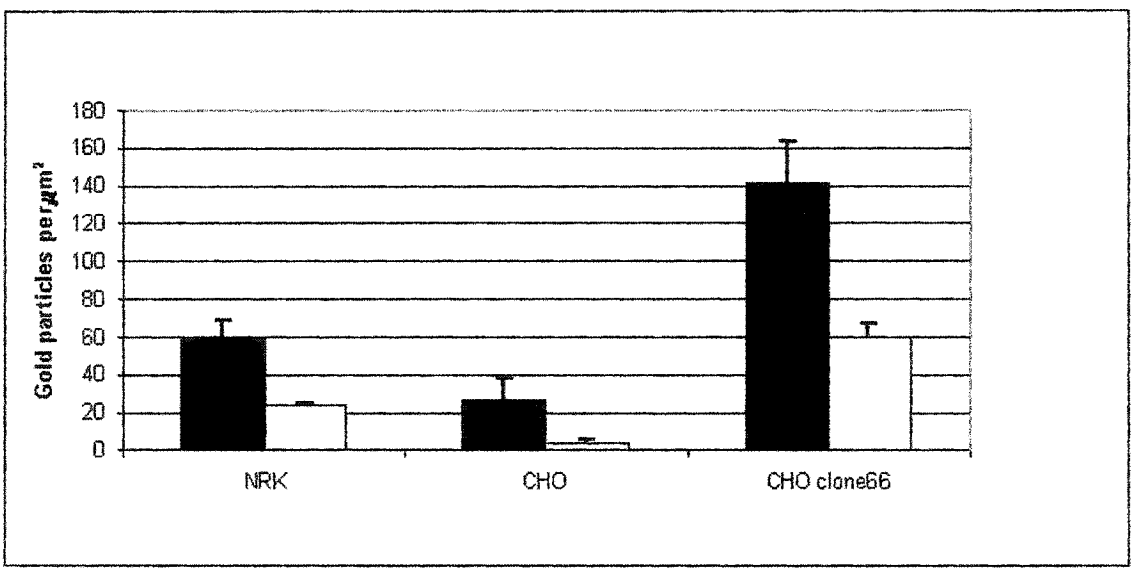

Gold particles per μm²
180
160
140
120
100
80
60
40
20
0
NRK
CHO
CHO clone66

Fig. 4 Northern blot analysis of prosaposin mRNA in amplified cell lines. Total RNA (5 μg) isolated from 4 transformants resistant to 0.065 μM methotrexate was electrophoresed on an agarose gel, transferred to nylon and hybridized to a prosaposin-DIG cDNA probe. Lanes 3 and 4 (designated clones 66 and 62 respectively) exhibit the presence of the bicistronic mRNA containing both endogenous prosaposin (3.15 kb) and prosaposin/dihydrofolate reductase sequence (3.50 kb).

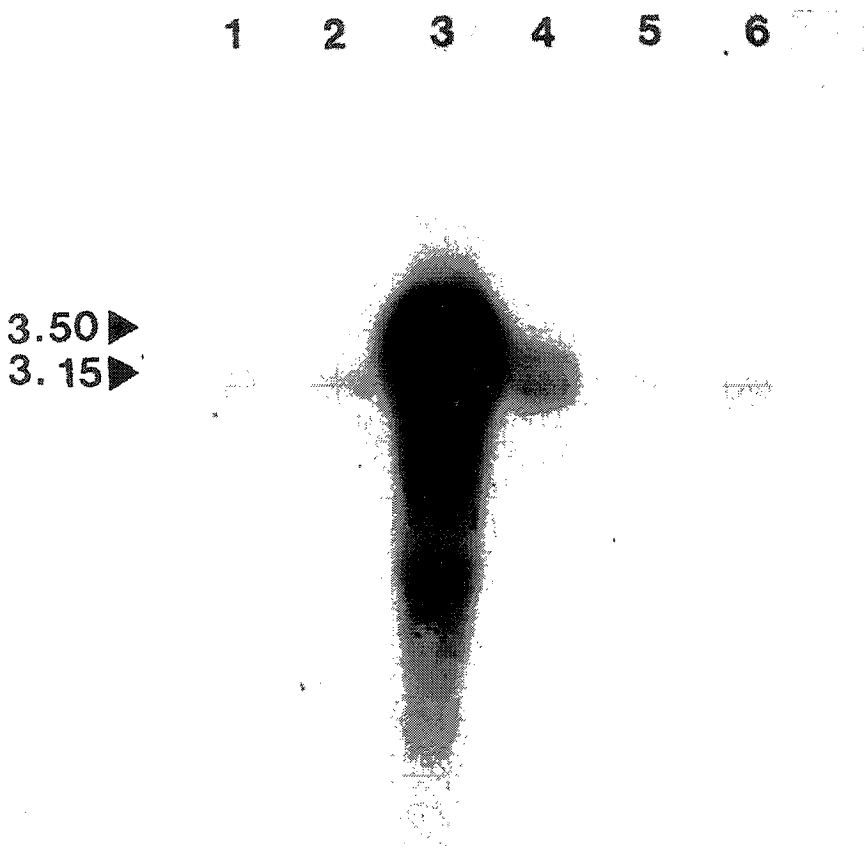

1
2
3
4
5
6
3.50
3.15

Fig. 5 Immunoprecipitation of prosaposin expressed in stably transformed CHO cells (clone 66) and control CHO cells. Control CHO (clone 74) cells were transformed with the vector p91093b without prosaposin cDNA and selected for resistance up to 0.5 μM of methotrexate. In both cases CHO cells were ^{35}S-methionine metabolically labeled for 90 min. The labeled proteins were immunoprecipitated using anti-prosaposin antibody and separated by SDS-PAGE (10%) under reducing conditions. Radiolabeled bands, along with ^{14}C-methylated protein markers used as molecular standards, were visualized by fluorography. Panel A: lane 1, standards; lane 2, cell lysate of clone 66 treated with 0.12 μM methotrexate; lane 3, cell lysate of clone 66 treated with 0.24 μM methotrexate; cell lysate of control CHO cells (clone 74) treated with 0.5 μM methotrexate. Panel B: lane 1, standards; lane 2, culture media of clone 66 treated with 0.12 μM methotrexate; lane 3, culture media of clone 66 treated with 0.24 μM methotrexate; lane 4, culture media of control CHO cells (clone 74) treated with 0.5 μM methotrexate. Note that fluorography revealed proteins corresponding to apparent molecular masses of 65 kDa in cell lysates and 70 kDa found in culture media.

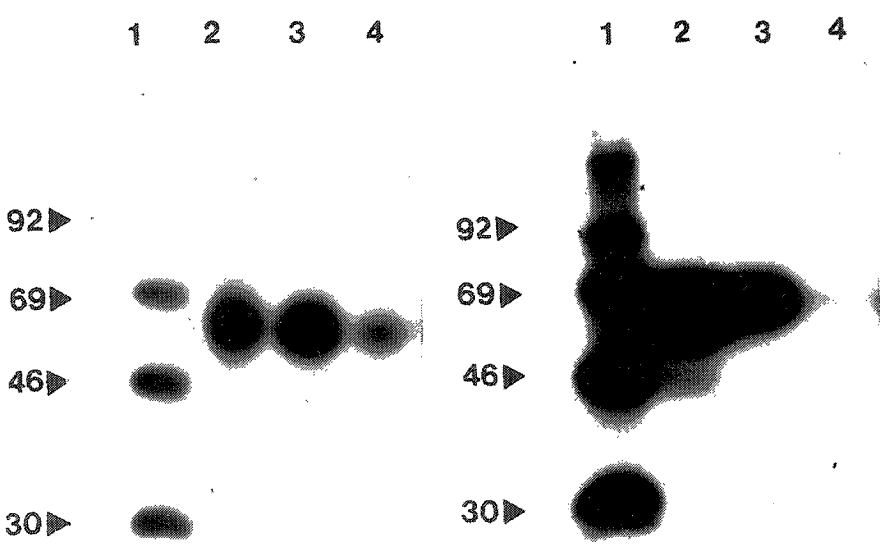
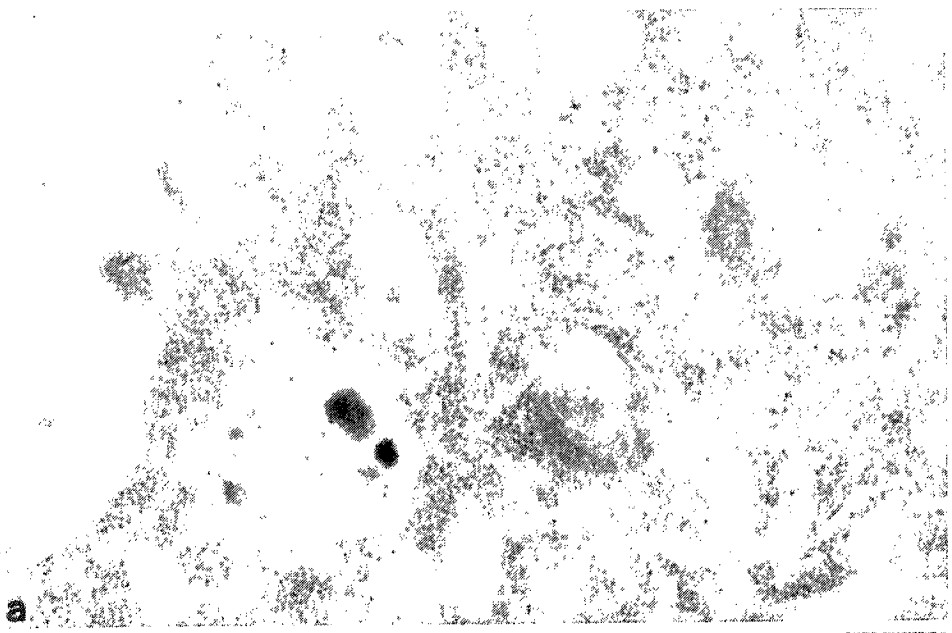
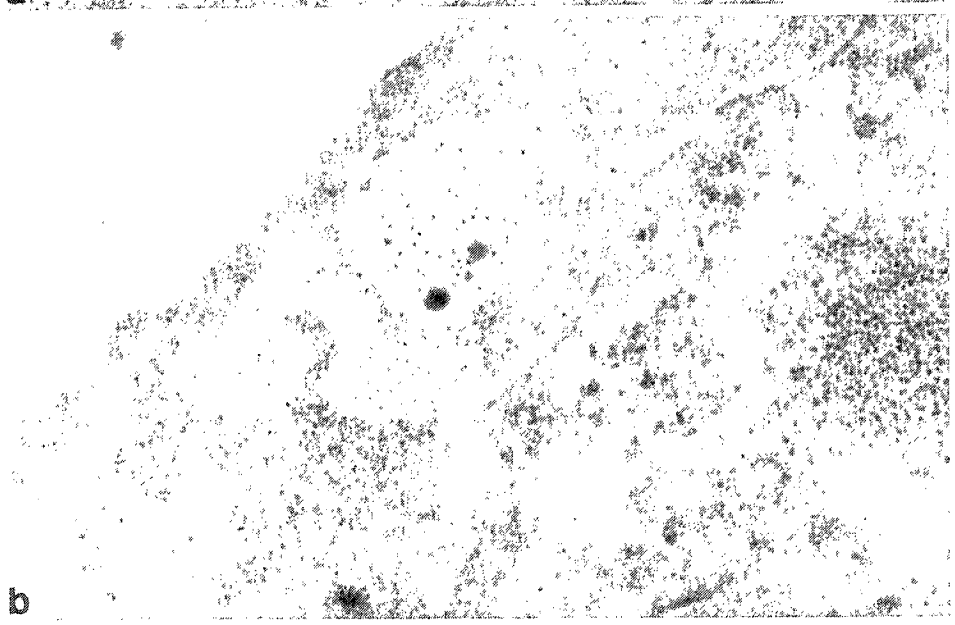

1 2 3 4
92
69
46
30
1 2 3 4
92
69
46
30

Fig. 6 Immunogold labeling with anti-prosaposin antibody of CHO cells transfected with the expression vector carrying (clone 66) or not (clone 74) the human prosaposin cDNA. (A) Lysosome (L) of control CHO cell (clone 74) showing weak labeling. (B) Lysosome (L) of clone 66 cell showing intense gold labeling (L). Magnification: x30000. (C) Bar graph representing the number of colloidal gold particles per μm^2 of lysosomes of CHO cells transformed with the expression vector carrying (clone 66) or not (clone 74) the prosaposin cDNA. Error bars represent standard deviation of the mean.


a


b

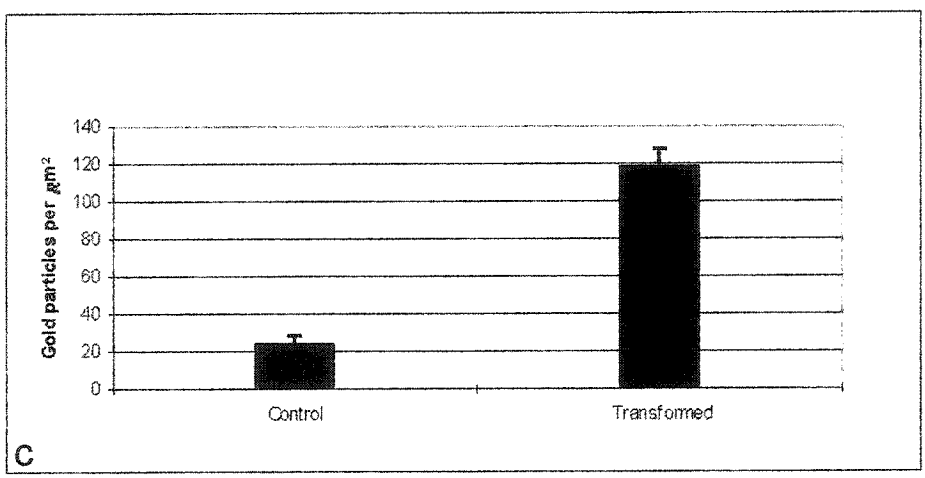

Gold particles per µm²
140
120
100
80
60
40
20
0
Control
Transformed
c

Fig. 7 (A) CHO (clone 66) grown in media without fumonisin B_1. Large lysosome (L) heavily labelled with anti-prosaposin antibody. x34 000. (B) CHO (clone 66) cell treated with fumonisin B_1 for 48 hours. Two large lysosome (L) moderately labelled with anti-prosaposin antibody. x34 000.

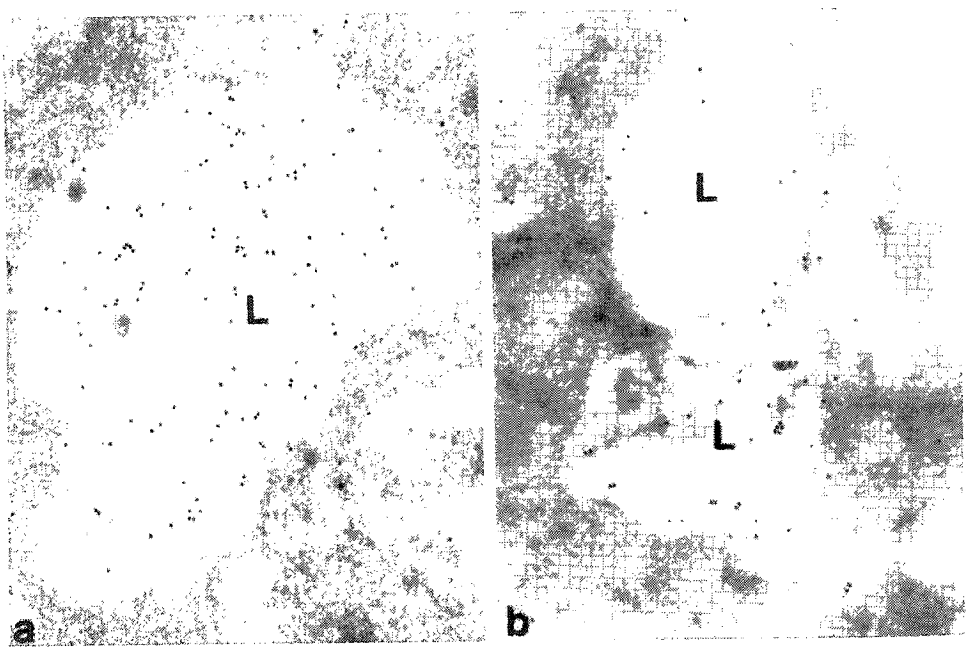
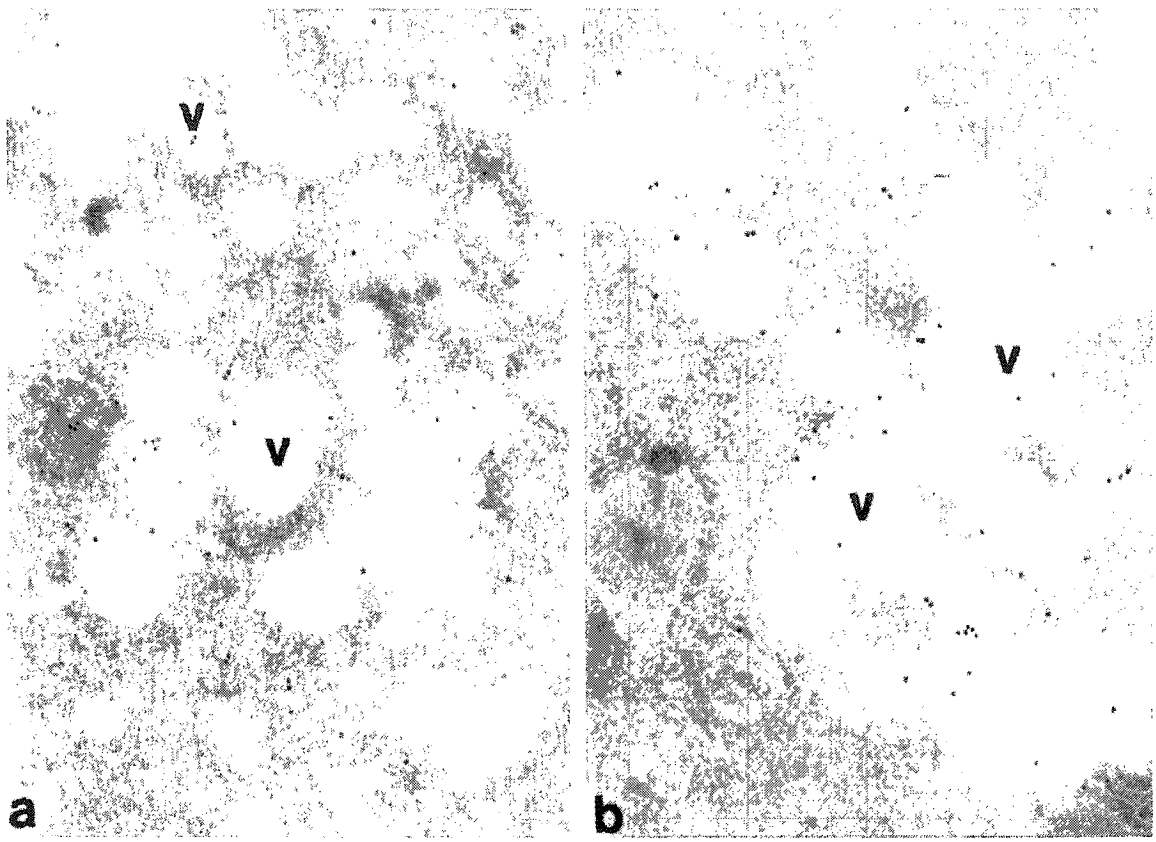

L
a
L
L
b

Fig. 8 (A) Control CHO cell (clone 66) labelled with anti-prosaposin antibody. Vesicles (V) are weakly labelled. x34 000. (B) CHO (clone 66) incubated with fumonisin B_1 and labelled with anti-prosaposin antibody. Vesicles (V) are moderately labelled. x34 000. (C) Bar graph representing number of colloidal gold particles per μm^2 of vesicles in the Golgi region of CHO cells (clone 66) non treated (control) or treated with fumonisin B_1. Error bars represent standard deviation of the mean.


v
v
a
v
v
b

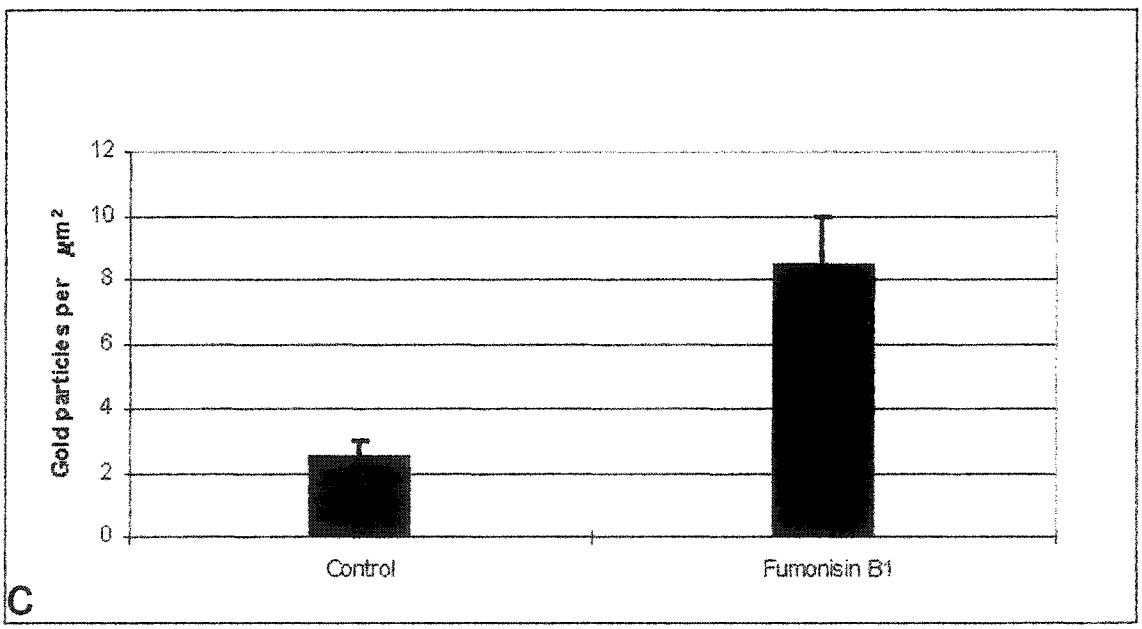

12
10
8
6
4
2
0
Gold particles per µm²
Control
Fumonisin B1
C

Fig. 9 Immunoblotting of culture media proteins from stably transformed CHO cells (clone 66) reacted with anti-prosaposin antibody. Culture media from clone 66 CHO cells untreated with fumonisin B_1 contains a 70 kDa band (lane 1). This band is stronger in fumonisin B_1 treated cells (lane 2). Densitometric analysis demonstrated a 3-fold increase of prosaposin in the media of fumonisin B_1 treated cells.

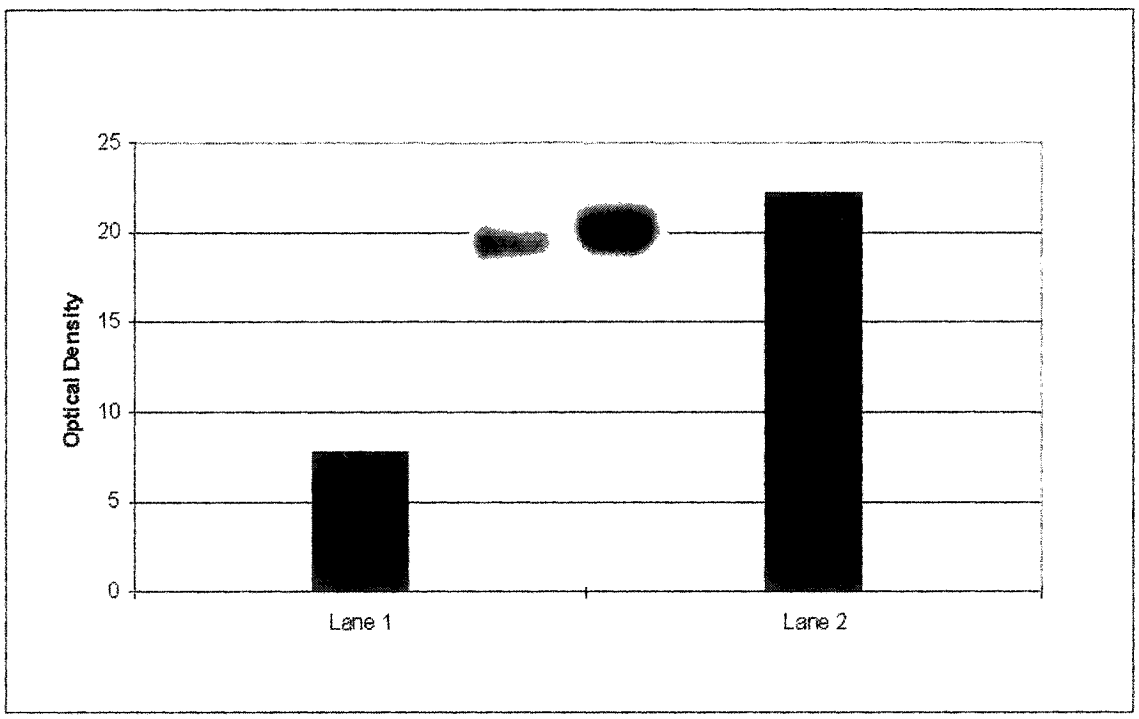

25
20
15
10
5
0
Optical Density
Lane 1
Lane 2

Fig. 10 Bar graph showing immunogold labeling density of cathepsin A antibody in lysosomes of cell lines treated (white bars) or not (black bars) with fumonisin B_1. Treatment with fumonisin B_1 did not cause any significant difference in the labeling density. Error bars represent standard deviation of the mean.

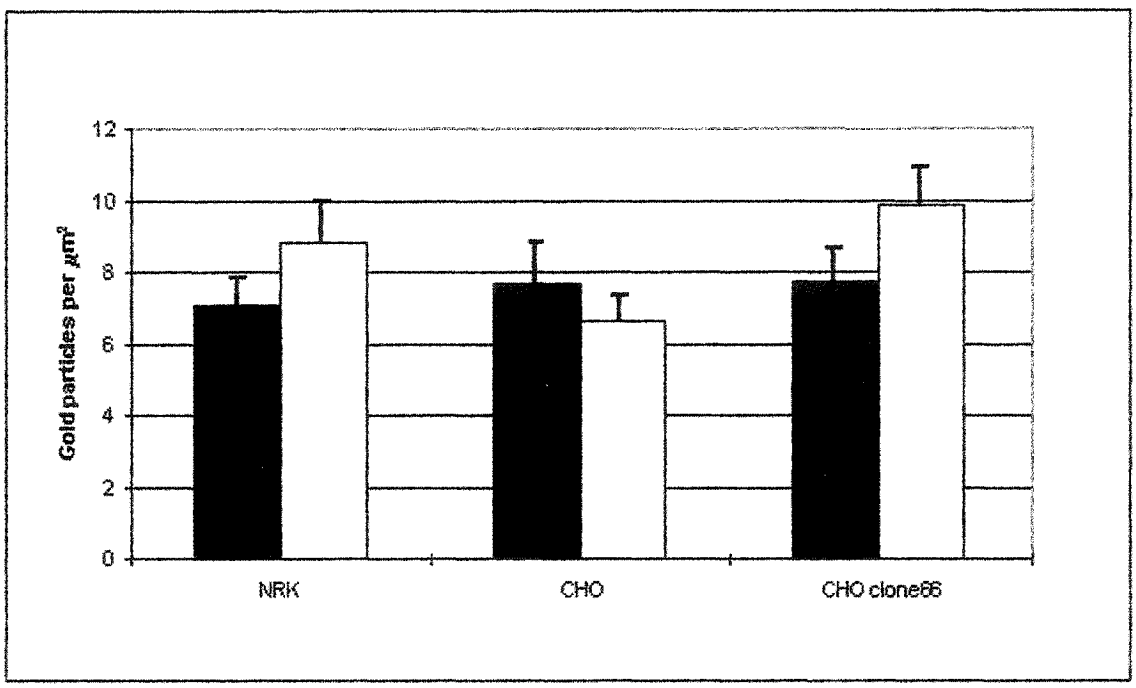

12
10
8
6
4
2
0
Gold particles per μm²
NRK
CHO
CHO clone66

Fig. 11 Confocal microscopy of cells immunostained with anti-prosaposin antibody. Control untreated CHO cells (clone 66) shows a granular reaction in the Golgi region of these cells. Fumonisin B_1 abolished the granular staining. PDMP treatment did not cause any change in the staining pattern. D609 incubation abolished both the Golgi and granular staining; x600; bar=10μm

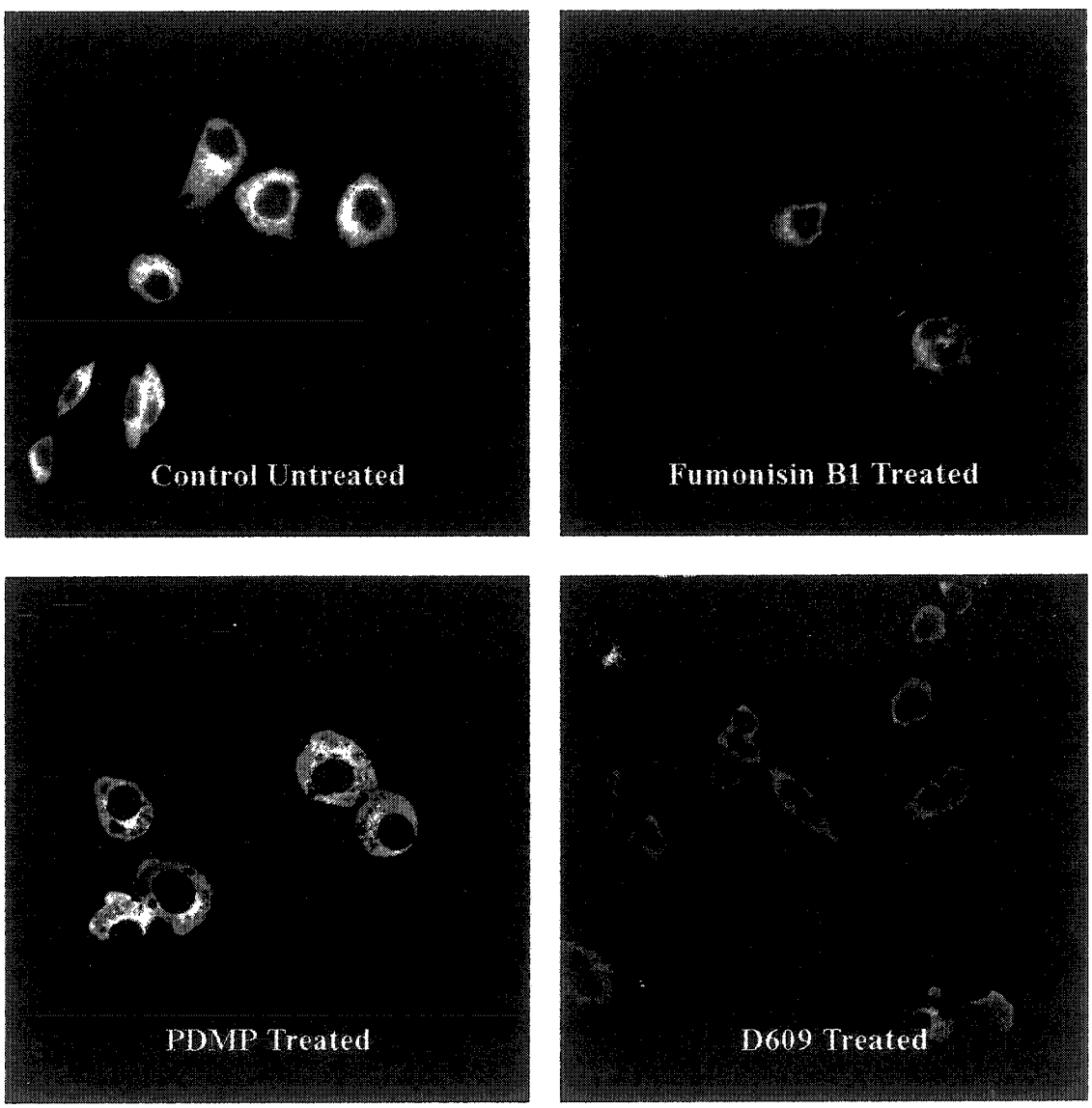

Control Untreated
Fumonisin B1 Treated
PDMP Treated
D609 Treated

Fig. 12 CHO cells over expressing prosaposin incubated with 5 μM ceramide (B), 5 μM dihydroceramide(C), or 5 μM sphingomyelin (D) for 12 hours. Untreated cells (A, no lipids) served as a control. All cells displayed a similar immunostaining pattern; showing a perinuclear reaction.

CHO cells over expressing prosaposin were treated with FB_1 (25 μg/ml) (E-H) followed or not by a 12-hour incubation with 5 μM ceramide (F), 5 μM dihydroceramide (G), or 5 μM sphingomyelin (H). Treated cells showed a decreased immunostaining pattern compared to CHO cells not treated with the inhibitor (E). Addition of ceramide, dihydroceramide, or sphingomyelin restored the labeling pattern similar to the untreated control cells (A).

CHO cells over expressing prosaposin were treated with GSL inhibitor PDMP (25 μg/ml) (I-L) followed or not by a 12-hour incubation with 5 μM ceramide (J), 5 μM dihydroceramide (K), or 5 μM sphingomyelin (L). All cells displayed a similar immunostaining pattern to untreated control cells (A).

CHO cells over expressing prosaposin were treated with D609 (100 μg/ml) (M-P) followed or not by a 12-hour incubation with 5 μM ceramide (N), 5 μM dihydroceramide (O), or 5 μM sphingomyelin (P). The D609 treated cells showed a decreased immunostaining pattern compared to CHO cells not treated with the inhibitor (M). Ceramide or dihydroceramide addition did not modify the weak labeling. Sphingomyelin addition displayed a weak perinuclear reaction. All cells

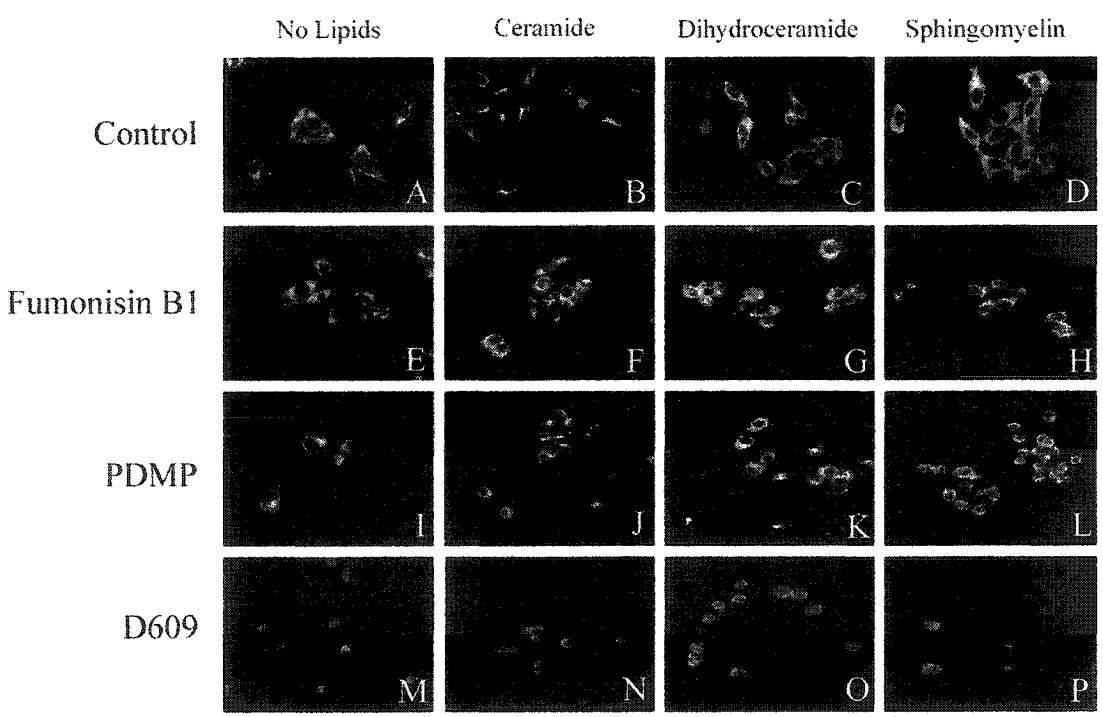

No Lipids
Ceramide
Dihydroceramide
Sphingomyelin
Control
Fumonisin B1
PDMP
D609
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P

Fig. 13 CHO cells over expressing prosaposin were incubated with 5 μM ceramide (B), 5 μM dihydroceramide (C), or 5 μM sphingomyelin (D) for 24 hours. Untreated cells served as a control (A). All cells displayed a similar immunostaining pattern, showing a perinuclear reaction.

CHO cells over expressing prosaposin were treated with FB_1 (25 μg/ml) (E-H) followed or not by a 24-hour incubation with 5 μM ceramide (F), 5 μM dihydroceramide (G), or 5 μM sphingomyelin (H). The FB_1 treated cells showed decreased immunostaining compared to CHO cells not treated with the inhibitor (E). The addition of exogenous ceramide, dihydroceramide, or sphingomyelin restored the immunostaining similar to the untreated control cells (A).

CHO cells over expressing prosaposin were treated with GSL synthesis inhibitor PDMP (25 μg/ml)(I-L) followed or not by a 24-hour incubation with 5 μM ceramide (J), 5 μM dihydroceramide (K), or 5 μM sphingomyelin (L). All cells displayed a similar immunostaining pattern to untreated control cells (A).

CHO cells over expressing prosaposin were treated with D609 (100 μg/ml) (M-P) followed or not by a 24-hour incubation with 5 μM ceramide (N), 5 μM dihydroceramide (O), or 5 μM sphingomyelin (P). The D609 treated cells showed a decreased immunostaining compared to CHO cells not treated with the inhibitor (M). Ceramide or dihydroceramide additions did not modify this low labeling pattern. In contrast, the addition of sphingomyelin restored the labeling pattern to one similar to the untreated control cells (A). All cells were labeled with anti-prosaposin antibody followed by a secondary antibody conjugated to FITC. (x400)

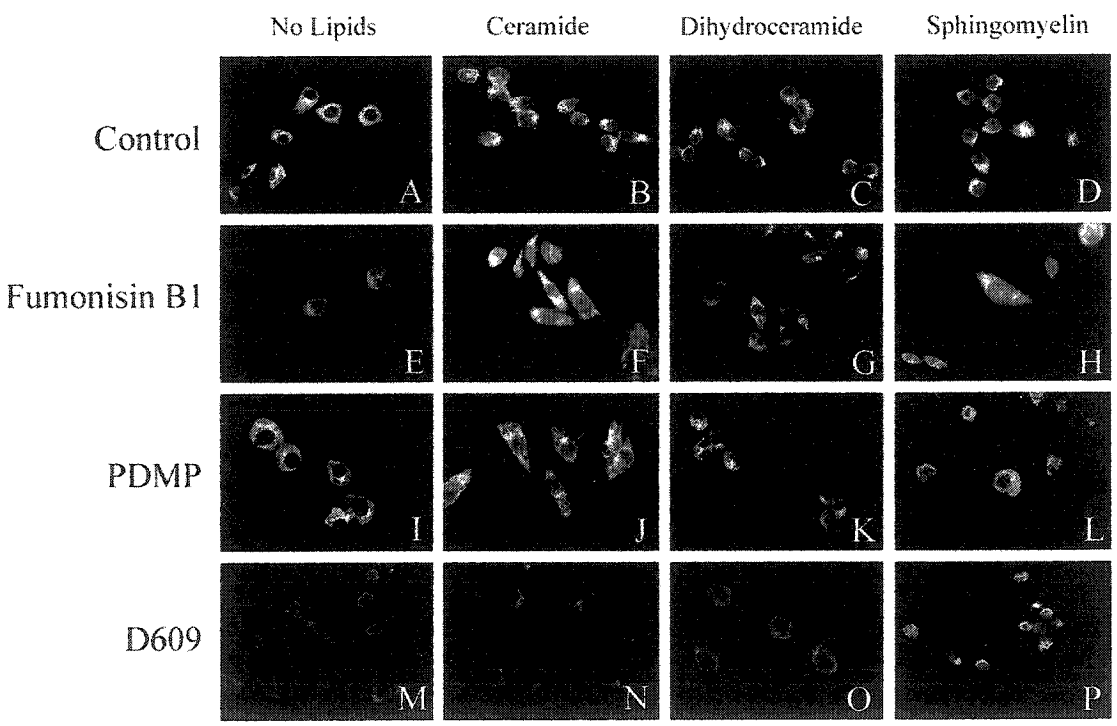

No Lipids
Ceramide
Dihydroceramide
Sphingomyelin
Control
Fumonisin B1
PDMP
D609
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P

Fig. 14 CHO cells over expressing prosaposin were incubated with 5 μM ceramide (B), 5 μM dihydroceramide (C), or 5 μM sphingomyelin (D) for 48 hours. Untreated cells served as a control (A). All cells displayed a similar immunostaining pattern; showing a perinuclear reaction.

CHO cells over expressing prosaposin were treated with FB_1 (25 μg/ml) (E-H) followed or not by a 48-hour incubation with 5 μM ceramide (F), 5 μM dihydroceramide (G), or 5 μM sphingomyelin (H). The FB_1 treated cells showed a decreased immunostaining pattern compared to CHO cells not treated with the inhibitor (E). Ceramide, dihydroceramide, or sphingomyelin additions restored the immunostaining similar to the untreated control cells (A).

CHO cells over expressing prosaposin were treated with GSL synthesis inhibitor PDMP (25 μg/ml) (I-L) followed or not by a 48-hour incubation with 5 μM ceramide (J), 5 μM dihydroceramide (K), or 5 μM sphingomyelin (L). All cells displayed similar immunostaining pattern to untreated control cells (A).

CHO cells were treated with the lipid synthesis inhibitor D609 (100 μg/ml) (M-P) followed or not by a 48-hour incubation with 5 μM ceramide (N), 5 μM dihydroceramide (O), or 5 μM sphingomyelin (P). The D609 treated cells showed a decreased immunostaining pattern compared to CHO cells not treated with the inhibitor (M). Addition of ceramide, dihydroceramide, or sphingomyelin did not modify this low labeling pattern. (x400)

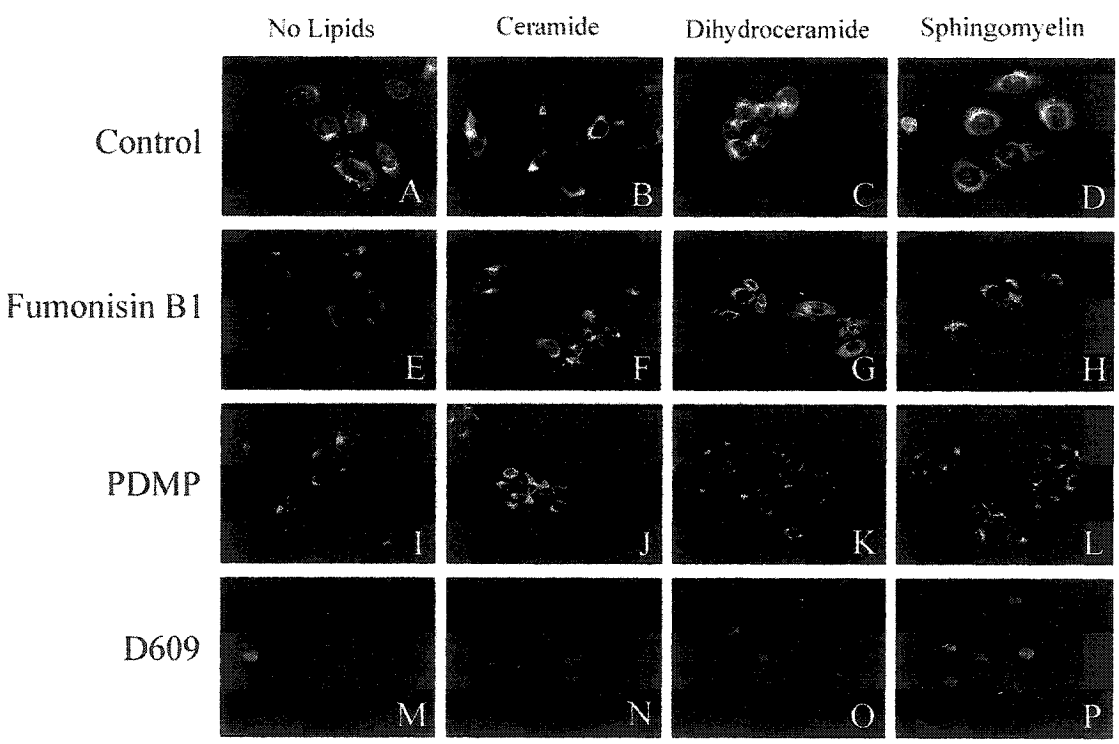

No Lipids
Ceramide
Dihydroceramide
Sphingomyelin
Control
Fumonisin B1
PDMP
D609
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P

Fig. 15 CHO cells over expressing prosaposin were treated or not with the lipid synthesis inhibitors 25 μg/ml FB_1 (E-H) or 100 μg/ml D609 (I-L) followed or not by a 24-hour incubation with 5 μM ceramide, 5 μM dihydroceramide, or 5 μM sphingomyelin. The cells were reacted with anti-cathepsin B antibody, labeled with a secondary antibody conjugated to FITC and viewed under a confocal microscope. All cells displayed a similar perinuclear immunostaining pattern. The addition of the inhibitors or exogenous lipids had no effect on the immunostaining pattern of these cells. (x400)

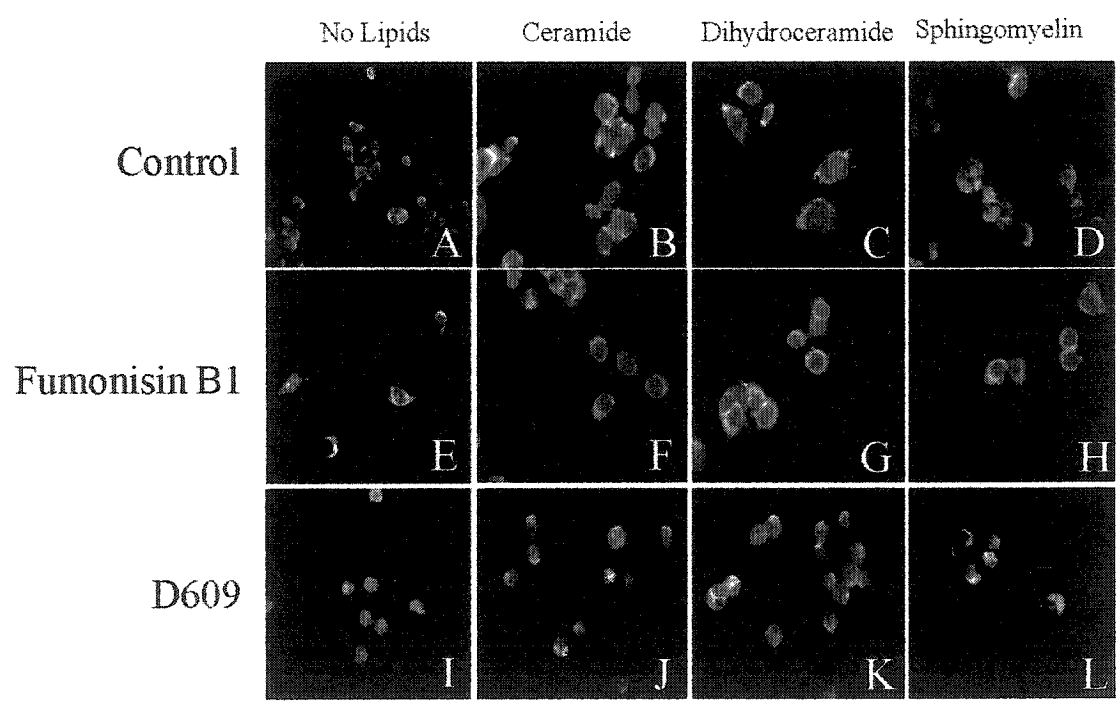

No Lipids
Ceramide
Dihydroceramide
Sphingomyelin
Control
Fumonisin B1
D609
A
B
C
D
E
F
G
H
I
J
K
L

Fig. 16 Seminiferous tubules supplemented or not with 25 μg/ml FB_1 or 100 μg/ml D609 were embedded in Lowicryl and labeled with anti-prosaposin antibody followed by a secondary antibody conjugated to 15 nm colloidal gold. Sertoli cell lysosomes were viewed under an electron microscope.

Control seminiferous tubules were left untreated and showed a specific localization of gold particles in the lysosomes. Control testis treated with ceramide (Control + C_2) or sphingomyelin (Control + Sm) showed no difference in the immunolabelling of lysosomes.

Seminiferous tubules treated with FB_1 showed a visible reduction in lysosomal immunogold staining compared to control lysosomes of Sertoli cells. The FB_1 treated tissue supplemented with 5 μM of ceramide (FB_1 + C_2) or sphingomyelin (FB_1 + Sm) restored the immunogold labeling in the lysosomes of these cells.

Seminiferous tubules treated with GSL inhibitor (PDMP). The immunolabelling pattern in the lysosomes of Sertoli cells was similar to that of untreated control cells. Cells treated with D609 displayed a marked decrease in lysosomal labeling compared to control cells. The addition of ceramide (D609 + C_2) did not restore the labeling of the lysosomes in these cells while the addition of exogenous sphingomyelin (D609 + Sm) did. (x40 000)

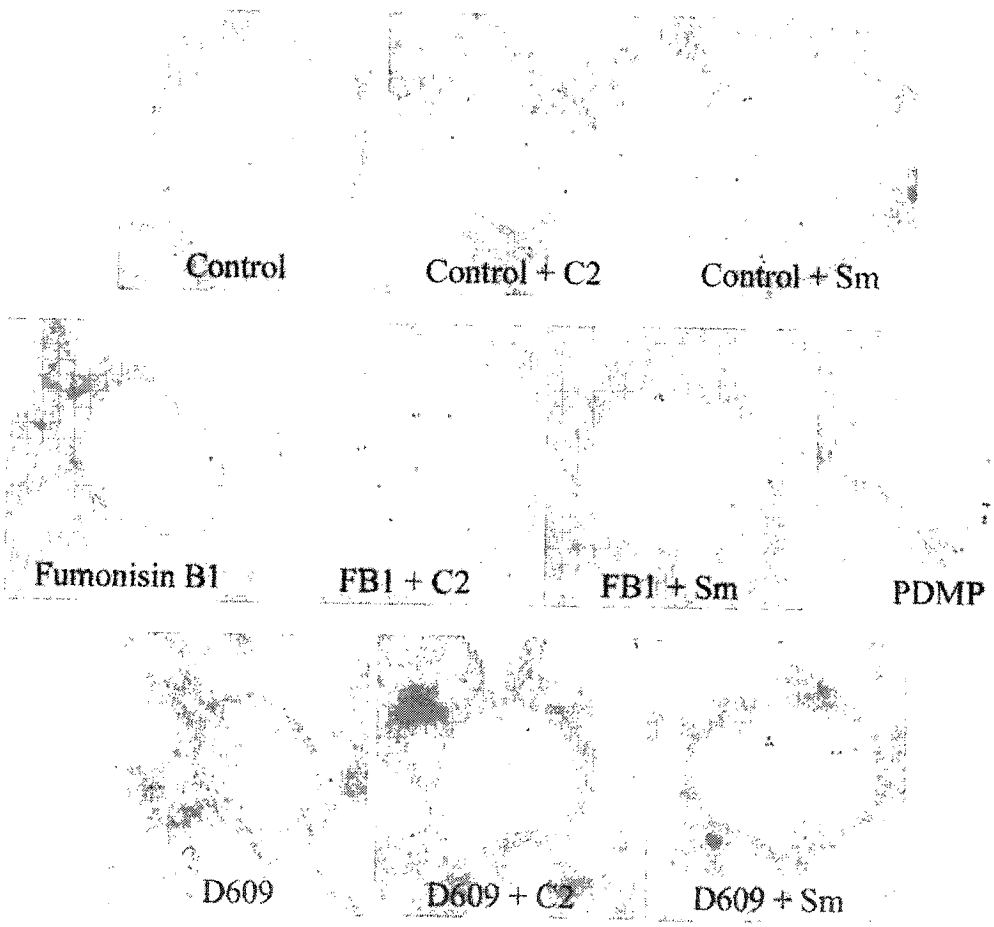

Control
Control + C2
Control + Sm
Fumonisin B1
FB1 + C2
FB1 + Sm
PDMP
D609
D609 + C2
D609 + Sm

Fig. 17 Quantitative analysis performed on cultured seminiferous tubules treated or not (Control) with 25 μg/ml FB_1 or 100 μg/ml D609 and labeled with anti-prosaposin antibody followed by a secondary antibody conjugated to 15 nm colloidal gold. The quantitative analysis was performed by dividing the number of gold particles in each lysosome by the lysosomal area. Mean lysosomal densities of 100 lysosomes from 3 separate experiments were plotted for each of the conditions. The average lysosomal density in control cells was 14.57 gold particles/μm^2 (Con) and was maintained in cells treated with ceramide (16.88 gold particles/μm^2) (Con + C_2) and sphingomyelin (15.87 gold particles/μm^2) (Con + Sm). The density was reduced in FB_1 treated cells (FB_1 + C_2) to 3.79 gold particles/μm^2, while the cells supplemented with ceramide (FB_1 + C_2) or sphingomyelin (FB_1 + Sm) were restored to 16.70 gold particles/μm^2 and 17.63 gold particles/μm^2 respectively. The lysosomal gold particle density of PDMP treated cells (PDMP) did not change when compared to untreated control cells (14.39 gold particles/μm^2). The lysosomal density of D609 treated cells (D609) was reduced to 3.16 gold particles/μm^2 and the addition of ceramide (D609 + C_2) did not restore the gold labeling in lysosomes, which remained at 2.38 gold particles/μm^2. The addition of exogenous sphingomyelin (D609 + Sm) restored the gold particle labeling in lysosomes to a value comparable to untreated control cells (17.69 gold particles/μm^2). * Values statistically different according to a student t-test.

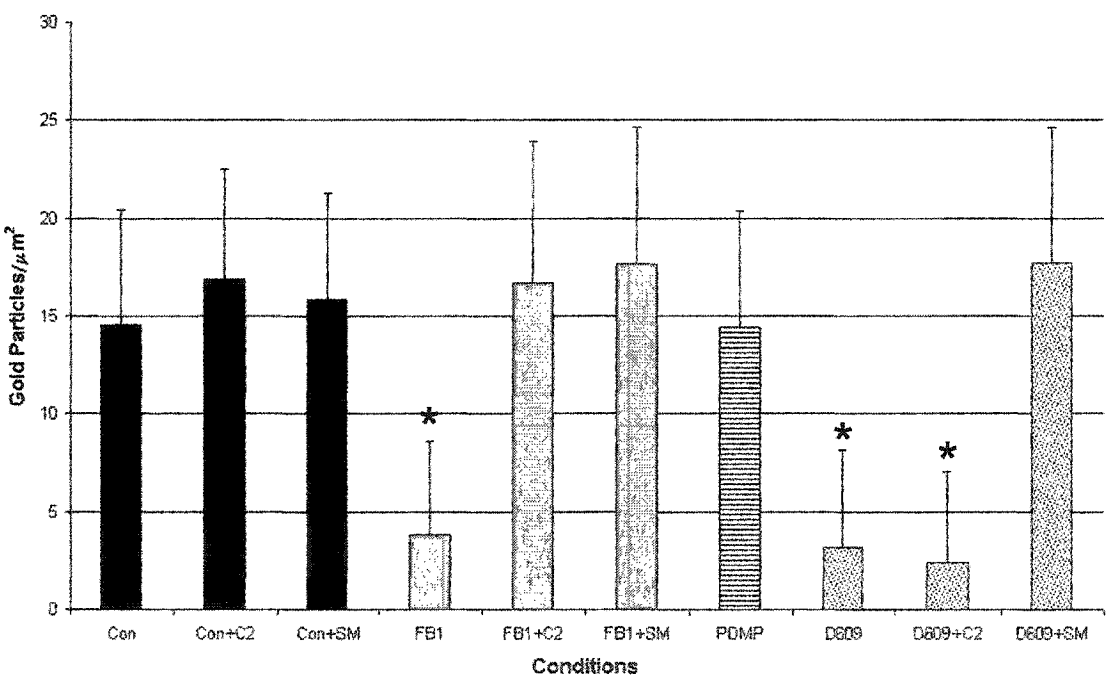

30
25
20
15
10
5
0
Gold Particles/μm^2
*
*
*
Con
Con+C2
Con+SM
FB1
FB1+C2
FB1+SM
PDMP
D609
D609+C2
D609+SM
Conditions

Fig. 18 COS-7 cells were transfected with a full-length wild-type prosaposin construct. The prosaposin construct was localized with an anti-myc antibody and FITC conjugated secondary antibody. LysoTracker, a red fluorescent acidic marker, was used to identify the lysosomal compartments of the cells. The control untreated cell (A) showed a perinuclear reaction with a strong punctate reaction. The LysoTracker staining (B) confirmed that the punctate structures were lysosomes. The construct was found in these structures as demonstrated by the overlaid image (C and inset). The addition of D609 eliminated most of the perinuclear staining and all of the punctate structures (D). As was verified by the LysoTracker stain (E) and overlaid image (F and inset), none of the constructs are in the lysosomal compartments. The addition of sphingomyelin to the D609 treated cells restored the perinuclear and punctate staining (G). The LysoTracker staining confirmed that the targeting of the construct was restored in the lysosomes as demonstrated by the overlaid image (I and inset). (x1000)

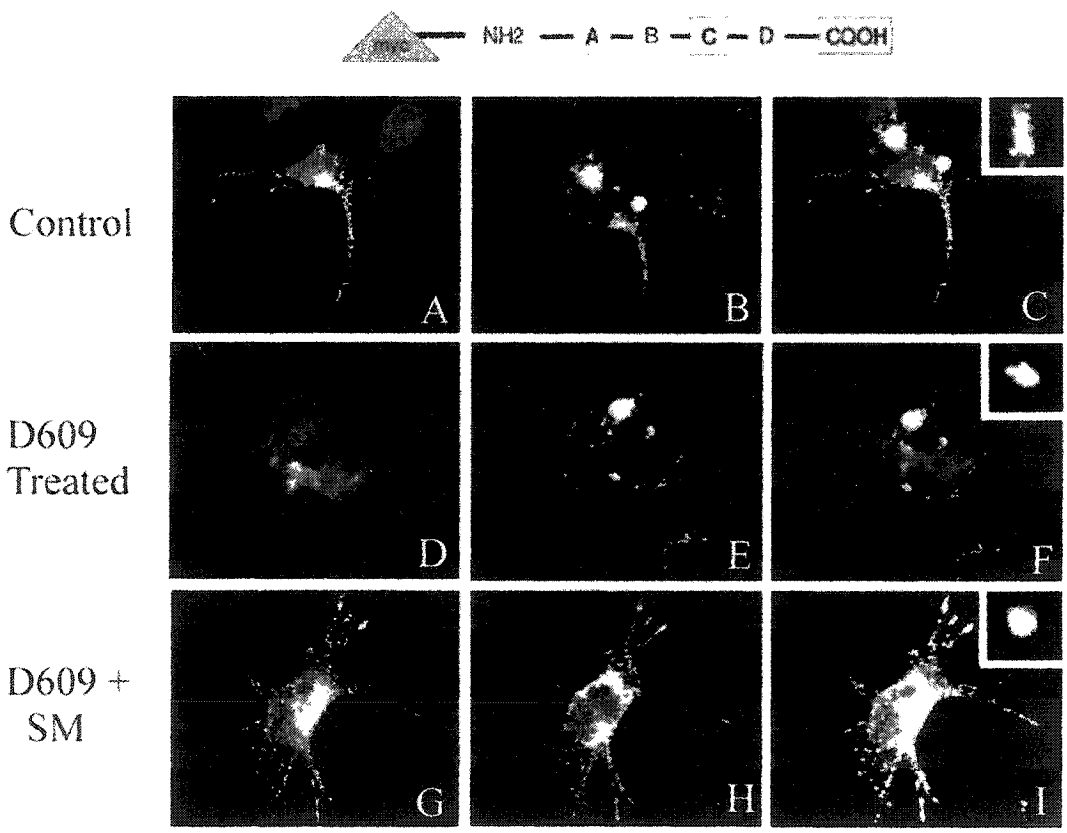

NH2 — A — B — C — D — COOH
Control
D609 Treated
D609 + SM
A
B
C
D
E
F
G
H
I

Fig. 19 COS-7 cells were transfected with an Albumin-prosaposin (Alb-D-COOH) construct. The construct was localized with an anti-myc antibody and localized with an FITC conjugated secondary antibody. LysoTracker, a red fluorescent acidic marker, was used to identify the lysosomal compartments of the cells. The control untreated cell (A) showed a perinuclear reaction with a strong punctate reaction. The LysoTracker staining (B) confirmed that the punctate structures were lysosomes and that the constructs were found in these structures as demonstrated by the overlaid image (C and inset). The addition of D609 eliminated most of the perinuclear staining and all of the punctate structures (D). As is verified by the LysoTracker stain (E) and overlaid image (F and inset), none of the construct was in the lysosomal compartment. The addition of sphingomyelin to the D609 treated cells restored the perinuclear reaction and punctate reaction (G). The LysoTracker staining confirmed that the targeting of the construct was restored in the lysosomes as demonstrated by the overlaid image (I and inset). (x1000)

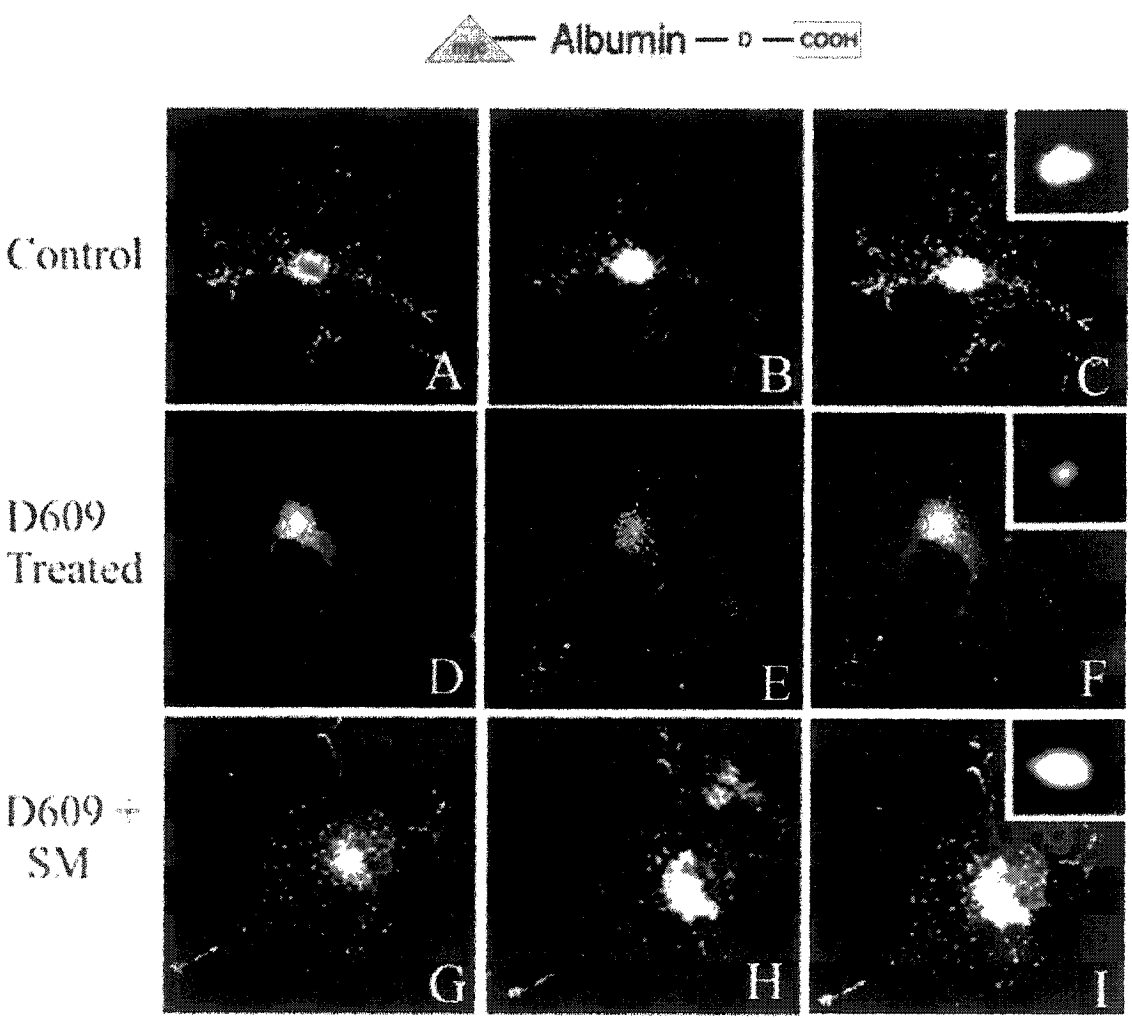

Albumin — D — COOH
Control
D609 Treated
D609 + SM
A
B
C
D
E
F
G
H
I

Fig. 20 Representative lysosomes showing the presence of endogenous prosaposin (15 nm colloidal gold particles (arrows), and the absence of anti-myc labeling in COS-7 cells transfected with an empty plasmid (Plasmid) or with Alb-D constructs (Alb-D). Representative lysosome showing a strong presence of Alb-D-COOH construct (encircled 10 nm colloidal gold particles) and a weak presence of endogenous prosaposin in lysosomes of transfected COS-7 cells (Alb-D-COOH). (x40 000) Graph above represents the density of gold particles per µm2 of lysosome in each of the conditions listed above. The black bars represent the average density of endogenous prosaposin while the gray bars demonstrate the Alb-D-COOH construct. In the plasmid only and Alb-D transfected cells, note the high concentration of endogenous prosaposin (14 and 16 particles/μm^2) and the very low amount of construct labeling (1 and 2 particles/μm^2). However, in the Alb-D-COOH, the construct labeling is very high (15 particles/μm^2) while endogenous prosaposin is very low (3 particles/μm^2). * Values statistically different according to a student t-test.

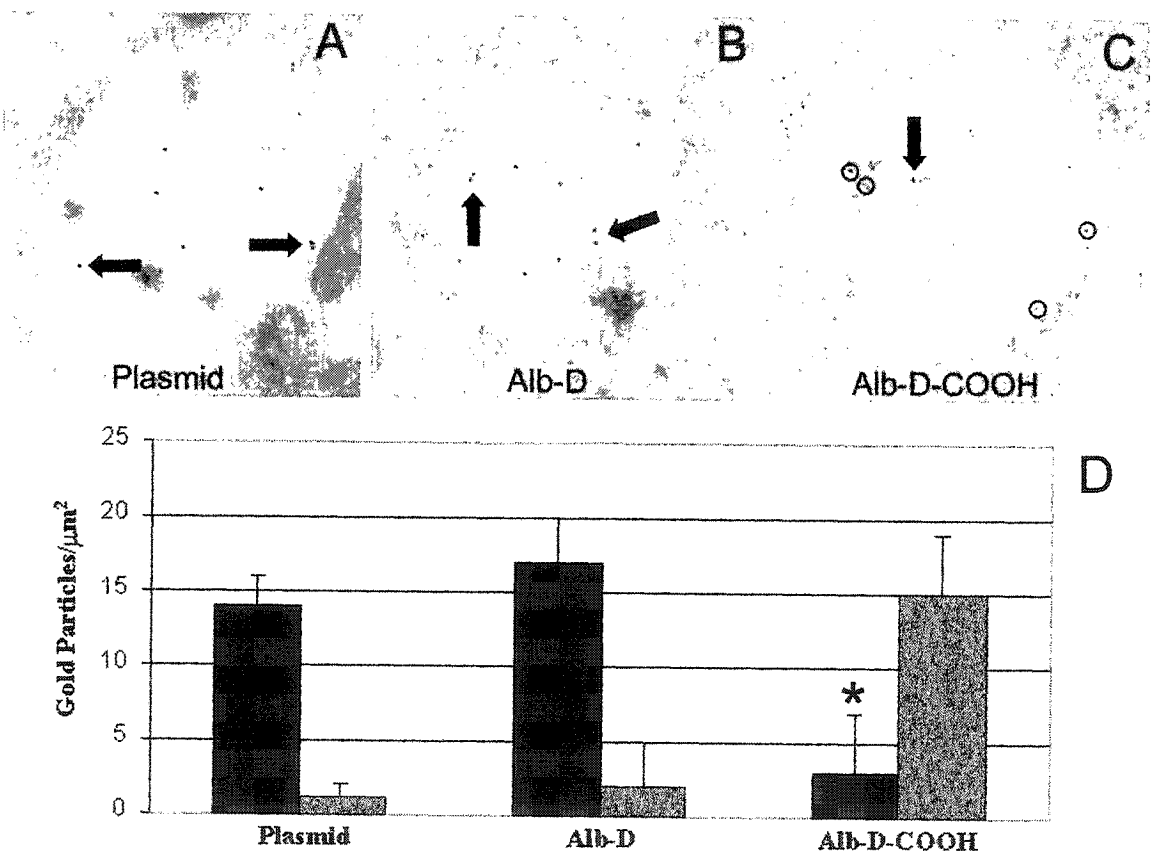

A
B
C
Plasmid
Alb-D
Alb-D-COOH
D
Gold Particles/μm²
25
20
15
10
5
0
*
Plasmid
Alb-D
Alb-D-COOH

Fig. 21 Representative multivesicular bodies (MVB) of COS-7 cells transfected with the Alb-D construct (A) with the plasmid only construct (B). The organelles show normal, wild type morphology. Representative lysosome (L) of COS-7 cells transfected with the Alb-D construct (C). This organelle also shows normal, wild type morphology. (x40 000)

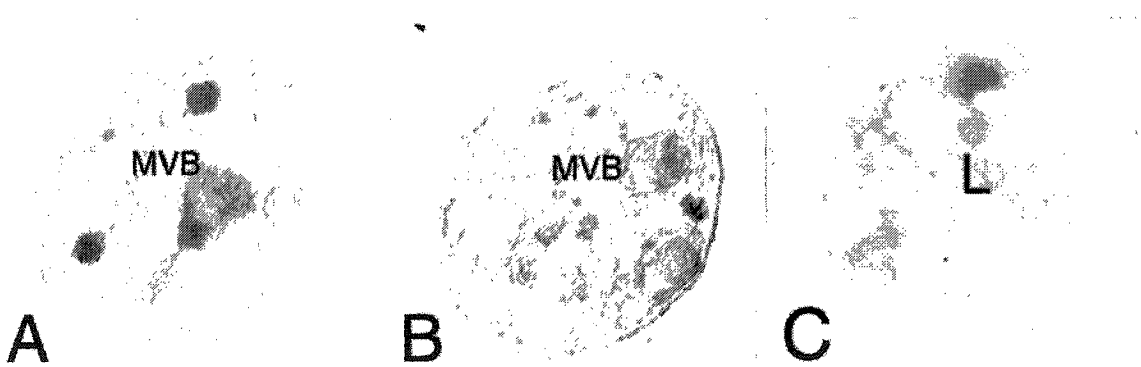

MVB
MVB
L
A
B
C

Fig. 22 Representative multivesicular body (mvb), mitochondria (m) of COS-7 cells transfected with the Alb-D-COOH construct (A +B). The multivesicular bodies show abnormal morphology due to the large accumulations of undegraded membranes. (x40 000)

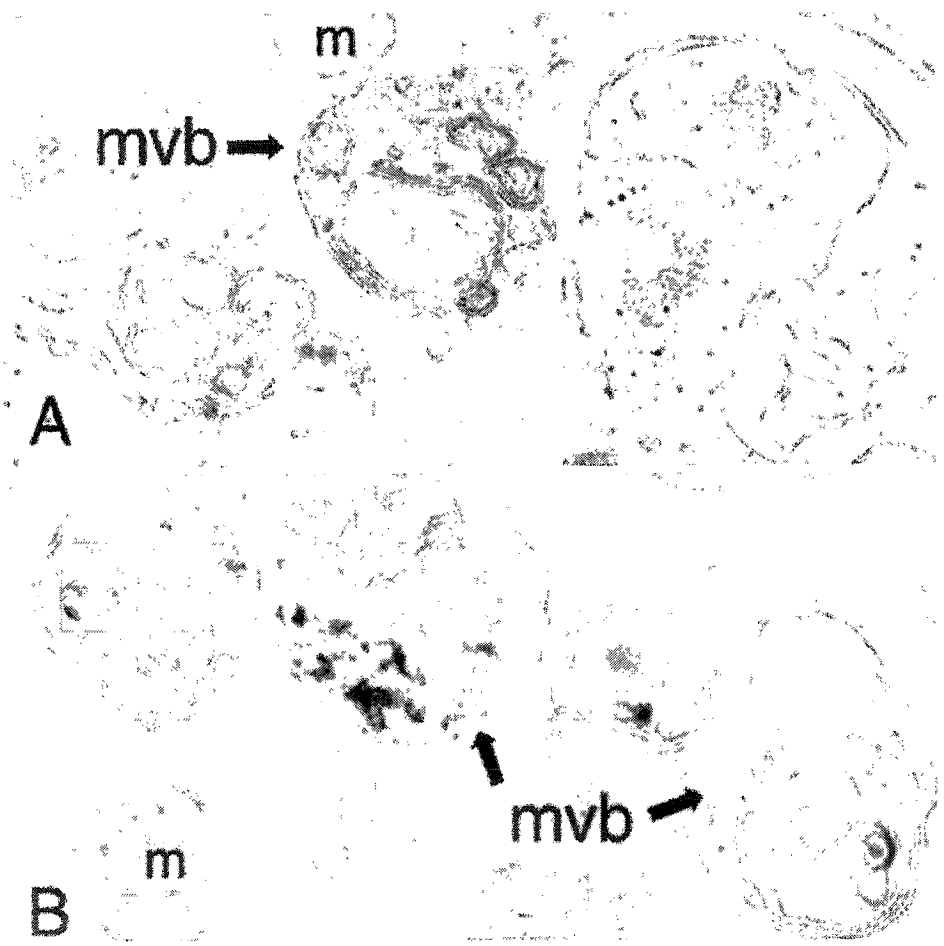

m
mvb
A
mvb
m
B

Fig. 23 Expression and localization of truncated sortilin. A full-length and truncated sortilin construct that lacked it cytosolic domain (A) where *in vitro* translated a run a 12.5% SDS-PAGE. The full-length construct ran to the standard 95 kDa (B, lane 1) while the truncated construct was slightly smaller (B, lane 2). The truncated construct was transfected into COS-7 cells and was expressed in a perinuclear region (C, anti-myc) of the cell. This staining pattern was merged to the Golgi staining with anti-Golgin that suggested that the truncated sortilin was being retained in the Golgi apparatus.

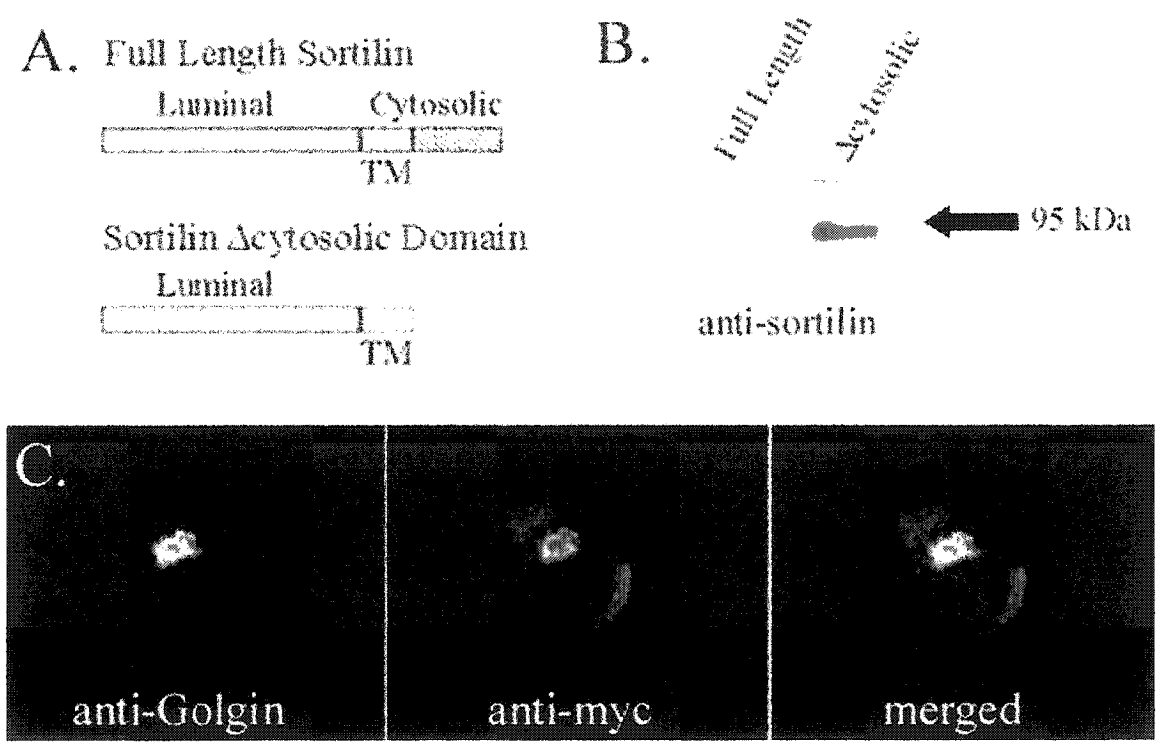

A.
Full Length Sortilin
Luminal
Cytosolic
TM
Sortilin Δcytosolic Domain
Luminal
TM
B.
Full Length
Δcytosolic
95 kDa
anti-sortilin
C.
anti-Golgin
anti-myc
merged

Fig. 24 Effect of dominant negative sortilin lacking its cytoplasmic domain in COS-7 cells immunostained with anti-prosaposin antibody. (x400) While anti-prosaposin antibody produced a green punctate staining in the cytoplasm of non-transfected cells (A), it did not stain the punctate structures of transfected cells (B and D). A perinuclear Golgi-like staining was observed in transfected cells suggesting that dominant negative sortilin disrupted the lysosomal trafficking of prosaposin (B) and retained the protein in the perinuclear Golgi region.

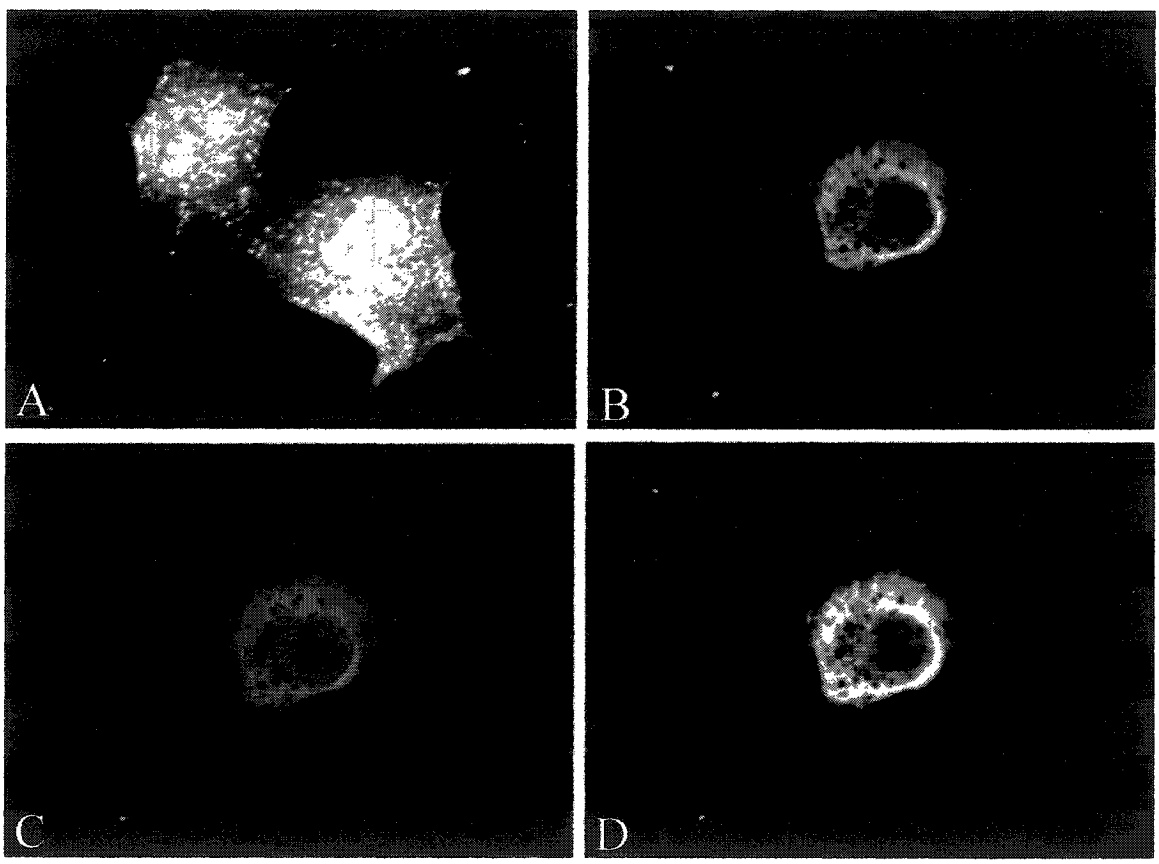

A
B
C
D

Fig. 25 Effect of dominant negative sortilin lacking the cytoplasmic domain in COS-7 cells immunostained with anti-$G_{M2}AP$ antibody. (x400) While anti-$G_{M2}AP$ antibody produced a green punctate staining in the cytoplasm of non-transfected cells (A), it did not stain the punctate structures of transfected cells (B and D). A perinuclear Golgi-like staining was observed in transfected cells suggesting that dominant negative sortilin disrupted the lysosomal trafficking of prosaposin (B) and retained the protein in the perinuclear Golgi region.

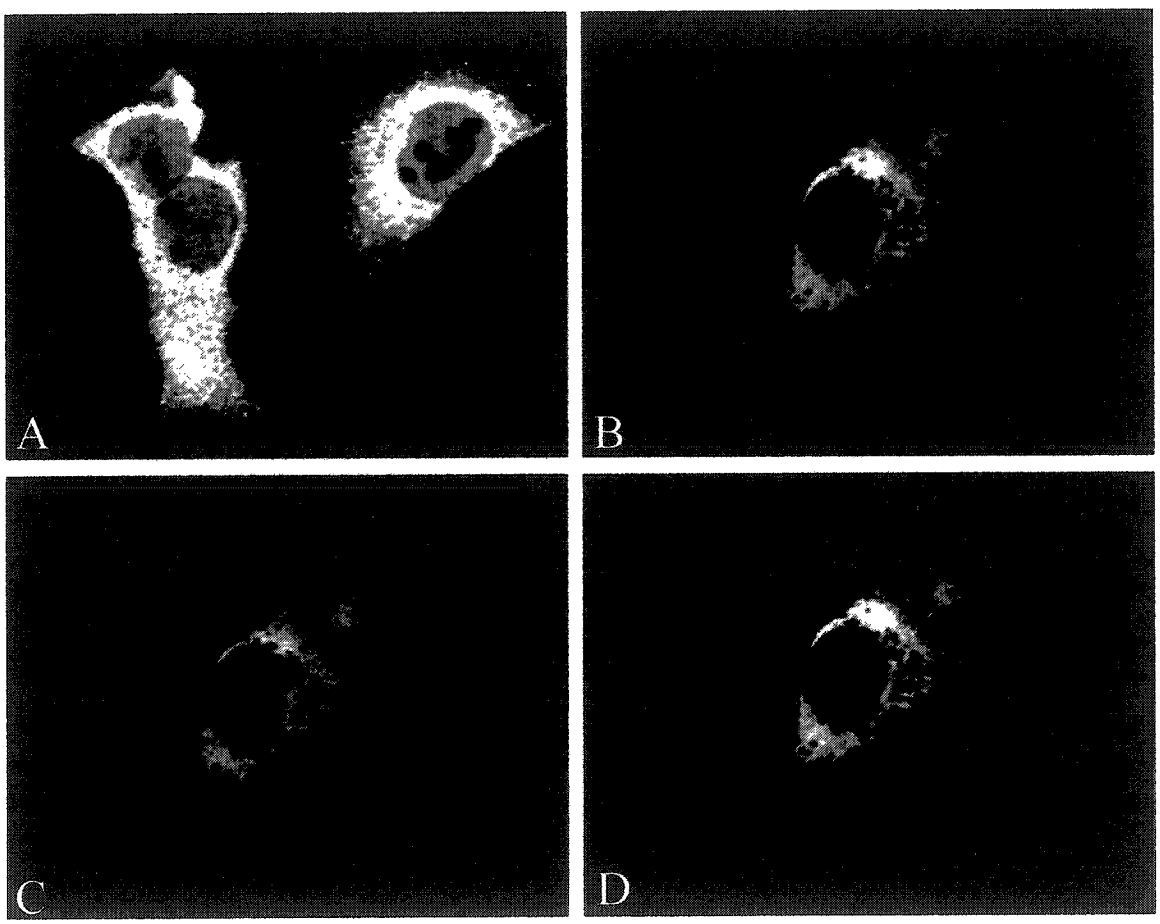

A
B
C
D

Fig. 26 Effect of dominant negative sortilin lacking the cytoplasmic domain in COS-7 cells immunostained with anti-cathepsin B antibody. (x400) While anti-cathepsin B antibody produced a green punctate staining in the cytoplasm of non-transfected cells (A), the truncated construct (C) had no effects on the staining pattern of anti-cathepsin B antibody (B and D).

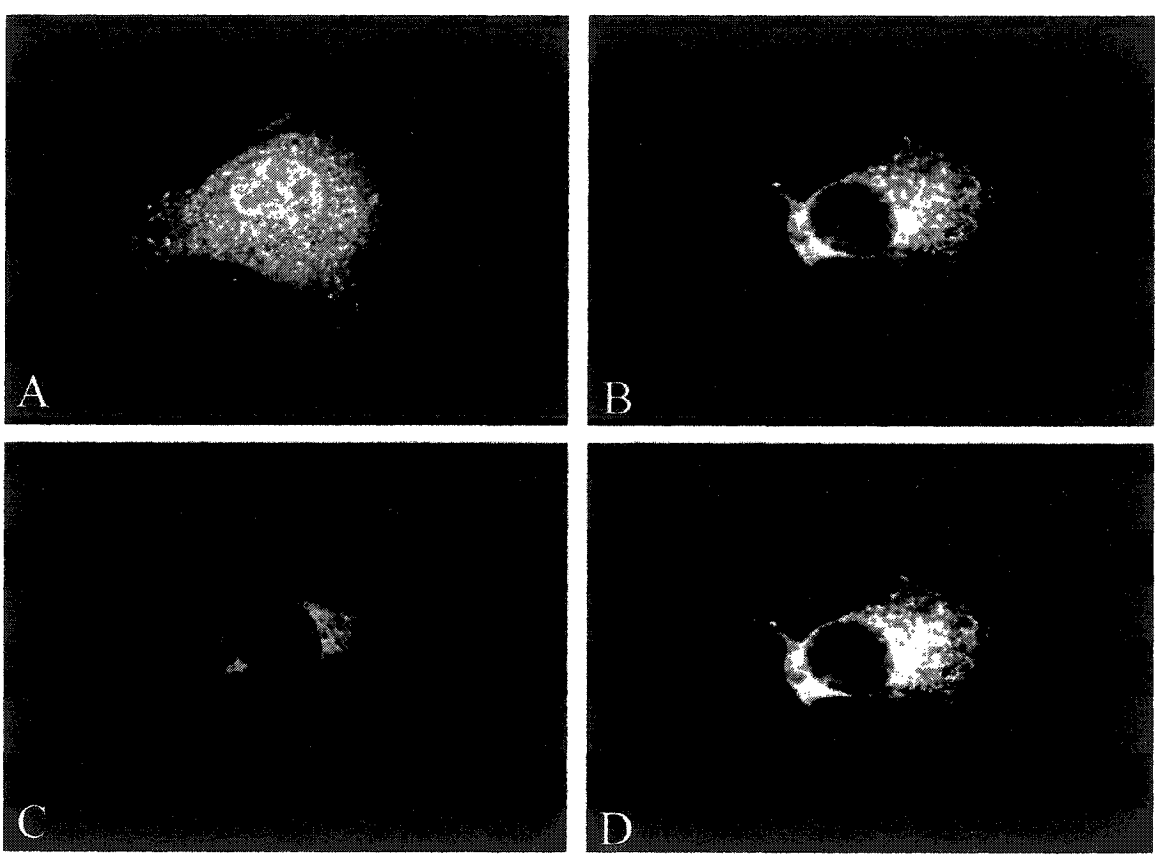

A
B
C
D

Fig. 27 Co-immunoprecipitation showing association of in vitro translated sortilin, truncated sortilin and sphingolipid activator proteins. Lanes 1 and 2 demonstrate that prosaposin pulls-down both full length and truncated sortilin. Lanes 3 and 4 show that $G_{M2}AP$ pulls-down full length and truncated sortilin while lanes 5 and 6 demonstrate that cathepsin B is unable to bind sortilin.

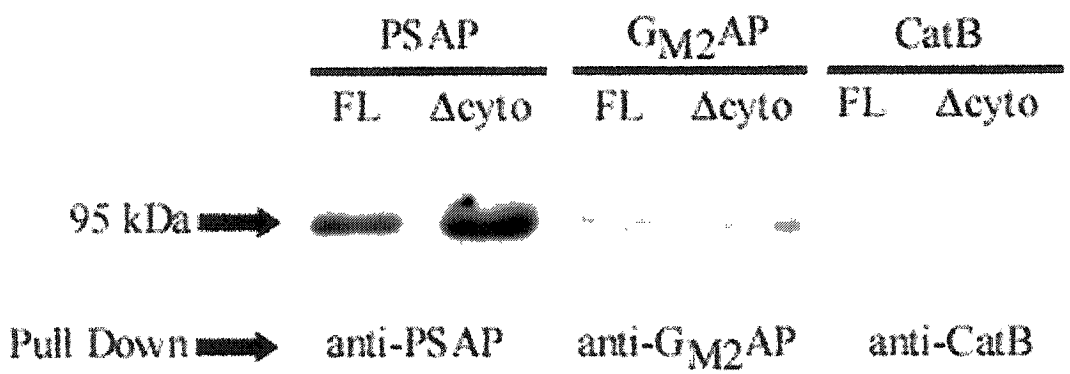

PSAP
$G_{M2}AP$
CatB
FL
Δcyto
FL
Δcyto
FL
Δcyto
95 kDa
Pull Down
anti-PSAP
anti-$G_{M2}AP$
anti-CatB

Fig. 28 Effect of dominant negative GGA-GFP construct in COS-7 cells immunostained with anti-prosaposin (A), anti-$G_{M2}AP$ (B), anti-cathepsin B (C) or anti-LAMP-2 antibody (D). (x400). GGA-GFP transfected cells are recognized by their green fluorescence. While anti-prosaposin, anti-$G_{M2}AP$ and anti-cathepsin B antibodies yielded an intense punctate staining, transfected cells (green fluorescence) are not stained. LAMP-2 staining is not affected.

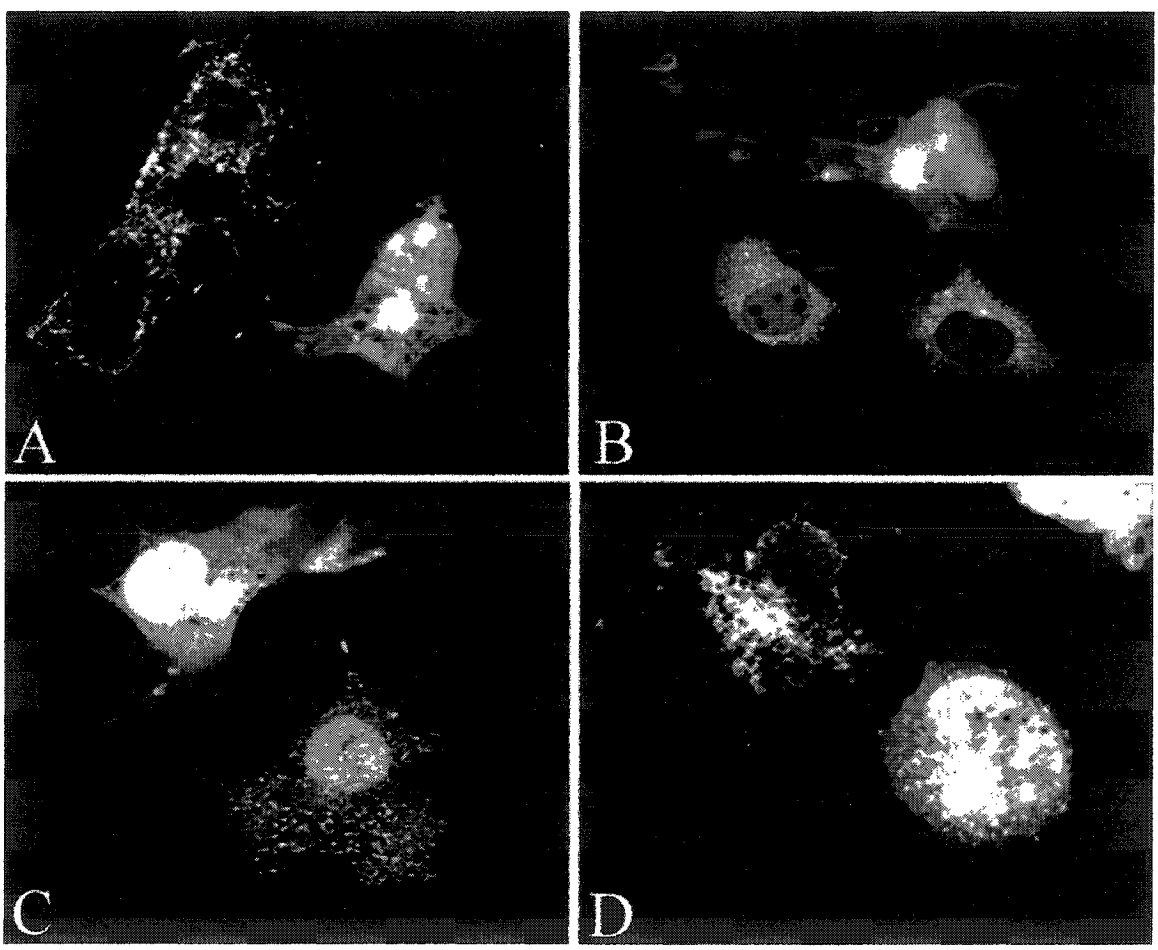
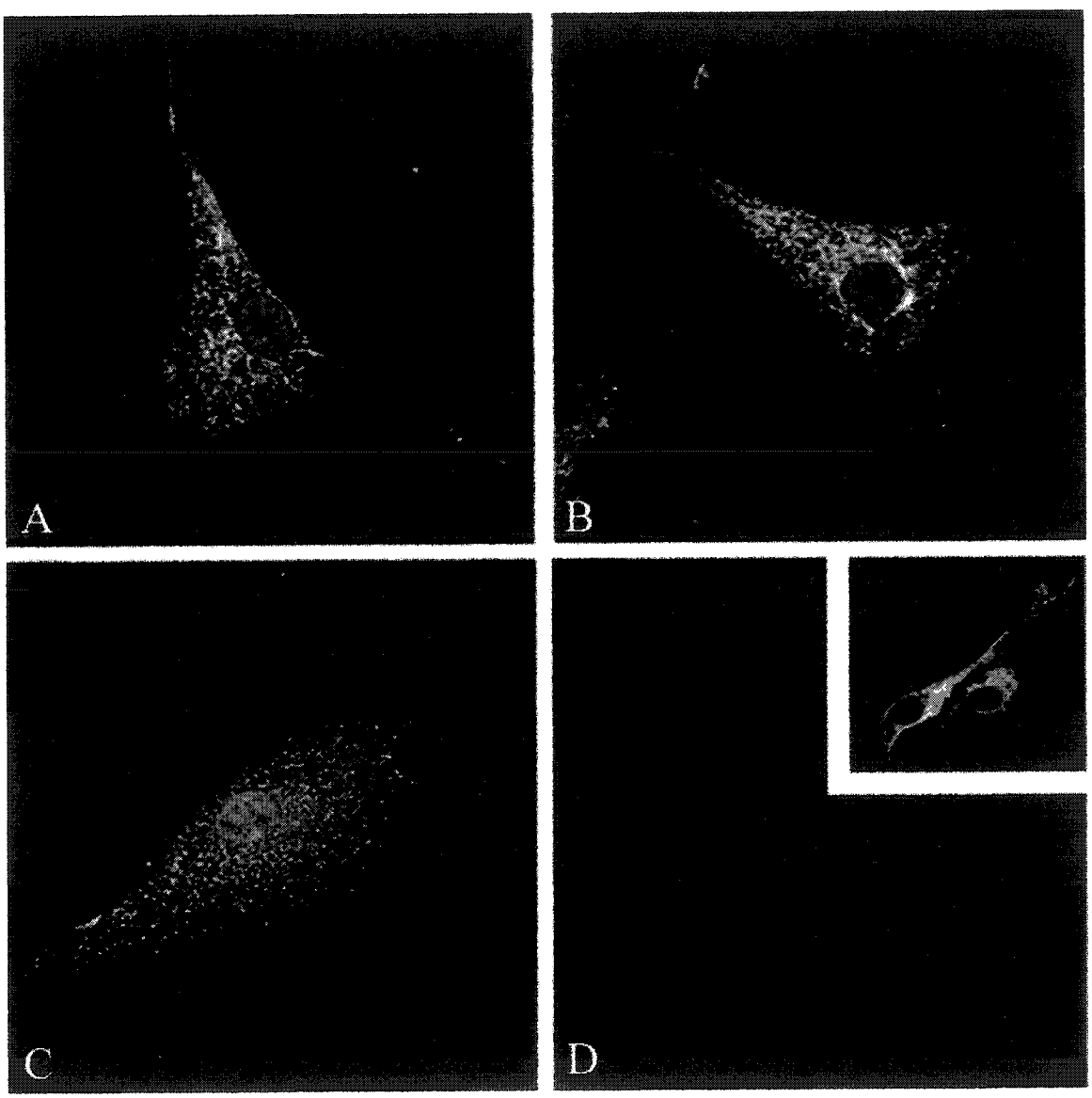

A
B
C
D

Fig.29 AP-3 mutant fibroblasts immunostained with anti-prosaposin (A), anti-$G_{M2}AP$ (B) anti-cathepsin B (C) or anti-LAMP-2 antibodies (D). (x400) Note punctate staining with anti-prosaposin, anti-$G_{M2}AP$ and anti-cathepsin B antibodies but not with anti-LAMP-2 antibody. Inset shows punctate LAMP-2 staining in a wild type fibroblast. (x100) These results demonstrate that AP-3 is only required for the trafficking of LAMP-2 and not for proteins that use either the M6P-R or sortilin


A
B
C
D

DISCUSSION

I. Effect of sphingolipid depletion in the targeting of prosaposin to the lysosomes

Protein targeting to the lysosomes most commonly occurs via the mannose 6-phosphate receptor that directs soluble hydrolases from the Golgi apparatus to the lysosomes (Kornfeld and Kornfeld, 1985). Trans-membrane lysosomal proteins are transported by an alternative pathway from the Golgi apparatus to the plasma membrane and then to the lysosomes via endocytosis (Braun et al., 1989; Fambrough et al., 1988; Rohrer et al., 1996).

Prosaposin exists as a 70 kDa protein that is secreted to the extracellular space and a 65 kDa intracellular protein that is routed to the lysosomes (Igdoura and Morales, 1995). Although lysosomal prosaposin is not a trans-membrane protein biochemical studies showed that this molecule is associated to Golgi membranes (Igdoura et al., 1996) and that it may bind sphingolipids (SLs) (Hiraiwa et al., 1992). Nevertheless, the mechanism of transport of prosaposin to the lysosomes is still unclear.

The present study indicates that the targeting of prosaposin to the lysosomes is mediated by SLs. In fact, fumonisin B_1, which inhibits the synthesis of sphingolipids (Mays et al., 1995), decreased the immunogold labeling of the lysosomes with anti-prosaposin antibody. When fumonisin B_1 at a concentration of 25 μg/ml of culture media was added to different cell lines for 48 hours it produced a

59 % to 85% gold density reduction. The dose used in this investigation was shown by others to disrupt the targeting of glycoprotein GP-2 in Madin-Darby canine kidney cells (Mays et al., 1995). Although all cell types did show a lysosomal decrease of colloidal gold labeling, the range of variation was relatively wide. This was attributed to different responses to fumonisin B_1 by the various cell types used. Total inhibition was never achieved, possibly, due to the presence of endogenous SLs before the initiation of the fumonisin B_1 treatment.

A major problem in assessing the labeling of prosaposin in the lysosomes was the low level of this protein in the different cell types utilized in this investigation. In order to overcome this problem, CHO cells were stably transfected with an expression vector containing a human prosaposin cDNA. It is noteworthy to indicate that the two processed (lysosomal and extracellular) forms of prosaposin were found in two positive clones. A clone designated 66 showed a significant increase of prosaposin mRNA and therefore was selected for further studies. After metabolic labeling of clone 66 CHO cells with ^{35}S-methionine, the lysosomal prosaposin (65 kDa) was found exclusively in cell lysates and extracellular prosaposin (70 kDa) was found in the culture media. In addition, clone 66 showed an over-expression of prosaposin as shown by the high level of immunogold labeling in the lysosomes.

When fumonisin B_1 was added to the culture media of clone 66 it produced a 55 % reduction of gold density in the lysosomes with anti-saposin A antibody. On the other hand, the gold particle density increased by 3 folds in the small vesicles found around the nucleus or near the Golgi apparatus in fumonisin B_1 treated cells. Similar results were obtained with the other cell lines. These vesicles were considered to be

secretory in nature due to their small size, their association with the Golgi apparatus and their lack of reactivity with anti-cathepsin L antibody. These results indicate that fumonisin B_1 treatment impaired the transport of prosaposin from the Golgi to the lysosomes and redirected the protein to the secretory pathway. To verify if this was the case, culture media of clone 66 treated or not with fumonisin B_1 was collected and examined by immunoblotting. The samples were taken from replica plates grown to confluence. Equivalent amount of protein loaded onto a SDS-PAGE gel revealed that fumonisin B_1 induced the released of larger amounts of the 70 kDa form of prosaposin into the culture media confirming the immunogold labelling results.

A recent investigation suggested that prosaposin may reach the lysosomes by a mechanism of secretion and endocytosis in I-cell disease fibroblasts (Vielhaber et al., 1996). Non-ciliated cells of the efferent ducts have also been shown to internalise prosaposin dissociated from spermatozoa (Igdoura et al., 1995), but this process was linked to a mechanism of clearance of the protein rather than to process of targeting of prosaposin to the lysosomes. Furthermore, previous investigations carried out in our laboratory indicate that prosaposin is transported to the lysosomes directly from the Golgi apparatus (Igdoura and Morales, 1995; Igdoura et al., 1996).

Prosaposin was initially found to be targeted to the lysosomes by a mannose-6-phosphate-independent pathway in various cell types (Igdoura et al., 1996; Rijnboutt et al., 1991; Vielhaber et al., 1996). Experiments that inhibited the glycosylation of proteins using tunicamycin, failed to impair the targeting of prosaposin to the lysosomes (Igdoura et al., 1996). When Golgi sub cellular fractions from Sertoli cells were permeabilized with mild detergents, the 70 kDa secreted form

of prosaposin was released but the 65 kDa lysosomal form of this protein was retained. Similarly when permeabilized Golgi fractions were incubated in excess of free mannose 6-phosphate, the 65 kDa protein remained associated to the Golgi membranes (Igdoura et al., 1996). Metabolic labeling of Sertoli cells demonstrated that prosaposin is first synthesized as a 65 kDa form which is post-translationally modified to a 70 kDa polypeptide that is secreted to the extracellular space. A fraction of the 65 kDa form is targeted to the lysosomes where it is cleaved into 15 kDa proteins (Igdoura and Morales, 1995). Finally, lysosomal subcellular fractions only contain the 65 kDa form of prosaposin (Igdoura and Morales, 1995). This evidence demonstrated that the 65 kDa protein but not the 70 kDa extracellular form of prosaposin is the precursor of the lysosomal saposins. Secreted prosaposin was found in various fluids such as milk (Kondoh et al., 1991), seminiferous tubular fluid (Igdoura and Morales, 1995), cerebrospinal fluid, bile and pancreatic juice (Hineno et al., 1991), but its function in extracellular compartments is still unknown.

In order to examine if the mannose 6-phosphate receptor pathway was affected by fumonisin B_1, treated and non-treated cells were reacted with an anti-cathepsin A antibody. Cathepsin A is a soluble lysosomal hydrolase that is targeted to the lysosomal compartment via the mannose 6-phosphate receptor system. Quantitative immunoelectron microscopy of NRK, CHO, and CHO clone 66 cell lines treated or not with fumonisin B_1 showed no significant differences in the immunogold labeling density. These results showed that the effect of fumonisin B_1 on the transport of prosaposin to the lysosomes was specific.

Based on published data which demonstrated that GSLs are involved in the

sorting and targeting of proteins to various destinations in the cell, and that prosaposin is capable of selectively binding to GSLs (Hiraiwa et al., 1992) we hypothesized that sphingolipids could either be responsible for the transport of prosaposin to the lysosomes or be constituents of raft microdomains that promote the anchoring of prosaposin to the Golgi membrane and its interaction with its putative receptor. Fumonisin B_1 that specifically inhibits sphingolipid biosynthesis reduced the amount of prosaposin in the lysosomes. In conclusion, our data supports our hypothesis and shows for the first time that SLs are required in the transport of the 65 kDa form of prosaposin to the lysosomal compartment. This study also raises the question as to whether or not other lysosomal activators of GLS degradation utilize a similar mechanism of transport to the lysosomes.

II. Role of sphingomyelin in the transport of prosaposin to the lysosomes

We presented direct evidence that sphingomyelin is an essential sphingolipid for the transport of prosaposin to the lysosomes. Sphingolipids are membrane components containing a ceramide moiety linked to a carbohydrate (glycosphingolipids /GSLs), or phosphocholine (sphingomyelin) (Schwarz et al., 1995). GSLs are found in the plasma membrane as integral components of the outer leaflet facing the extracellular space (Conzelmann and Sandhoff, 1978; Sandhoff, 1996; Sano et al., 1994). Sphingomyelin is also found on the outer leaflet of the plasma membrane and on the luminal aspect of membranes enclosing intracellular organelles (Sandhoff, 1996). GSLs and sphingomyelin are synthesized in the Golgi apparatus (Schwarz et al., 1995). Sphingolipids and cholesterol may be found in microdomains called lipid rafts that are implicated in sorting and vesicle formation

(Ikonen, 2001). Degradation of sphingolipids occurs in lysosomes by the concerted activity of hydrolases and sphingolipid activator proteins (Conzelmann and Sandhoff, 1978; Sano et al., 1994). Saposins A, B, C and D are four activators derived from partial proteolysis of a common precursor, prosaposin (Kishimoto et al., 1992). Studies in our laboratory demonstrated the existence of two forms of prosaposin, a lysosomal isomer (65 kDa) and a secreted form (70 kDa) found in extracellular fluids (Igdoura and Morales, 1995; Igdoura et al., 1996). Therefore, prosaposin follows two distinct trafficking pathways: a) a direct delivery of prosaposin from the Golgi apparatus to the lysosomes, and b) a secretory routing from the Golgi apparatus to the extracellular space.

This section encompasses three objectives. The first one deals with the role of sphingomyelin in the targeting of prosaposin to the lysosomes. Lysosomal prosaposin (65 kDa) is a Golgi membrane bound glycoprotein that is transported to the lysosomes in a mannose 6-phosphate independent manner (Igdoura and Morales, 1995; Igdoura et al., 1996; Rijnboutt et al., 1991). The use of inhibitors of sphingolipid synthesis, PDMP, Fumonisin B_1 (FB_1) and D609 suggested that prosaposin does not depend on GSLs but on sphingomyelin for its transport to the lysosomal compartment (Lefrancois et al., 1999). However, the analysis of these data was based on the suppression of sphingolipid synthesis and therefore on negative results. The use of these inhibitors alone did not provide direct evidence since these compounds may have side effects on the treated cells. To overcome these hurdles we decided to examine the role of sphingolipids, and more specifically sphingomyelin, in the transport of prosaposin to the lysosomes. This objective was accomplished by

altering endogenous lipid synthesis in Chinese Hamster Ovary (CHO) cells, COS-7 cells and testis explants followed by the addition of exogenous lipids.

When CHO cells were treated with the exogenous sphingolipid precursors the cells exhibited an immunostaining pattern similar to the untreated control cells. These results indicate that the exogenous lipids did not interfere with cell functions or with lysosomal transport in general.

Treatment of prosaposin transfected CHO cells with each of the inhibitors alone yielded results that were consistent with those reported in previous studies (Lefrancois et al., 1999). Supplementation with exogenous sphingomyelin restored the prosaposin immunostaining. Although it was difficult to determine which compartment was stained at the resolution of the confocal microscope in CHO cells, the results suggest that sphingomyelin was required to restore the immunostaining pattern observed in control cells. This observation was supported by electron microscopic results showing an inhibition of lysosomal prosaposin transport in the presence of D609. FB_1 caused a decrease in the immunostaining pattern of CHO cells when labeled with anti-prosaposin antibody. PDMP, a glycosphingolipid inhibitor, which does not affect sphingomyelin synthesis, was also used in this investigation to serve as a negative control for CHO cells. Confocal microscopic images of cells treated with PDMP, followed or not with supplementation of exogenous lipids, displayed a similar immunostaining pattern to the untreated control cells when reacted with anti-prosaposin antibody. These results indicate that glycosphingolipids are not required for the transport of prosaposin to the lysosomes.

Cathepsin B is a soluble lysosomal protein that is known to use the mannose

6-phosphate receptor system to be targeted from the Golgi to the lysosomes. Confocal microscopy showed an unchanged immunostaining pattern of cathepsin B in cells treated with FB_1 or D609. Since the half-life of cathepsin B is less than 24 hours (Mort and Buttle, 1997), the strong immunostaining with the anti-cathepsin B antibody indicates that this pathway was not altered by FB_1or D609. Thus our results indicate that none of the inhibitors used in this experiment nor the exogenous lipids interfered with the mannose 6-phosphate receptor system.

Prosaposin transfected CHO cells were also incubated with exogenous sphingolipid precursors at various time intervals. The optimal incubation time was determined to be 24 hours since the turnover rate of sphingomyelin, in vitro, was determined to be 25-26 hours (Koval and Pagano, 1991). Incubation at 12 hours yielded similar results in the confocal microscope as the results obtained from cells incubated with the sphingolipid precursors for 24 hours. The intensity of labeling was in some cases fainter than the intensity observed in the 24-hour incubation. This was attributed to the optimal time required for the exogenous lipids to enter the different compartments of the cells. The 48-hour incubation yielded varying results. The control cells and the PDMP treated cells exhibited a similar immunostaining pattern as cells treated for 24 hours. Cells treated with FB_1 followed by addition of exogenous lipids for 48 hours displayed similar immunostaining pattern to their 24-hour counterparts, including the case of sphingomyelin supplementation. The restoration of labeling seen when sphingomyelin was added to FB_1 treated cells in the 48-hour incubation was attributed to the de novo synthesis of endogenous sphingomyelin from breakdown products of exogenous sphingomyelin, which is

metabolized in 25-26 hours. On the other hand, D609 treated cells incubated with any of the exogenous lipids for 48 hours, showed no fluorescent immunostaining under the confocal microscope. The addition of sphingomyelin did not maintain the perinuclear labeling because the lipid was metabolized within the first 26 hours and the resulting ceramide from sphingomyelin breakdown could not be converted back to sphingomyelin due to the inhibition of sphingomyelin synthase by D609. In fact, exogenous ceramide or dihydroceramide added to the medium could not be converted to sphingomyelin due to the sphingomyelin synthase enzymatic blockage.

The data generated in the CHO cell line demonstrated a relationship between the trafficking of prosaposin and the presence of sphingomyelin. However, the exact compartment in which prosaposin was targeted was difficult to determine by confocal microscopy. Previously when studying the same transfected CHO cell line in conjunction with electron microscopy, it was demonstrated that the final destination of recombinant prosaposin was the lysosomal compartment (Lefrancois et al., 1999). In this investigation we used an additional model system, the seminiferous tubules from the mouse testes, to verify the role of sphingomyelin in the targeting of prosaposin to the lysosomes. Sertoli cells, the somatic components of the seminiferous epithelium lining these tubules, are professional phagocytes that have large amounts of lysosomes containing endogenous prosaposin (Igdoura and Morales, 1995). Using a similar experimental approach (i.e. depletion and supplementation of sphingolipid precursors), and immunogold labeling, it was possible to study the effects of sphingomyelin on the targeting of prosaposin to lysosomes. This was best accomplished using the electron microscope and

quantitative analysis on the lysosomes of Sertoli cells. The results confirmed the confocal microscopy data. While PDMP did not decrease the immunogold labeling of the Sertoli cell lysosomes, Fumonisin B_1 and D609 produced a significant reduction in labeling. Dihydroceramide, ceramide and sphingomyelin restored the labeling in Fumonisin B_1 treated tubules and only sphingomyelin did in D609 treated tubules.

III. Role of functional domain D and the carboxy-terminus in the targeting of prosaposin to the lysosomes

Another objective of this investigation was to determine if an albumin-prosaposin chimeric construct used the same lysosomal pathway as prosaposin and consequently employ this construct as a dominant negative competitor. Thus, COS-7 cells were transfected with a prosaposin cDNA or with a chimeric construct composed of albumin, the prosaposin D domain and its adjacent COOH-terminal region (termed Albumin/SAP-D/COOH). Both recombinant proteins were linked to a myc-tag and displayed targeting to cytoplasmic punctate structures (green fluorescence) that also reacted with LysoTracker (red fluorescence), a dye specific for acidic organelles such as endosomes and lysosomes. When these cells were incubated with D609 and stained with the α-myc antibody they lost the punctate green staining. This staining was restored after supplementation of sphingomyelin. In conclusion, the three experimental systems (stable transfection of CHO cells, transient transfection of COS-7 cells, and Sertoli cells from seminiferous tubules explants) demonstrated that exogenous sphingomyelin was required to restore the transport of prosaposin to the lysosomes in sphingomyelin-depleted cells.

We wanted to determine the role of the prosaposin D domain by testing the

hypothesis that the Alb-D-COOH chimeric construct uses the same lysosomal targeting mechanism as, and competes with, endogenous prosaposin. This competition should result in the depletion of prosaposin from the lysosomes of COS-7 cells.

Immunogold labeling of cells transfected with the Alb-D-COOH construct, linked to a myc-tag, showed well-labeled lysosomes with the α-myc antibody and negligible lysosomal labeling with the α-prosaposin antibody. Conversely, COS-7 cells transfected with the Alb-D construct without the COOH-terminal region showed no immunostaining with the α-myc antibody and strong labeling with the α-prosaposin antibody. This type of immunogold labeling was also observed in control cells transfected with the plasmid only. These results indicate that the dominantly expressed chimeric protein (Alb-D-COOH construct) established preferential use of the targeting mechanism of endogenous prosaposin. The prosaposin antibody was raised to recognize domains A and B of prosaposin. Hence, the antibody does not cross react with the albumin constructs which contains the D domain and the COOH region, but did recognize mature saposins A and B. Thus the weak immunogold labeling of the prosaposin antibody is attributed to the competitive effect of the construct and the presence of residual saposins. Along with the adjacent COOH-terminal region, the D domain is the most conserved region of prosaposin (Zhao et al., 1997) and it has been suggested to interact with sphingomyelin (Morimoto et al., 1988) and acidic phospholipids (Ciaffoni et al., 2001). Thus, it appears that the role of the prosaposin D domain is to bind to sphingomyelin in the membrane of the *cis*-Golgi compartment to allow the binding of the COOH-terminal domain of prosaposin

to a targeting protein (see below).

Interestingly, Alb-D-COOH transfected cells presented prominent accumulation of perinuclear multivesicular bodies and an absence of mature lysosomes. It appears that lysosomal progression becomes arrested at this stage of maturation as a result of the Alb-D-COOH over-expression. The resulting multivesicular bodies presenting multi-layered membranes and prominent accumulation of undegraded lipid matter is a strong indication of a dominant negative competition of the chimeric construct. This displacement of endogenous prosaposin resulted in lysosomal storage disorders similar to MLD and Gaucher's disease.

In conclusion, the Alb-D-COOH construct used the same mechanism of transport as endogenous prosaposin and acted as a dominant negative competitor that displaced prosaposin from the lysosomes inducing the retention of undigested membranes in multivesicular bodies. These results support the notion of a novel mechanism of lysosomal targeting, involving a simultaneous interaction of the prosaposin D domain with sphingomyelin, and the COOH-terminal region of prosaposin with an unknown targeting protein (Lefrancois et al., 1999; Zhao and Morales, 2000). A putative compartment where this interaction could occur is the *cis/medial* region of the-Golgi apparatus, which has been implicated in the synthesis of sphingomyelin (Futerman et al., 1990).

IV. Role of sortilin in the targeting of prosaposin and $G_{M2}AP$ to the lysosomes

To exit from the trans-Golgi network (TGN), soluble lysosomal proteins bind the cation independent mannose 6-phosphate receptor (CI-MPR) (Lobel et al., 1989; Nielsen et al., 2001). The CI-MPR is a well-characterized trans-membrane protein

containing a cytoplasmic sorting signal that interacts with monomeric adaptor proteins termed GGAs (Lobel et al., 1989). GGAs are composed of a VHS domain that binds to the tail of the sorting receptor, a GAT domain which interacts with ARF, and a HINGE and an EAR domain that bind clathrin and accessory proteins to form coated vesicles (Hirst et al., 2000; Puertollano et al., 2001a; Puertollano et al., 2001b; Zhu et al., 2001).

Integral lysosomal membrane proteins do not require sorting receptors since their cytoplasmic tails interact directly with multimeric adaptor proteins (Peden et al., 2002). Multimeric adaptor proteins (AP-1 to AP-4) are involved in several trafficking processes such as endocytosis, basolateral targeting and lysosomal targeting (Robinson and Bonifacino, 2001). Multimeric adaptors are composed of four polypeptide chains that form a functional complex (Peden et al., 2002; Robinson and Bonifacino, 2001). Sorting of integral lysosomal membrane proteins, such as lysosomal associated proteins (LAMPs) from the TGN to lysosomes is mediated by AP-3 (Robinson and Bonifacino, 2001).

Analyses of human fibroblasts from patients with I-cell disease (ICD) showed that sphingolipid activator proteins (SAPs) use an alternative targeting mechanism to reach the lysosomes (Rijnboutt et al., 1991). ICD results from a mutation in the phosphotransferase that adds mannose 6-phosphate to newly synthesized hydrolases which is recognized by CI-MPR (Reitman et al., 1981).

Sphingolipid activator proteins are five non-enzymatic cofactors required for the lysosomal degradation of glycosphingolipids with short oligosaccharide chains (Bierfreund et al., 2000). Four of them (saposins A, B, C, D) are small homologous

glycoproteins derived from a common precursor protein (prosaposin) encoded by a single gene (Hiraiwa et al., 1997). Saposins increase the catalytic rate of lysosomal hydrolases by forming water-soluble complexes with specific sphingolipids. Hence, lysosomal hydrolases can cleave sugar residues to yield ceramide, which is deacylated to sphingosine. The fifth activator, the G_{M2} activator protein ($G_{M2}AP$), is the product of a separate gene and is an essential cofactor to β-hexosaminidase A in the degradation of G_{M2} to G_{M3} ganglioside (Yamanaka et al., 1994). The lysosomal trafficking of prosaposin is dependant on the D domain along with its highly conserved carboxyl-terminal region (Zhao and Morales, 2000). Deletion of either of these regions causes the misrouting of prosaposin to the secretory pathway (Zhao and Morales, 2000). Sphingomyelin present in the inner leaflet of the Golgi membrane is also required to target prosaposin to the lysosomes (Lefrancois et al., 2002). Depletion of this lipid by specific inhibitors causes the misrouting of prosaposin to the secretory pathway (Lefrancois et al., 1999). Based on these results, we have hypothesized that the D domain of prosaposin interacts with sphingomyelin to bring this protein into close contact to the Golgi membrane and to facilitate the binding of its C-terminal region to an unknown sorting receptor (Lefrancois et al., 2002). Little is known about the trafficking mechanism of $G_{M2}AP$, but experimental data suggest that the lysosomal sorting of this protein is CI-MPR independent (Glombitza et al., 1997).

Recent evidence demonstrated that the trans-membrane Golgi protein, sortilin, may be implicated in the transport of proteins to the lysosome. Chimeric constructs containing the luminal domain of the CI-MPR linked to the trans-

membrane and cytosolic portion of sortilin restored the lysosomal trafficking of soluble hydrolases in CI-MPR deficient cells (Nielsen et al., 2001). While the mannose 6-phosphate receptor binds the majority of soluble hydrolases, no lysosomal proteins are known to interact with sortilin (Nielsen et al., 2001). Interestingly, the cytosolic portion of sortilin and CI-MPR contain an "acidic cluster-dileucine motif" that interacts with the monomeric adaptor proteins (Nielsen et al., 2001). Based on these results we tested the hypothesis that sortilin is the trafficking receptor of prosaposin and $G_{M2}AP$.

Deletion of the acidic-cluster-dileucine signals from the CI-MPR tails abolished interactions with the VHS domain of GGA. Thus, we used a similar approach to investigate the role of sortilin in the transport of prosaposin and $G_{M2}AP$. First, a wild type and a truncated sortilin lacking the acidic-cluster-dileucine signals (Δcytosolic) were in vitro translated in a reticulocyte system, run on a 12.5% acrylamide gel and transferred to nitrocellulose paper. The membrane was stained with an anti-sortilin monoclonal antibody. The full-length sortilin produced a band at approximately 95 kDa while the truncated sortilin was slightly lower. COS-7 cells were then transiently transfected with both constructs linked to a myc-tag that allowed to examine its intracellular localization and particularly to distinguish the mutant form of sortilin from endogenous sortilin. The cells were subsequently immunostained with an anti-myc antibody and with an anti-Golgin antibody. The immunostaining demonstrated that both constructs were present within the Golgi apparatus.

Mutant sortilin lacking the acidic-cluster-dileucine signal was overexpressed

in COS-7 and used as a dominant negative competitor of endogenous sortilin. The cells were then immunostained with anti-prosaposin, anti-$G_{M2}AP$ or anti-cathepsin B antibodies followed by incubation with a secondary antibody conjugated to Alexa 488 (green fluorescence). To detect mutant sortilin, the same culture cells were immunostained with an anti-myc antibody followed by a secondary antibody conjugated to Alexa 568 (red fluorescence). Non-transfected cells showed the characteristic anti-prosaposin and anti-$G_{M2}AP$ immunostaining of the Golgi region as well as of cytoplasmic punctate structures characteristic of lysosomes. Transfected cells with the dominant negative sortilin did not abolish the Golgi staining with anti-prosaposin, anti-$G_{M2}AP$ and the anti-myc antibodies but eliminated the immunostaining of the punctate structures. On the other hand, the immunolabeling of anti-cathepsin B antibody was not different in the transfected or non-transfected cells. Therefore, our results indicate that truncated sortilin competed for and retained prosaposin and $G_{M2}AP$ in the Golgi apparatus. As expected, overexpression of truncated sortilin had no effect on cathepsin B immunostaining since this hydrolase is trafficked to the lysosomal compartment via the CI-MPR (Mort and Buttle, 1997).

To confirm whether prosaposin or $G_{M2}AP$ were interactive partners of sortilin, an *in vitro* co-immunoprecipitation assay was conducted. Truncated and wild type-sortilin as well as prosaposin, $G_{M2}AP$ or cathepsin B were translated and labeled in a reticulocyte lysate system. Each lysosomal protein was incubated with full-length or truncated sortilin, and immunoprecipitated with anti-prosaposin, anti-$G_{M2}AP$ or anti-cathepsin B antibody. The immunoprecipitated complex was pulled down with protein A covered sepharose beads, electrophoresed, transferred to nitrocellulose

paper and visualized with a non-radioactive detection assay. The results demonstrated that both full-length and truncated sortilin co-immunoprecipitated with both prosaposin and $G_{M2}AP$ but not with cathepsin B.

The recent use of a dominant negative GGA construct lacking the HINGE and EAR domains abolished the recruitment of clathrin and caused the retention of CI-MPR in the Golgi preventing proper intracellular sorting of several lysosomal proteins (Puertollano et al., 2001a). Since the cytosolic portion of sortilin also contains "acidic cluster-dileucine motif" that interacts with GGAs (Nielsen et al., 2001) we tested the effect of mutant adaptor proteins on the transport of prosaposin and $G_{M2}AP$ to lysosomes. Thus, COS-7 cells were transfected with a dominant negative GGA construct lacking the HINGE and EAR domains and immunostained with anti-prosaposin, anti-$G_{M2}AP$, anti-cathepsin B or anti-LAMP-2 antibodies. The GGA construct lacking the HINGE and EAR domains was linked to the green fluorescence protein (GFP). Thus, transfected cells were recognized from non-transfected cells by their green fluorescence. While transfected cells did not exhibit immunostaining for anti-cathepsin B, anti-prosaposin, or anti-$G_{M2}AP$ antibodies, the anti-LAMP-2 antibody produced a strong granular and perinuclear immunostaining. On the other hand, non-transfected COS-7 cells were immunostained by the four antibodies.

To evaluate the specificity of our assay, human fibroblasts carrying a mutation in the μ subunit of AP-3 that prevents the transport of lysosomal integral membrane proteins were immunostained with anti-prosaposin antibody, anti-$G_{M2}AP$, anti-cathepsin B antibody, or anti-LAMP-2 antibody. While anti-cathepsin B, anti-prosaposin and anti-

$G_{M2}AP$ antibodies yielded a perinuclear Golgi-like reaction and a cytoplasmic punctate reaction typical of lysosomes; anti-LAMP-2 antibody did not generate immunostaining. As expected, anti-LAMP-2 antibody immunostained punctate structures in wild-type fibroblasts.

In conclusion, these results demonstrated that prosaposin and $G_{M2}AP$ immunostaining were absent in the lysosomes of cells transfected with the dominant negative GGA construct lacking the HINGE and EAR domains, and that their transport is dependent on the presence of GGAs. The absence of immunostaining in the lysosomes with anti-cathepsin B antibody was expected because GGAs are required to traffic CI-MPR bound proteins including cathepsin B. Finally, our experiment with the mutant fibroblast cell line lacking functional AP-3, demonstrated that this adaptor protein was not involved in the transport of prosaposin to the lysosomes since both the anti-prosaposin and anti-cathepsin B antibodies immunostained the perinuclear Golgi region and cytoplasmic punctate structures characteristic of lysosomes in this cell line.

Prior to this study, the mechanism of lysosomal targeting of sphingolipid activator proteins (prosaposin and $G_{M2}AP$) was unknown. Earlier reports from several laboratories demonstrated that both sphingolipid activator proteins can reach the lysosomes in a MPR independent manner (Rigat et al., 1997; Rijnboutt et al., 1991). Immunocytochemical analysis of tunicamycin treated cells showed that non-glycosylated prosaposin can be targeted more efficiently to lysosomes and biochemical analysis revealed that prosaposin is associated to Golgi-membrane fractions and that this interaction is not disrupted by mannose 6-phosphate (Igdoura

et al., 1996). Also, a MPR independent pathway of $G_{M2}AP$ has been recently characterized (Rigat et al., 1997). Taken together, all these reports suggested the existence of an alternative mechanism of sorting and transport of lysosomal proteins in addition to the mannose 6-phosphate receptor pathway.

Based on structural features and experimental evidence, sortilin was proposed to traffic lysosomal proteins from the trans-Golgi network (TGN) to the lysosomes (Nielsen et al., 2001). Sortilin belongs to a growing family of multiligand type-1 receptors with homology to the yeast receptor Vps10p (Nielsen et al., 2001). However, no lysosomal proteins were known to interact with sortilin. In this investigation, we tested the hypothesis that sortilin is involved in the sorting of sphingolipid activator proteins (prosaposin and $G_{M2}AP$) and that the lysosomal trafficking of these proteins requires monomeric adaptor protein GGA. We have identified specific interactions between sphingolipid activator proteins and sortilin, and functional interactions between sortilin and monomeric GGA. We have also demonstrated that deletion of the acidic-cluster-dileucine signals from sortilin abolish the transport of sphingolipid activator protein to lysosomes.

In a recent study, the selective deletion of prosaposin functional domains demonstrated that the D domain and the C-terminus are required for its targeting to lysosomes. When the D domain and the C-terminus of prosaposin were added to albumin, the chimeric construct was routed to the lysosomes (Zhao and Morales, 2000). Interestingly the C-terminus of prosaposin contains a saposin-like motif that forms a short α-helix stabilized by two conserved disulfide bonds (Zhao et al., 1998a). This region is significantly similar to the N-terminus of surfactant-B protein

(SP-B) (Zaltash and Johansson, 1998). SP-B requires the presence of the N-terminal region for its transient routing to multivesicular and lamellar bodies in neumocytes type II (Lin et al., 1996; Stahlman et al., 2000). Members of the superfamily of aspartic proteinases, also contain a common saposin-like motifs (Kervinen et al., 1999). Other proteins containing saposin-like motifs are acyloxyacyl hydrolase and acid sphingomyelinase (Muniz and Riezman, 2000). Evidence from several laboratories suggests that saposin-like motifs in aspartic proteinases promote vacuolar targeting (Ponting and Russell, 1995).

To our surprise, the over-expression of truncated sortilin abolished the transport of the G_{M2} activator protein to the lysosomes. Although the G_{M2} AP does not contain a saposin-like motif, a striking feature of its structure is the presence of a short α-helix stabilized by a disulfide bridge between C112 and C138. Patients with a C138 substitution are associated with the AB variant form of G_{M2} gangliosidosis characterized by the absence of G_{M2} AP in lysosomes (Mahuran, 1998).

Taken together, out results demonstrate the existence of a novel mechanism of sorting and transport of lysosomal proteins mediated by sortilin. Although sortilin is involved in the sorting and trafficking of both prosaposin and G_{M2}AP, this receptor may play a broader role in the targeting of other soluble lysosomal proteins.

Summary

We have demonstrated that the inhibition of sphingomyelin synthesis with fumonisin B_1 and D609 abolishes the transport of prosaposin to the lysosomal compartment.

We showed that sphingomyelin restores the transport of prosaposin to the lysosomes in cells treated with fumonisin B_1 and D609.

We showed that the precursors of sphingomyelin restored the transport of prosaposin to the lysosomes in fumonisin B_1 treated cells but not in those treated with D609.

We presented strong evidence using chimeric constructs and dominant negative competition that the D domain may be responsible for this lipid protein interaction.

We induced a phenotype of lysosomal storage disorder in COS-7 cells with our dominant negative experiment.

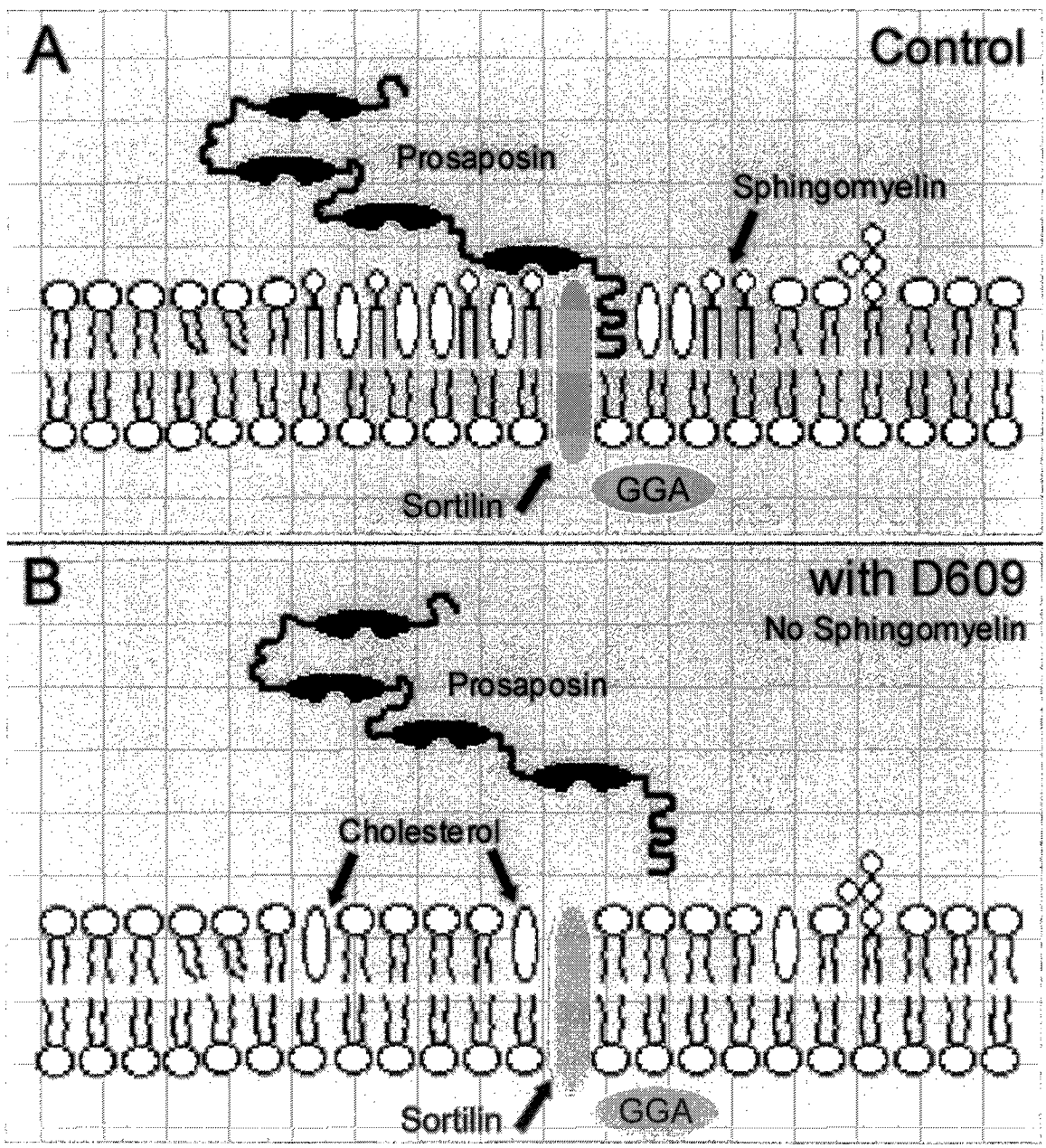
We presented strong evidence demonstrating that sortilin and prosaposin/G_{M2}AP are interactive partners.

We presented strong functional evidence using a chimeric truncated sortilin that this sorting receptor is implicated in the lysosomal transport of prosaposin and G_{M2}AP.

In conclusion, we demonstrated a novel lysosomal protein trafficking mechanism mediated by sortilin. To our knowledge, this is the first demonstration implicating sortilin as a sorting receptor for lysosomal proteins. We have also shown that this process requires the GGA proteins. Based on the data we have collected, we

propose that the D domain of prosaposin interacts with sphingomyelin present in the inner leaflet of the Golgi membrane with sphingomyelin and that the carboxy-terminus interacts with sortilin (See figure C). According to our model, the addition of D609 removes sphingomyelin and eliminates the protein/lipid interaction causing the release of prosaposin into the secretory route.

Fig. C The diagrams illustrate our hypothesis of the events occurring on the Golgi membrane. Our results suggest that prosaposin binds with sphingomyelin found in the inner leaflet of the Golgi membrane (yellow circle) and then interact with sortilin (pink oval)and then in turn to GGA (red oval) to traffic to the lysosomes (A). The depletion of sphingomyelin by D609 interferes with the initial binding of prosaposin to the Golgi membrane, which prevents its interaction with the targeting receptor. The protein cannot be routed to the lysosome, and therefore follows the default secretory pathway (B).


A
Control
Prosaposin
Sphingomyelin
Sortilin
GGA
B
with D609
No Sphingomyelin
Prosaposin
Cholesterol
Sortilin
GGA

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70% ethanol. All materials used to clean the contaminated area will be placed in biohazardous waste bags and autoclaved.

All spills, will be reported in writing to the laboratory supervisor or acting alternate as soon as circumstances permit; the supervisor will in turn file this report with the Biosafety Committee.

7. Does the protocol present conditions (e.g. handling of large volumes or high concentrations of pathogens) which could increase the hazards of the infectious agent(s)?
No

8. Do the specific procedures to be employed involving genetically engineered organisms have a history of safe use?
Yes

9. What precautions are being taken to reduce production of infectious droplets and aerosols?
Mechanical pippeting devices will be used for all procedures. Eating and drinking is not permitted in the laboratory. Manipulations of biohazardous material will be carried out in a laminar flow hood. Lab bench tops will be covered with protective absorbent paper before the experiment begins. After completion of the experiments, all surfaces where biohazardous material has been used will be cleaned with 1% detergent, distilled water and 70% ethanol. Biohazardous material to be discarded will be placed in waste bags and autoclaved. The bags will be stored in waste drums until picked up by the McGill waste disposal centre.

10. List the biological safety cabinets to be used.

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