Targeting Exogenous β-Defensin to the Endolysosomal Compartment Via a Vehicle Guided System

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Summary

A number of pathogens for which there are no effective treatments infect the cells via endocytosis. Once in the endosomes, the pathogens complete their life cycle by overriding normal lysosomal functions. Recently, our laboratory identified the lysosomal targeting signal of prosaposin, which is recognized by the sorting receptor "sortilin". Based on this evidence, we tested whether the antimicrobial peptide β-Defensin linked to the targeting sequence of prosaposin (βD-PSAP) could be redirected from its secretory pathway to the endolysosomal compartment. To this effect, βD-PSAP was transfected into COS-7 cells. The sub-cellular distribution of βD-PSAP was analyzed by confocal microscopy and differential centrifugation. Confocal microscopy demonstrated that βD-PSAP overlaid with the lysosomal marker LAMP1, indicating that the construct reached endosomes and lysosomes. Differential centrifugation also showed that βD-PSAP was in the lysosomal fractions. In addition, our binding inhibition assay demonstrated that βD-PSAP bound specifically to sortilin. Similarly, the delivery of βD-PSAP was abolished after overexpressing a truncated sortilin. These results indicate that the prosaposin C-terminus and D/C-domain (prosaposin targeting sequence) was an effective "guidance system" to redirect βD-PSAP to the endolysosomal compartment. In the future, this and other fusion proteins with antimicrobial properties will be assembled to our "biotic vehicle" to target pathogens growing within these compartments.

Introduction

Viral, bacterial and protozoal infections are a preoccupation with respect to the evolution of virulence and iatrogenic enhancement due to cross-species transmission and antibiotic resistance. Furthermore, a number of bacteria, viruses and protozoa take advantage of receptor-mediated endocytosis, a critical function of eukaryotes, by binding to surface proteins and receptors and by using their endocytic pathway to infect cells. Intracellular survival of *Salmonella typhimurium* (Buchmeier and Heffron, 1991) and *Brucella spp*. (Starr et al., 2008) are attributed to failure of bacteria containing phagosomes and endosomes to become or deliver their cargo to lysosomes. The bacteria actively inhibit this maturation process and preferentially divide within these prelysosomal compartments (Buchmeier and Heffron, 1991; Starr et al., 2008). Moreover, a number of life threatening pathogens such as the *Hepatitis C Virus* (Meertens et al., 2006), *Human Immunodeficiency Virus, Dengue Virus* (Zaitseva et al., 2010), *Trypanosome cruzi* (Gruenberg and van der Goot, 2006), *Mycobacterium tuberculosis* (Koul et al., 2004), *Mycobacterium leprae* (Alves et al., 2004), *Leishmania donovani* (Lofgren et al., 2008), for which there are no effective treatments in all the cases, enter the cell via endocytosis and through endosomes and lysosomes. Once inside the cell, pathogens evade lysosomal degradation by different mechanisms, most notably by the selective retention of Rab GTPases in the membranes of the vacuoles that they occupy in host cells during infection, inhibiting the fusogenic function and acidification of these organelles and/or the activity of the lysosomal hydrolases (Fratti et al., 2001; Rzomp et al., 2003; Brumell and Scidmore, 2007; Cardoso et al., 2010; Sherwood and Roy, 2013). Once inside, the pathogens divide within these compartments and infect other cells (Fratti et al., 2001; Brumell and Scidmore, 2007).

Given the specific site of entry of most pathogens, the objective of this work is to develop a biotic vehicle able to target molecules with antimicrobial properties to the endolysosomal compartment. In this respect, we have identified a sequence of seventeen amino acids within the C-terminus of the prosaposin molecule that is

necessary for its endolysosomal targeting (Zhao and Morales, 2000). Prosaposin is a nonenzymatic sphingolipid activator protein involved in the hydrolysis of glycosphingolipids. The seventeen amino acid sequence is located within the first half of its C-terminus (Yuan and Morales, 2010), and binds the lysosomal sorting receptor "sortilin" (Tiszlavicz et al., 2011).

In recent years an increasing number of proteins, lectins and peptides with antimicrobial properties have been discovered in mammals, amphibians, invertebrates, insects and bacteria (Chen et al., 2006; Lofgren et al., 2008; Huskens and Schols, 2012; Akkouh et al., 2015). Since these biologically active substances, such as "β-Defensin" (Tiszlavicz et al., 2011), have an intrinsic capacity to kill pathogenic microorganisms, we think that they can be used to substitute and/or enhance existing antibiotic and antiviral treatments. However, the effectiveness of antimicrobial proteins could be enhanced if they are targeted to the site of cellular entry and propagation of pathogens. To overcome this problem, we propose to deliver the antimicrobial protein directly to the endolysosomal compartment with the intent of destroying them therein.

Thus, the objective of this investigation was to test the hypothesis that the antibacterial peptide "β-Defensin", linked to a fragment of prosaposin containing the sortilin binding sequence is directed to the endolysosomal compartment. We believe that the validation of this hypothesis will constitute a proof of concept for the delivery of other fusion proteins with antimicrobial properties to endosomes and lysosomes.

Materials and Methods

Constructs

The sortilin cDNA was a gift from Dr. C. Petersen (University of Aarhus) that was subcloned into the pcDNA 3.1C/Myc/His vector, Invitrogen (Carlsbad, CA, USA). In addition, we used the cDNAs of one control and two fusion proteins that were subcloned in the pcDNA 6A/V5-His vector to express V5-tagged proteins. The constructs were as follow: 1) A mouse prosaposin cDNA (PSAP), which expressed a recombinant mouse prosaposin and was used as positive delivery

control. 2) A fusion protein consisting of the mouse PSAP signal peptide, the active domain of β-Defensin, followed by the domains C/D and the C-terminus of PSAP (βD-PSAP). 3) A fusion protein consisting of the mouse PSAP signal peptide and the active domain of β-Defensin alone (βD), which was used as a negative delivery control.

Truncated sortilins were generated in our lab by including the luminal and trans-membrane regions without the cytosolic domain subcloned into the pcDNA3.1B/Myc/His (Lefrancois et al., 2003) or pEGFP (Ni and Morales, 2006) expression vectors.

Antibodies

Antibodies used for fluorescence microscopy: anti V5, Invitrogen (Burlington, ON, Canada); anti LAMP1, Abcam Inc. (Toronto, ON, Canada); anti TGN46, Novus Biologicals (Oakville ON, Canada); polyclonal anti mouse Alexa 488, Invitrogen (Burlington, ON, Canada); polyclonal anti rabbit Cy3, EMD Millipore (Billerica, MA, USA) and polyclonal anti mouse Cy3, Invitrogen (Burlington, ON, Canada).

Antibodies used for differential centrifugation: anti V5 and anti cathepsin D, Santa Cruz Technology (Dallas, TX, USA); anti TGN46 and anti goat antibody conjugated to horseradish peroxidase (HRP), EMD Millipore (Billerica, MA, USA); anti rabbit and anti mouse antibodies conjugated with HRP, Sigma-Aldrich (Oakville, ON, Canada).

A monoclonal antibody against the luminal domain of sortilin purchased from BD Bioscience (Franklin Lakes, NJ, USA) was used for the binding inhibition assay. The immunoblots were developed with anti V5, anti sortilin and anti mouse antibody conjugated with HRP, Sigma-Aldrich (Oakville, ON, Canada).

Cell Culture

COS-7 cells (Gluzman, 1981) were cultured in DMEM supplemented with 10% FBS, 5% penicillin and streptomycin, and L-glutamine overnight at 37°C and 5% CO_2 to approximately 70% confluence for both immunofluorescence and differential centrifugation. All transfections were carried out using the PolyFect Transfection kit, QIAGEN (Hilden, Germany) and left for a 24 hour period.

Indirect Immunofluorescence and Confocal Microscopy

COS-7 cells were cotransfected with pcDNA 3.1C/Myc/His vector containing the sortilin cDNA (sortilin-myc) and pcDNA 6/V5-His vectors containing the three different fusion constructs (PSAP, βD-PSAP and βD) for 24 hour. After 24 hour following transfection, the cells were fixed with 4% paraformaldehyde solution for 20 minutes. After washing with 1X phosphate buffer saline (PBS), the cells were permeabilized with 0.1% saponin in PBS solution for 20 minutes. The cells were then blocked by incubation with 5% goat serum in 1X PBS. Primary antibody incubation was done overnight. Secondary antibody incubation was done for a 2 hour period the following day, after which confocal microscopy was carried out. Immunostaining with anti LAMP1 was done to verify the localization of the fusion constructs to the endolysosomal compartment. Immunostaining with anti TGN46 was used to verify the presence of fusion constructs in the Golgi apparatus.

To verify that the lysosomal targeting βD-PSAP was mediated by sortilin, COS-7 cells were co-transfected with the pcDNA 3.1B/Myc/His vector containing the truncated sortilin cDNA (Lefrancois et al., 2003) and the pcDNA 6A/V5-His vectors PSAP and βD-PSAP for 24 hour and processed as described above. The truncated sortilin vector has been shown to inhibit the transport of prosaposin to the lysosomes by competitive inhibition (Lefrancois et al., 2003). In addition, COS-7 cells were transfected with a truncated sortilin cDNA subcloned in the pEGFP vector (Ni and Morales, 2006).

Due to the low retention of β-Defensin and the secreted nature of this molecule, the transfected cells were incubated with 0.5% tannic acid, a substance known to block exocytosis (Polishchuk et al., 2004). To this extent, 6 hour after transfection, the dish was incubated from 37°C to 40°C. After another 4 hour, the dish was incubated at 20°C and left overnight. The following day, 10 minutes prior to culturing the cells to 32°C, 0.5% tannic acid was added. The cells were fixed after 1 hour incubation period at 32°C (Polishchuk et al., 2004). Each temperature block served to retain the fusion protein in ER, Golgi and post Golgi compartments respectively.

Differential Centrifugation

COS-7 cells were cultured and co-transfected with sortilin-myc and one of the three fusion constructs (PSAP, βD-PSAP and βD) inserted into either the pcDNA 3.1C/Myc/His vector or the pcDNA6A/V5-His vector respectively for 24 hour. Again, for the βD / sortilin-Myc co-expression, 0.5% tannic acid was added following the same protocol as the one used for confocal microscopy (Polishchuk et al., 2004). The cells were washed with 1X PBS buffer and scrubbed from the dish, after which they were pipetted into falcon tubes. Then the cells were scrubbed and put into the corresponding tubes, followed by centrifugation for 7 minutes at 800xg to collect the pellet. The buffer was then removed and the pellet was resuspended in H buffer (10mM Tris acetate, 0.25M Sucrose, 1% EDTA, 0.02% sodium azide, 5mM glycerophosphate, and a protease inhibitor cocktail). The H buffer and resuspended pellet were transferred to an Eppendorf tube, after which a 25 gauge needle was used to further resuspend the pellet and to break up the cells. The homogenate was then submitted to several rounds of centrifugation (de Duve C. et al., 1955). After each centrifugation, the supernatant was removed and submitted to a new round of centrifugation. The remaining pellets N (enriched in nuclei and unbroken cells), M (enriched in endosomes, lysosomes and mitochondria), L (enriched in lysosomes), and P (enriched in microsomes containing membranes derived from Golgi, ER and plasma membrane), were divided into three equal volumes and prepared for immunoblotting with anti V5, anti cathepsin D and anti TGN46 antibodies. The immunoblot also included 40 μg of protein from the S fraction (i.e., the remaining supernatant from last centrifugation).

Binding Inhibition Assay

To examine if the βD-PSAP fusion protein binds specifically to sortilin, we applied a binding inhibition assay. The assay was modified from Carvelli et al. (Carvelli et al., 2010), consisting of two samples in parallel (n=5), one of which was given the opportunity to bind in the absence of anti-sortilin antibody, and a control which has the anti-sortilin antibody present. The procedure is divided into three steps: 1) Purification of ligand; 2) Membrane purification of overexpressed receptor; 3) Binding inhibition assay.

Ligand purification step. COS-7 cells were transfected with PSAP or βD-PSAP pcDNA6A expressing vectors. After 24 hour, the cells were harvested and homogenized with lysis buffer (10 mM Tris acetate pH 7.2, 0.25 M sucrose and 0.1% EDTA). The cell suspension was passed through a 25 gauge needle 30 times and centrifuged for 20 minutes at 800xg. The supernatant was ultracentrifuged at 130 000xg, at 4°C for 25 minutes. The supernatant (S1) was kept and the pellet resuspended in saponin buffer (50 mM Tris acetate pH 7.2, 0.5% saponin, 50 mM EDTA), sonicated and incubated for 15 minutes on ice. The sample was centrifuged at 130 000xg, at 4°C for 25 minutes. Again, the supernatant (S2) was kept and the pellet resuspended in ionic saponin buffer (0.6 M potassium chloride in saponin buffer, pH 7.2), sonicated and incubated for 15 minutes on ice. The sample was further centrifuged at 130 000xg, at 4°C for 25minutes. The supernatant (S3) and the pellet were conserved at -20°C. S3, rich in either PSAP or βD-PSAP were dialyzed overnight with Spectra/Pore Membrane (MWCO 3.5 kDa) submerged in the binding buffer at 4°C. The protein concentration was estimated by the Bradford assay and the dialyzed supernatants were conserved at -20°C until use.

Membrane purification of overexpressed receptor. COS-7 cells were transfected with sortilin pcDNA 3.1C. After 24 hour, the cells were harvested and homogenized with lysis buffer. The suspension was passed through a 25 gauge needle 30 times and centrifuged for 20 minutes at 800xg. The supernatant was ultracentrifuged at 130 000xg, at 4°C for 25 minutes. The pellet was resuspended in saponin buffer (50 mM Tris acetate pH 7.2, 0.5% saponin, 50 mM EDTA), sonicated and incubated for 15 minutes on ice. The sample was centrifuged at 130 000xg, at 4°C for 25 mins. The pellet was resuspended in ionic saponin buffer (0.6 M potassium chloride in saponin buffer pH 7.2), sonicated and incubated for 15 minutes on ice. The sample was centrifuged at 130 000xg, at 4°C for 25 minutes. The pellet was resuspended in binding buffer (10 mM Tris acetate pH 6.8), sonicated and incubated for 15 minutes on ice. The sample was centrifuged at 130 000xg, at 4°C for 25 mins. The final pellet containing membranes with overexpressed sortilin was resuspended in binding buffer, sonicated and

conserved at -20°C until use. The protein concentrations were estimated by the Bradford assay (Bradford, 1976).

Binding inhibition assay. Membrane protein (60 µg) was incubated with 30 µg of S3 containing either PSAP or βD-PSAP, in presence or absence of 0.2 or 0.5 µg of monoclonal antibodies against the luminal domain of sortilin in a final volume of 0.25 ml binding buffer (10 mM Tris acetate pH 6.8, containing 0.5 mM CaCl2 and 0.5 mM MnCl2). After 60 minutes incubation at 4°C, 0.75 ml of binding buffer was added and the samples were centrifuged at 17 000xg for 30 minutes at 4°C. The supernatants were discarded and the pellets washed in 1 ml of binding buffer. After a new centrifugation at 17 000xg for 30 minutes at 4°C the pellets were resuspended in 30 µl of loading buffer and immunoblotted with anti V5 and anti sortilin antibodies. The binding of the ligand to the overexpressed receptor, followed by its inhibition by the anti sortilin antibody was considered specific binding.

Results

Indirect Immunofluorescence and Confocal Microscopy

Since β-Defensin is a secretory protein, we used the cell's endogenous trafficking machinery to build a controlled vehicle that targets the antimicrobial molecule to the endolysosomal compartment. To achieve this objective, we created a fusion protein consisting of β-Defensin, followed by the C/D domains and the C-terminus of PSAP. The C/D domains including the C-terminus are critical for the trafficking of PSAP to the lysosomal compartment (Canuel et al., 2009; Yuan and Morales, 2010; Yuan and Morales, 2011). The resulting fusion construct termed βD-PSAP was cotransfected with wild type sortilin and visualized with a commercial anti V5 antibody. To examine whether the fusion protein reached endosomes and lysosomes the transfected cells were costained with a LAMP1 antibody. The results showed a colocalization of βD-PSAP (green fluorescence) with LAMP1 (red fluorescence) within vesicular structures indicating that the βD-PSAP construct reached endosomes and lysosomes (**Fig. 1**). As expected, the positive control PSAP also co-localized with LAMP1 (**Fig. 1**). On the other hand,

the negative control βD did not colocalize with LAMP1 and it was not seen within the cell (data not shown). Based on this observation we hypothesized that the βD construct was rapidly secreted. To test this hypothesis, we used the tannic acid procedure to impair the secretion of βD from the COS7 cells. Within this procedure, the cultures are synchronized for their processing pathway of newly synthesized ER luminal proteins. At 40°C the proteins are arrested in the ER, at 20°C in the Golgi complex and at 32°C in presence of tannic acid, the proteins are released to a post Golgi compartment. Incubation with tannic acid showed retention of βD within punctate structures dispersed in the cytoplasm of the transfected cells. However, βD did not colocalize with LAMP1 in the cells, indicating that the fusion protein was trafficked to a compartment other than endosomes and lysosomes (**Fig. 1**).

Since PSAP was shown to transiently passage through the Golgi apparatus, we used a TGN46 antibody as a Golgi marker. The immunofluorescence results showed a perfect match between the TGN46 antibody (red fluorescence), and the V5 antibody (green fluorescence) in the perinuclear region of the cells were co-transfected with the βD-PSAP or PSAP and wild type sortilin constructs (**Fig. 2**). Again, the negative control βD was not seen within the cell (data non shown). As expected, the tannic acid treatment induced the reappearance of βD in punctate structures that did not colocalize with TGN46 (**Fig. 2**).

To determine if the lysosomal targeting βD-PSAP was mediated by sortilin, COS-7 cells were cotransfected with the pcDNA 3.1B/Myc/His vector expressing truncated sortilin and the pcDNA 6A/V5-His vectors expressing either PSAP or βD-PSAP. Truncated sortilin has been shown to abolish the transport of prosaposin to lysosomes (Lefrancois et al., 2003). Immunostaining with anti V5 and LAMP1 antibodies showed both fusion proteins to be present in the Golgi perinuclear region but absent from the punctate structures highlighted by the LAMP1 antibody (**Fig. 3**). The cells transfected with truncated sortilin-EGFP also showed a perinuclear retention of both PSAP or βD-PSAP that colocalized with the fluorescence produced by truncated sortilin (**Fig. 4**).

Subcellular Localization of fusion proteins

Differential centrifugation is a procedure used to separate organelles from whole cells. In the process, a cell sample is first lysed to break the plasma membrane. The lysate is then subjected to repeated centrifugations, each time removing the pellet and increasing the centrifugal force. Thus, from lower to higher centrifugal forces we obtained the following fractions: N (enriched in nuclei and unbroken cells), M (enriched in endosomes, lysosomes and mitochondria), L (enriched in lysosomes), P (enriched in microsomes containing membranes derived from Golgi, ER and plasma membrane), S (remaining supernatant from last centrifugation).

Differential centrifugation was carried out to examine the subcellular distribution of fusion proteins and to further verify the transport of βD-PSAP to lysosomes. Immunoblotting with anti V5 antibody detected βD-PSAP within the N, M, L and P fractions (**Fig. 5a**). Positive control PSAP was also seen within fractions M, N, L and P (**Fig. 5a**). Negative control, βD was not seen in any of the fractions alluding to the possibility that it had been secreted from the cells. To test this possibility, cells were treated with the tannic acid procedure and submitted to differential centrifugation. Treatment with tannic acid produced detection of βD protein within fractions N and M (**Fig. 5a**).

We performed immunoblots with anti cathepsin D and anti TGN46 antibodies to assess the purity of the subcellular fractions. The anti cathepsin D antibody was employed to determine the purity of fraction L enriched with lysosomes. As expected, mature cathepsin D (33 kDa) was seen in fractions N, M and L but not in fraction P which is enriched in microsomes, Golgi and ER elements (**Fig. 5b,** upper two panels). On the other hand, fraction P presented three bands corresponding to the newly synthesized non-glycosylated (47 kDa) form of cathepsin D, the immature (52 kDa) form, and a lower unspecific band. Unlike the 33 kDa mature cathepsin D observed in COS-7 cells synchronized with temperature and tannic acid, the 47 kDa and 52 kDa bands were absent (**Fig. 5b,** lower panel), since the end point of the treatment liberated the enzyme to a post-Golgi compartment.

Immunoblots with TGN46 were used to test for the presence of Golgi elements in fraction P and Golgi contamination in faction L. For all fractionations (i.e., all cells transfected with the three vectors), TGN46 was seen in fractions N, M and P (**Fig. 5c**). It is also worth noting the absence of TGN46 within fraction L (**Fig. 5c**).

Binding inhibition assay

To confirm that lysosomal targeting of βD-PSAP was mediated by sortilin, we performed a binding inhibition assay. To obtain cellular membranes enriched in sortilin, COS-7 cells were transfected with the pcDNA 3.1C/Myc/His vector expressing the wild type receptor. For the purification of PSAP or βD-PSAP, COS-7 cells were transfected with pcDNA 6A/V5-His vectors expressing either PSAP or βD-PSAP. The purified peptides were incubated with membranes overexpressing sortilin receptor in presence or absence of 0.2 and 0.5 μg of anti sortilin antibody (Carvelli et al., 2010), which recognizes the luminal domain of sortilin. The result demonstrated that the membranes enriched with sortilin had affinity for PSAP and βD-PSAP (**Fig. 6**). This observation was further substantiated by the inhibition of PSAP and βD-PSAP binding to sortilin by the anti sortilin antibody (**Fig. 6**).

Discussion

The objective of this work was to test whether β-Defensin linked to the targeting sequence of prosaposin (PSAP) is directed to the endolysosomal compartment. We applied the principles of a guided missile using a biotic approach. A guide missile is a “warhead” carrying vehicle. Moved by an “engine” rocket in a controlled flight path, the intent of the missile is the destruction of the target. We divided our biotic vehicle into the following parts or components. The “warhead” was the antipathogenic protein β-Defensin. The “guidance system” was the targeting sequence of PSAP, and the “engine” the sortilin receptor along with the accessory proteins associated to its cytoplasmic tail, required for its transport to the lysosomal compartment (Nielsen et al., 2001; Takatsu et al., 2001; Puertollano et al., 2001; Shiba et al., 2002; Lefrancois et al., 2003; Canuel et al., 2008; Braulke and Bonifacino, 2009). In addition, a missile requires a “deployment system”. In our

particular case the “deployment system” was pcDNA 3.1C/Myc/His and pcDNA 6A/V5-His vectors.

In the present investigation we tested what were considered the two more important components of our biotic missile: the “guidance system” and the “engine”. For operational simplicity we have chosen the PSAP lysosomal targeting sequence over the mannose-6-phosphate residue (MP6) as our preferred “guidance system” and sortilin instead of any of the two M6P receptors as our preferred “engine”.

PSAP is the precursor of four lysosomal sphingolipid activator proteins needed for the hydrolysis of glycosphingolipids in the lysosomes (O'Brien et al., 1988; Hiraiwa et al., 1997; Schuette et al., 2001). The C-terminus of PSAP has been identified as the interacting region with the sorting receptor, sortilin, an interaction that leads to the activation of the receptor and the endolysosomal trafficking of prosaposin (Lefrancois et al., 2003). Previous work done in our laboratory demonstrated that deletion of the C-terminal domain inhibited the trafficking of PSAP to the lysosomes (Zhao and Morales, 2000). A chimeric construct of albumin fused with the C-terminus and D-domain of PSAP was trafficked to the lysosomes of COS-7 cells (Zhao and Morales, 2000). The results indicated that these domains were sufficient and necessary to redirect the secretory protein albumin to the lysosomes.

It is well known that some bacteria, viruses and protozoa, for which there is no effective treatment or cure in all cases, take advantage of receptor-mediated endocytosis by binding to cell surface proteins and receptors leading to cell infection and completion of their life cycle within the endolysosomal compartments (Gruenberg and van der Goot, 2006). We predict that targeting these pathogens with specific antimicrobial proteins at the site of entry would be an excellent therapeutic approach and a real alternative to antibiotic treatment and antibiotic resistance, such as multidrug resitant tuberculosis (Gunther, 2014; Matteelli et al., 2014). In recent years, several antimicrobial proteins, peptides and lectins have been discovered in a variety of species. However, their effectiveness depends on the concentration, tissue distribution and species specificity.

To circumvent these hurdles, we fused a segment of PSAP that included its C/D domains and the C-terminus, hereto termed as the "guidance system", to a "warhead", in this case the antimicrobial protein β-Defensin. The construct is referred to throughout the paper as βD-PSAP. β-Defensin is a physiologically secreted antimicrobial peptide that is part of the innate immune response system, mostly found in epithelial tissues (Tiszlavicz et al., 2011). Although the scope of this investigation was not to test the biological effect of β-Defensin, we chose this protein for various reasons: 1) to probe or dismiss the approach; 2) β-Defensin is a mammalian protein and thus physiologically closer to the cell system used in this investigation; 3) β-Defensin fulfill the "warhead" concept given that it targets bacteria, fungi and some enveloped viruses (Ganz, 2003). In addition, the proof of concept would allow us to assemble new antimicrobial warheads to our biotic missile and test their effectiveness.

In this study, we transfected COS-7 cells with the construct β-Defensin linked to the targeting segment of PSAP and examined whether the addition of this segment redirected the antimicrobial peptide to the endolysosomal compartment. We observed by confocal microscopy punctate green structures highlighted by the anti V5 antibody, which colocalized with the red fluorescence of anti LAMP1 antibody. When the cells were cotransfected with a dominant negative vector expressing truncated sortilin, βD-PSAP and PSAP were absent from LAMP1 positive vesicles, indicating that the transport of the fusion proteins required a functional sortilin trafficking pathway (Lefrancois et al., 2003).

To verify the presence of the chimeric construct within lysosomes, we homogenized the transfected cells and fractionated by differential centrifugation (de Duve C. et al., 1955). The subcellular fractions were immunoblotted with anti V5 primary antibody. While the positive control, PSAP, was also present in the lysosomal fraction, the βD negative control was not found associated to this or other fractions. In conclusion, both immunofluorescence and differential centrifugation indicated that βD-PSAP reached endosomes and lysosomes. The absence of βD in the confocal microscopy studies raised three possibilities: a) lack of expression, b) rapid secretion or c) rapid degradation of the construct. Based on

previous studies, it was tempting to assume that βD was secreted from the cell (Ganz et al., 1993). To test if this was the case, we incubated the transfected COS-7 at different temperatures and tannic acid, a drug shown to inhibit exocytosis (Polishchuk et al., 2004). Following tannic acid addition βD was found in fractions N and M. These results were supported by confocal microscopy, showing accumulation of a multitude of vesicles positively stained by the anti V5 antibody at the periphery of the cell. To be more specific, the technique entailed the incubation of the transfected cells at 20°C and 32°C (Polishchuk et al., 2004). According to the protocol (Polishchuk et al., 2004) when the temperature is adjusted to 20°C, proteins accumulate within the Trans-Golgi Network (TGN) and are released from this compartment at 32°C. It is possible that the band observed in fraction M was the result of cross contamination with a post-Golgi compartment. Most importantly, the results show that the L and P fractions were devoid of βD, suggesting that β-Defensin was released from the Golgi but did not reach the lysosomal compartment.

The validity of differential centrifugation depends on the purity of the subcellular fractions. In this case, it was important to have a fraction enriched in lysosomes and free of Golgi components. To assess this issue we used anti cathepsin D antibody. Cathepsin D is a soluble lysosomal hydrolase whose immature forms are found in the Golgi apparatus and its mature form only exists within late endosomes and lysosomes (Gieselmann et al., 1983). Immunoblotting of the subcellular fractions with cathepsin D revealed the mature form in the enriched lysosomal fraction (L) but not in the enriched Golgi fraction (P). TGN46 was also used to assess the purity of our fractions. TGN46 is a marker of the trans-Golgi network. As expected, TGN46 did not stain our lysosomal fraction (L) suggesting that it was largely free of Golgi contaminants.

We also found that the recognition of βD-PSAP fusion protein by sortilin was specific. To accomplish this aim we used a binding inhibition assay (Carvelli et al., 2010). As expected, both βD-PSAP and PSAP bound sortilin and a monoclonal antibody against the luminal domain of sortilin antibody inhibited the binding of these proteins to the receptor.

In conclusion, this study allowed us to confirm a proof of concept in which β-Defensin, linked to a fragment of prosaposin containing the sortilin binding sequence was directed to the endolysosomal compartment. The system constitutes, to our knowledge, the first biotic "guided missile system" where the "warhead" was an antimicrobial peptide. Given that most pathogens enter the cell via endocytosis to the endolysosomal system, that there is a growing list of antimicrobial proteins and that the "guidance system" can accommodate other antimicrobial "warheads", the present approach may have a great relevance in the treatment against the infection of viruses, parasites, and superbugs resistant to antibiotics.

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Figure Legends

Fig. 1. Immunolocalization of βD-PSAP in Lysosomes. COS7 cells were co-transfected with wild type sortilin pcDNA 3.1C/Myc/His and with PSAP, βD-PSAP or βD pcDNA 6/V5-His vectors ± tannic acid treatment. After transfection, cells were immunostained with anti V5 antibody, followed by a secondary Alexa 488 conjugated goat anti mouse antibody (green fluorescence). Lysosomes were immunostained with anti LAMP1 antibody, followed by a secondary Cy3 conjugated goat anti rabbit antibody (red fluorescence). The nuclei were counterstained blue with Hoechst 33342. Unlike βD, PSAP and βD-PSAP colocalize with LAMP1. After tannic acid treatment βD was released to a LAMP1-negative post Golgi compartment. The inset shows the merged figure at higher magnification. The bar is equal to 7 μm and applies to all figures.

Fig. 2. Immunolocalization of βD-PSAP in the Golgi compartment. COS7 cells were co-transfected with wild type sortilin pcDNA 3.1C/Myc/His and with PSAP, βD-PSAP or βD pcDNA 6/V5-His vectors ± tannic acid treatment. After transfection, cells were immunostained with V5 antibody, followed by a secondary Alexa 488 conjugated goat anti mouse antibody (green fluorescence). The Golgi complex was immunostained with a TGN46 antibody, followed by a secondary Cy3 conjugated goat anti rabbit antibody (red fluorescence). The nuclei were counterstained blue with Hoechst 33342. Unlike βD, PSAP and βD-PSAP colocalized with TGN46. After tannic acid treatment βD appeared juxtanuclearly and colocalized with TGN46. The inset shows the merged figure at higher magnification. The bar is equal to 4 μm and applies to all figures.

Fig. 3. Immunolocalization of βD-PSAP after Competitive Inhibition with Truncated Sortilin. COS-7 cells were co-transfected with truncated sortilin pcDNA 3.1B/Myc/His and PSAP or βD-PSAP pcDNA 6/V5-His vectors. After transfection, cells were immunostained with anti V5 antibody, followed by a secondary Alexa 488 conjugated goat anti mouse antibody (green fluorescence). Lysosomes were

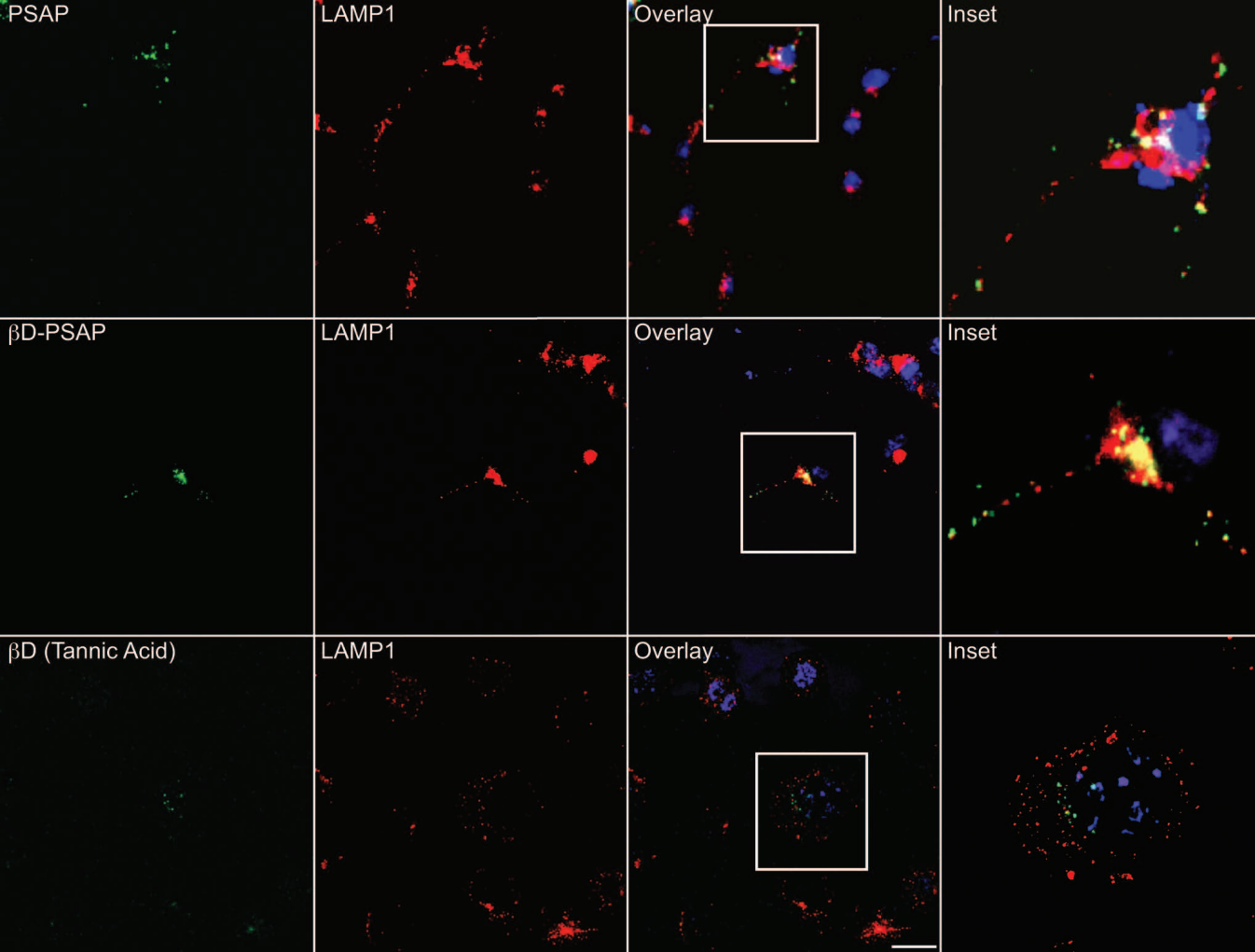
immunostained with anti LAMP1 antibody, followed by a secondary Cy3 conjugated goat anti rabbit antibody (red fluorescence). The nuclei were counterstained blue with Hoechst 33342. Truncated sortilin arrested both probes in a juxtanuclear compartment. The inset shows the merged figure at higher magnification. The bar is equal to 7 μm and applies to all figures.

Fig. 4. Effect of Truncated Sortilin on the Trafficking of βD-PSAP. COS-7 cells were co-transfected with truncated sortilin pEGFP vector (green fluorescence) and either PSAP or βD-PSAP pcDNA 6/V5-His vectors. After transfection, cells were immunostained with a V5 antibody, followed by a secondary Cy3 conjugated goat anti mouse antibody (red fluorescence). The nuclei were counterstained blue with Hoechst 33342. Note that PSAP, βD-PSAP and truncated sortilin colocalize in a juxtanuclear compartment. The inset shows the merged figure at higher magnification. The bar is equal to 7 μm and applies to all figures.

Fig. 5. Differential Centrifugation. COS7 cells were co-transfected with wild type sortilin pcDNA 3.1C/Myc/His and with PSAP, βD-PSAP pcDNA 6/V5-His vectors or βD pcDNA 6/V5-His vector (with tannic acid treatment). The cells were homogenized and subjected to differential centrifugation. Immunoblotting with anti V5 antibody determined the sub-cellular localization of PSAP (65 kDa), βD-PSAP (27kDa) and βD (8 kDa) (**a**). While PSAP and βD-PSAP were seen in all fractions, βD was absent in L, P and S. To assess the purity of the L fraction, we examined the presence of the mature lysosomal form of cathepsin D using a cathepsin D antibody (**b**), and a TGN46 antibody to assess the purity of the fraction enriched in Golgi elements (**c**). Mature cathepsin D (33 kDa) was absent from fraction P and present in fractions N, M and L. The Golgi marker TGN46 was seen in fractions N, M and P and was absent in fraction L.

Fig. 6. Binding Inhibition Assay. Membrane proteins overexpressing wild type sortilin (top panel) bound purified PSAP (**a**) and βD-PSAP (**b**). The specific binding was confirmed by its inhibition in presence of 0.2 or 0.5 μg of monoclonal

antibodies against the luminal domain of sortilin. The samples were immunoblotted with mouse monoclonal anti V5 and mouse monoclonal anti sortilin antibodies. An extra band of 50 kDa was present, which corresponds to the IgG heavy chain of the antibody used to inhibit the binding of sortilin to the ligands.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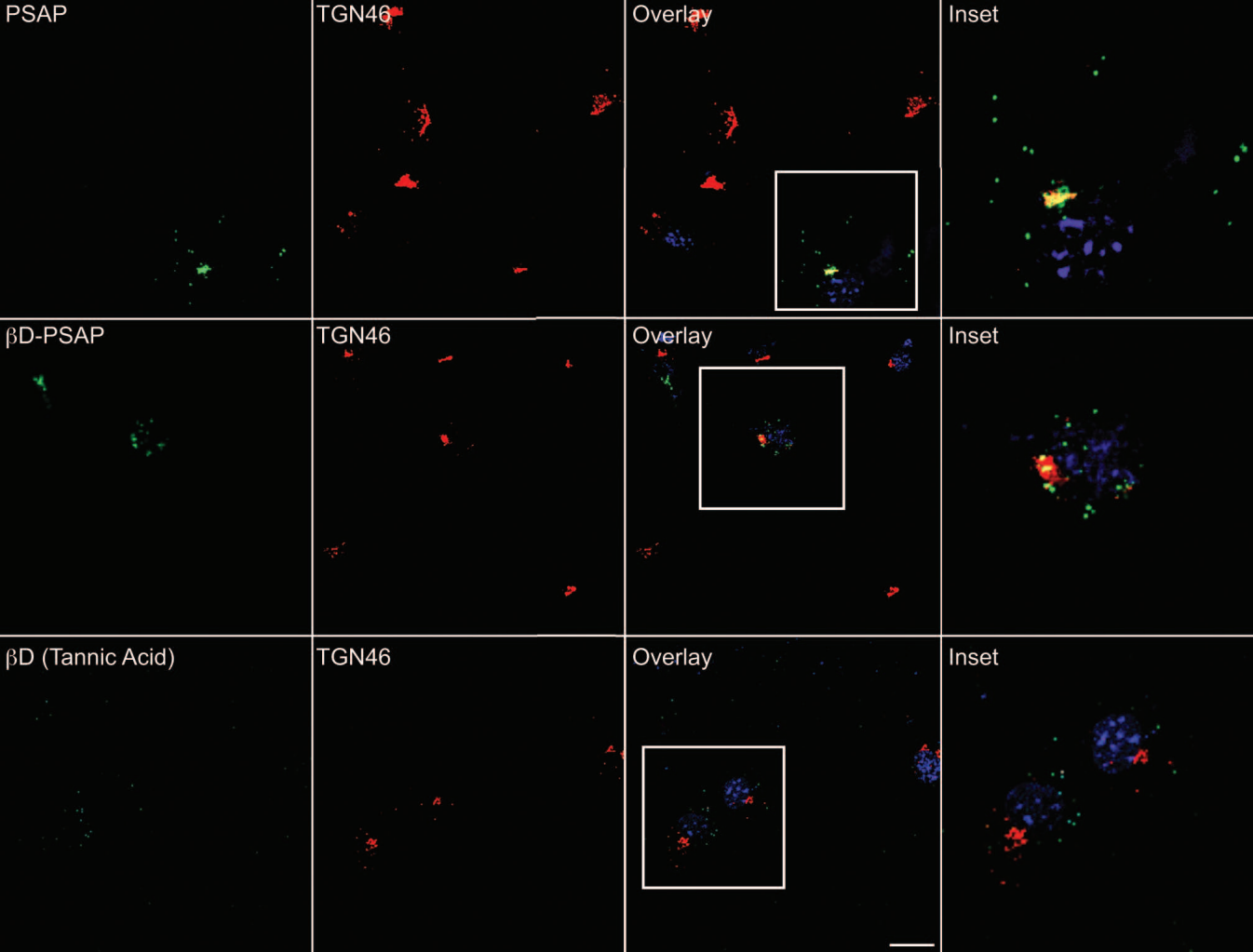
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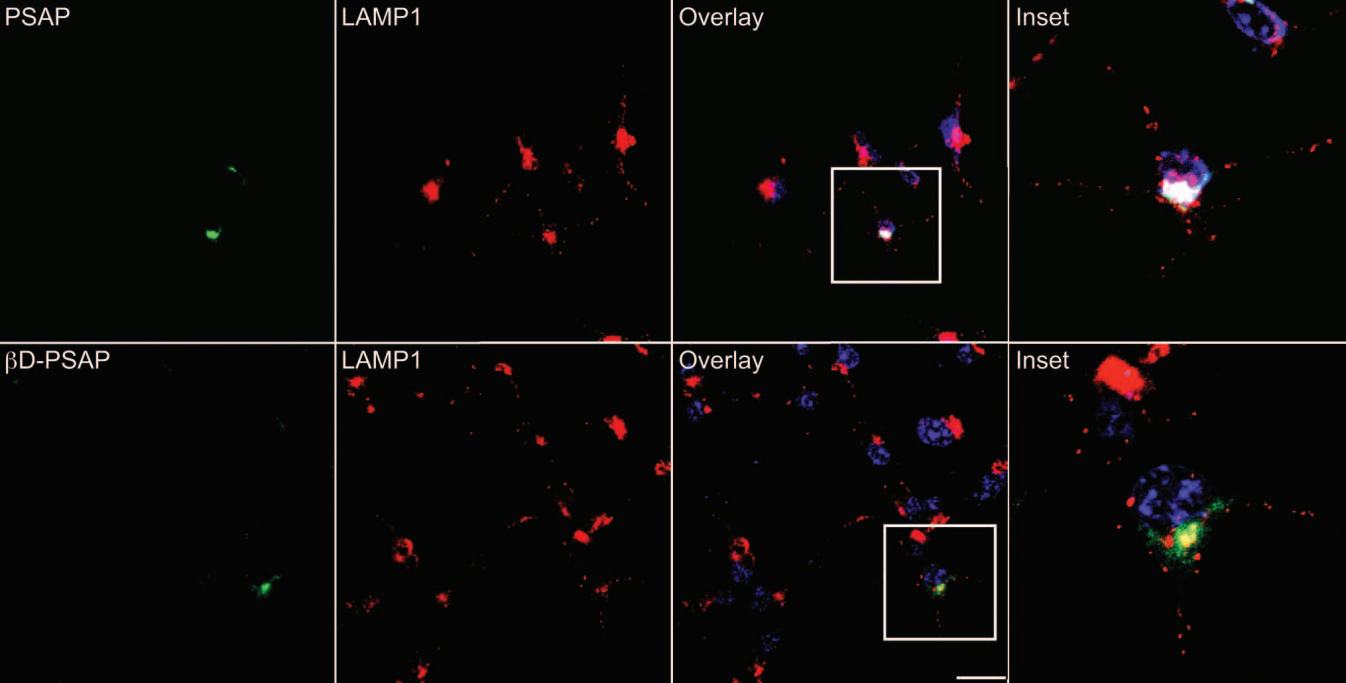
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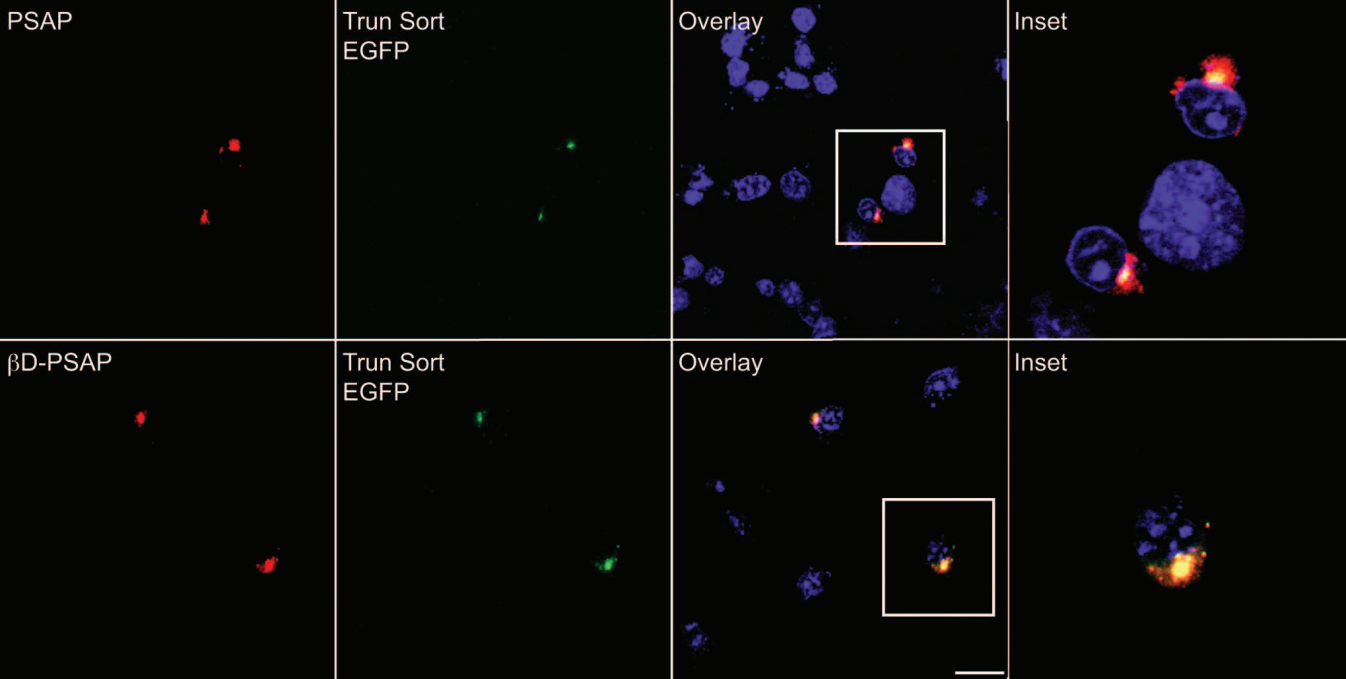
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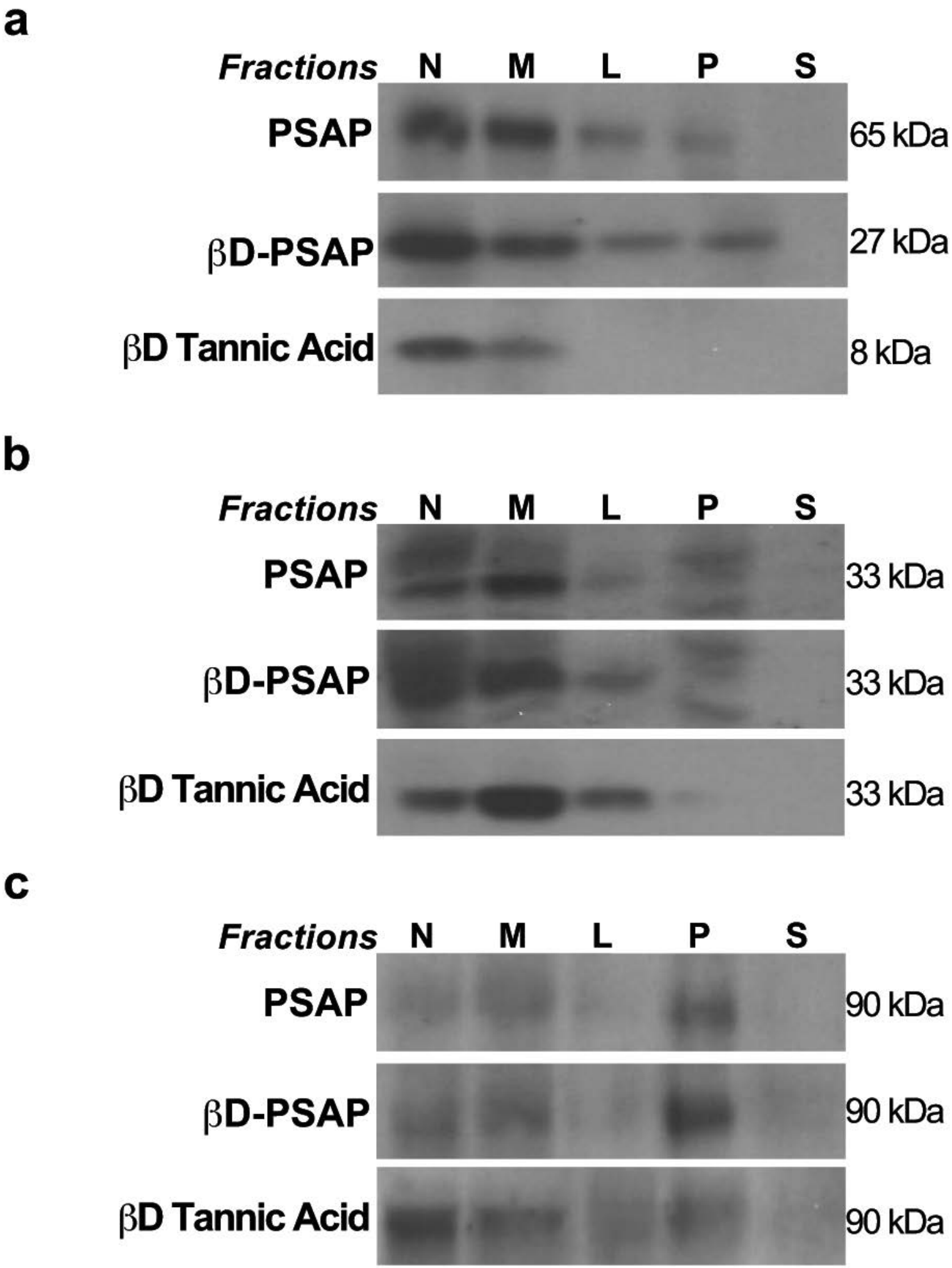
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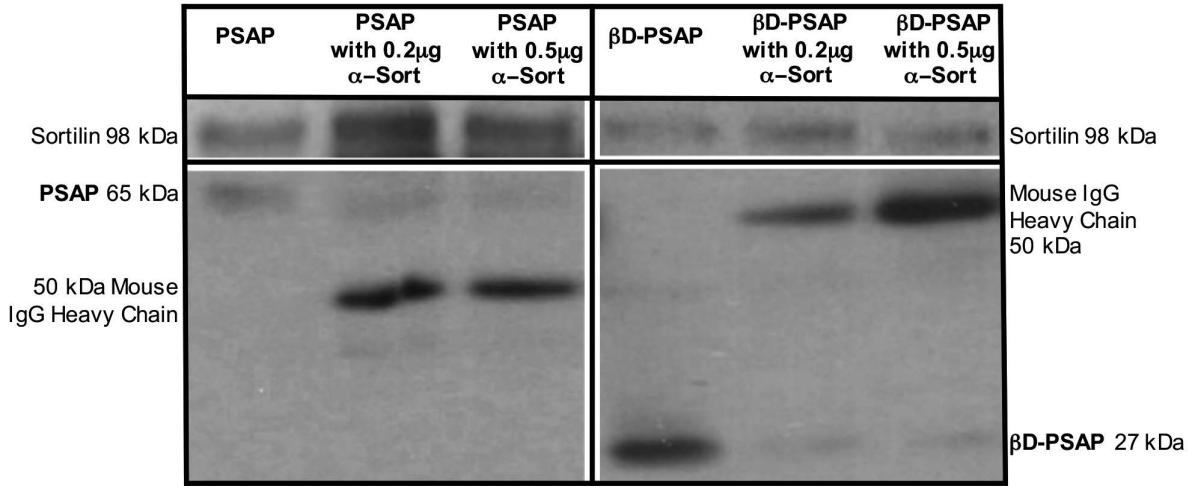
PSAP
PSAP with 0.2µg α–Sort
PSAP with 0.5µg α–Sort
βD-PSAP
βD-PSAP with 0.2µg α–Sort
βD-PSAP with 0.5µg α–Sort
Sortilin 98 kDa
PSAP 65 kDa
50 kDa Mouse IgG Heavy Chain
Sortilin 98 kDa
Mouse IgG Heavy Chain 50 kDa
βD-PSAP 27 kDa