INTRODUCTION

Chapter 1. Introduction

1.1 Prelude: Endocytosis and endocytic vesicles

Endocytosis is defined as a cellular process of energy dependent uptake of extracellular material(s) into cells and their multiphasic, vesicle-enclosed transport into lysosomes for complex enzymatic processing. It includes pinocytosis, phagocytosis and receptor mediated endocytosis.

In their part, endocytic vesicles (endosomes) are described as a discrete group of subcellular, vesicular, cytoplasmic organelles with spherical and tubuloreticular morphology. The organelles originate via internal invagination of host plasma membrane and terminate by fusing with lysosomes. Along the route - termed as the 'endocytic pathway' the organelles undergo dynamic changes in morphological, biophysical and biochemical characters that affect their function(s) in sorting, enzymatic processing and lysosome- targeted-transport of endocytosed material(s).

1.2. The endocytic process: molecular components and mechanism

1.2.1 Molecular components of endosomes and their functions

The detailed profiles of the lipid, protein and carbohydrate components of endosomes and their biological functions are yet to be completely elucidated. Studies showed, that unique membrane domains, often characterized by distinctive protein or lipid components offer unique biophysical and functional properties associated with endosomes (138, 186). Further, such membrane domains, unique to an endosomal class (early, recycling or late) coexist in respective endocytic compartment(s), suggesting that endocytic organelles are composed of a mosaic of structural and functional regions. Whereas some of these molecular assemblies can be found in more than one compartment, a given combination seems to be unique to each compartment, indicating that membrane organization might be modular.

1.2.1.1 The endosomal proteins

A plethora of different proteins and enzymes play important role in endosome biogenesis and function. Many of these are unique to respective classes of endosomes (early, recycling or late) or endocytic processes (pinocytosis or phagocytosis). The following are among the best characterized endosomal proteins:

1.2.1.1.1 Proteins specific to coat formation

(i) Clathrin: These are fibrous proteins that form the scaffolding around vesicles. The tertiary structure of clathrin resembles a triskelion and has three arms, each of which is comprised of a clathrin light chain and a clathrin heavy chain. These triskelions oligomerize to form a polygonal lattice with intrinsic curvature, which results in a more or less spherical shape about 50 - 100 nm in diameter. Each lattice is composed of 12 pentagons and a variable number of hexagons (28, 117, 125).

(ii) Caveolin: These are the principal proteins of caveolae membranes. They form hair pin like structure in the membrane and bind cholesterol. Multiple forms of caveolin have been identified: caveolin 1α, caveolin 1β, caveolin 2 and caveolin 3. Caveolin 1/VIP 21 (an integral membrane protein) is the major component of the caveolar coat while caveolin 2 functions as an accessory protein in conjunction with caveolins 1 (44, 230).

(iii) Adaptor proteins: These proteins reside in the 20 nm space between the clathrin coat and the membrane of the vesicle. AP (Adaptor proteins) mediate clathrin attachment and recruit membrane proteins that are localized to clathrin coated vesicles. For this, the adaptor proteins are assembled into particles consisting of four individual polypeptides, each of which binds to the clathrin heavy chain. The other end of the adaptor protein binds to a motif in the cytoplasmic domain of the protein to be incorporated into the vesicle. The 'heterotetrameric AP family' has five members: AP 1, AP 2, AP 3, AP 4 and the α, β, δ, ζ subcomplex of COP I (there are also subtypes of each complex due to the presence of tissue-specific isoforms of some of

the subunits. All AP complexes comprise of two large subunits (α and β 2 in AP 2), a medium subunit (μ 2 in AP 2) and a small subunit (σ 2 in AP 2) (44, 117, 200).

(iv) Epsin: Epsin is a curvature-forming molecule. It is first recruited to the PtdIns (phosphatidylinositol) (4,5) P2 rich sites of the plasma membrane where it induces membrane budding and facilitates recruitment of clathrin triskelions. Epsin molecule then associates with AP 2 complex and becomes displaced when AP 2 molecule triggers clathrin polymerization (67, 117).

(v) Eps 15: Eps 15 (EFGR-pathway substrate 15), an interacting partner of epsin is located at the edges of forming coated pits where it is in complex with AP 2. The Eps 15 - AP 2 complex becomes disrupted upon clathrin assembly (44, 67).

(vi) AP 180/CALM: The neuronal adaptor protein AP 180 (previously known as AP 3) and its ubiquitous isoform CALM (clathrin assembly lymphoid myeloid leukaemia) are monomeric proteins with binding sites for both clathrin and AP 2. They act synergistically with epsin and AP 2 to recruit clathrin triskelions and form clathrin coated vesicles respectively. AP 180/CALM and AP 2 have also been shown to play important roles in the regulation of vesicle size (68).

(vii) Dab 2: Dab 2 (Disabled 2) is a widely expressed protein. It plays a role in receptor sorting [LDL (low density lipoprotein) receptor, ApoER 2 (apolipoprotein E receptor 2) and the scavenger receptor - megalin] and in the general endocytic machinery (134).

Several other molecules have also been identified as components of coated pits including: HIP 1 (huntingtin interacting protein), hStn 2 (human stoning 2), intersectin/Ese and numb to name only a few (44, 117, 172).

1.2.1.1.2 Proteins specific to the fission machinery

(i) Dynamin: Dynamin is a high molecular weight protein with GTPase activity. It plays an important role in vesicle scission during phagocytosis, caveolae mediated endocytosis, clathrin mediated endocytosis as well as some clathrin and caveolae independent endocytic pathways. Thus it is a master regulator of membrane trafficking events at the cell surface. It is suggested that dynamin functions as a regulatory GTPase or mechanochemical enzyme or a combination of both (44, 211).

(ii) Endophilin: Endophilin has acyl transferase activity. It mediates conversion of (lyso phosphatidic acid) LPA-to-PA (phosphatidic acid) and hence acts jointly with dynamin to induce vesicle fission (44, 111).

(iii) Amphiphysin: Amphiphysin is needed for dynamin recruitment to the coated pits and functions as a linker between dynamin and clathrin coats (211).

1.2.1.1.3 The SNARE(s) family

The term SNARE was coined by J. Rothman and colleagues to describe receptors which could participate in the binding of soluble factors e.g. NSF (*N*-ethylmaleimide-sensitive fusion protein) and SNAPs (its membrane-attachment proteins) to membranes derived from bovine brain homogenates (receptor to SNAPs hence the name SNA*RE*) (175, 199). They are found throughout the secretory pathway and participate in a number of membrane-trafficking function(s), including endosome fusion events. SNAREs include distinct families of conserved membrane-associated proteins which facilitate membrane fusion in eukaryotes. The SNARE protein family has been broadly categorized into two subgroups. SNAREs present on the vesicle (donor) compartment are known as v-SNAREs, while those on target (acceptor) compartment are known as t-SNAREs. Functionally, SNAREs act in a lock and key mechanism to specify the docking of vesicles with their target membrane and even to catalyze their fusion downstream of Rab proteins. Along with other proteins, the function of SNARES are assisted primarily by two soluble proteins in the membrane complex: the ATPase NSF and its cofactor SNAP (62, 143, 193).

1.2.1.1.4 The Ras GTPase family

The Ras superfamily of small GTPases (~30 kDa) include a large group of membrane phosphatases that perform GTP-GDP driven functions critical for growth, motility, signalling and membrane transport (135). The Ras superfamily consists of five families, Ras, Rho, Rab, Rac, Arf, Sar 1/ARF and Ran (159, 166). The largest group in the superfamily is the family of the Rab GTPases.

(i) The Rab GTPase family: Rab proteins form the largest reported sub-group of the Ras GTPase family with almost 60 members identifed in the mammalian system (147). They are present on the cytoplasmic surface of organelles in the biosynthetic/secretory and endocytic pathways. They play crucial role in homotypic fusion between organelles as well as in the specific targeting/docking of transport intermediates with their acceptor membranes. Their binding to GTP and its hydrolysis result in conformational changes in the protein (Rabs) directing their interaction with a wide range of effector proteins (135, 162, 173, 187).

Rab GTPases are major players in the endocytic pathway. Several of them are known to be remarkably specific with stage-specific endosome morphology/function(s) and are recognized as markers for endosomes at definite stage(s) of endocytic pathway. Rab 4, Rab 5, Rab 20, 21 and 22 are associated with early endosomes and differentially regulate membrane transport through this compartment (195). Rab11 is on the pericentriolar recycling endosome and the TGN (trans-golgi network). It regulates recycling of the transferrin receptor to the plasma membrane and endosome to TGN trafficking (173). Rab 15 co-localizes with Rab 4 and Rab 5 on early endosomes and with Rab 11 on the pericentriolar recycling endosome and seems to function as an inhibitory GTPase in early endocytic trafficking. Rab 7 and Rab 9 have been shown to localize on to the late endosomes. The latter GTPase mediates transport from early to late endosome/lysosome and from late endosome to the TGN network respectively in mammalian cells and in yeast. Other Rab proteins not directly connected to endosomal traffic are Rab 17, Rab 18 and Rab 25, all of which are specific to polarized cells and participate in transport of

macromolecules across epithelia. Interestingly, Rab 22 is uniquely present both in early and late endosomes and facilitates sorting of endocytosed material along the endocytic pathway (173, 187).

Newly synthesized Rab proteins are post-translationally modified on carboxy-terminal cysteine residues. Following synthesis, Rab proteins associate with the cytosolic Rab escort protein (REP) and are geranylated. The doubly geranylated Rab protein remains associated with REP. The latter delivers the GTPase to a specific organelle or transport vesicle (164). Rab proteins can be further modulated by their interaction with a multitude of effector proteins like rabphilin 3, rabaptin 5, rabaptin 4, phosphatitylinositol-3 kinases, EEA1, rim, rabkinesin 6, p 40, rabenosyn 5 and other FYVE domain proteins to facilitate discrete steps in membrane transport (173).

***(ii) Rho GTPase*:** Recent studies point to multiple functions for these signalling proteins in endocytosis. Rho GTPases play a pivotal role in the dynamic regulation of the actin cytoskeleton and through this control cell morphology, motility as well as adhesion. Insulin-stimulated translocation of the GLUT 4 (glucose transporter) vesicles also require Rho GTPases. Rho A helps in both clathrin dependent and independent mechanisms. Rho D GTPases are localized to plasma membrane and early endocytic vesicles, while the RhoB GTPases are present in multivesicular endosomal membranes. Rho GTPases also coordinate cytoskeletal rearrangement with intracellular traffic to generate polarity during cell migration (166)

1.2.1.1.5 LAMP

LAMPs (LAMP-1 and LAMP-2) are the major protein constituents of late endolysosomal membranes. Phagosomes, endosomes, autophagic vacuoles and lysosomes are all enriched in LAMP (lysosome associated membrane proteins) and LIMPs (lysosome associated integrated membrane proteins). 50% of total proteins found on lysosomal membranes are LAMPs/LIMPs. They protect the membranes from hydrolysis and prevent leakage of the endo/lysosomal constituents into the cytoplasm. LAMPs are type-I membrane proteins with a short cytoplasmic tail, a transmembrane region and a large luminal domain (60).

1.2.1.1.6 Vacuolar H^+-ATPase(s)

The proton pumping activity of a vacuolar-type H^+-ATPase is responsible for the increasing acidity of the endocytic organelles from early endosomes to lysosomes. The acidic environment is responsible for two essential roles. (i) It promotes dissociation of ligands from receptors and of Fe^{3+} from transferrin in endosomes. (ii) It is essential for the activity of many hydrolases in endosomes and lysosomes (39, 206).

1.2.1.1.7 Actin

The cytoskeletal protein actin helps in scaffold assembly, early endosome motility, fusion and transfer of cargo from multivesicular bodies to lysosomes in clathrin mediated processes. Ligand uptake in clathrin-independent routes like macropinocytosis and phagocytosis are driven by actin polymerization (44, 166, 181).

Other endocytic proteins are annexins, flotillin, protein kinase C (PKC), PI 3-kinase, tubulin, myosin I, myosin V, myosin IX, paxillin, talin, vinculin, α actinin, protein kinase Cα, NECAP 1 and NECAP 2, MARCKS and MacMARCKS to name a few. Many more proteins are being worked upon (3, 44, 117, 143, 166, 172).

1.2.1.2 The endosomal proteases

Considering proteolytic processing is the major function of the endocytic pathway, endosome/lysosome mediated proteolysis has attracted significant attention. Proteases, lysosome-associated membrane proteins, H^+-ATPases and possibly cysteine transporters are the major components of the endosomal hydrolytic environments. The lysosomes are the main storage organelles of the hydrolases, perhaps in an inactivated form. Most lysosomal enzymes are probably introduced into the endocytic pathway at the level of early endosomes and some even at the level of plasma membrane. Some of these enzymes, whose final destination is the late endocytic structures, may be active in early endosomes, as it has been shown that lysosomal proteolysis may be involved in activation of enzymes, hormones and in the production of antigenic peptides (18, 33, 65). Though the lysosomes have the highest

concentration of proteolytic enzymes in the endocytic pathway, late endosomes are assumed to be the main degradative compartment in the cell. Lysosomes store mature lysosomal enzymes and inject them to late endosomes and autophagosomes by fusion (214). Biochemical and morphological studies have defined the thiol-dependent cysteine protease Cathepsins B and H, and the pepstatin A-sensitive aspartic protease Cathepsin D as responsible for the majority of proteolytic activities in endosomes (219).

Some of the important proteases of the endolysosomal system are given in Table 1.

A few other novel, tissue-specific cathepsins have been reported in mammals, *i.e* rat and mouse Cathepsins Q, M, P, and R. These are primarily expressed in placenta and therefore may play important roles in implantation and embryonic development (198).

1.2.1.3 The endosomal lipids

An array of recent report indicates membrane constituent lipids play an important role in the organization and functions of the vacuolar apparatus. These studies revealed that constituent lipids vary significantly between early, recycling and late endosomes. Further the lipid components of the endosomal membrane are not randomly distributed within the endosome(s); discrete membrane populations with defined composition can be distinguished relative to the endosome stage and domain function (138, 186). Kobayashi *et al.* reported that the internal membranes of particularly late endosomes consist of at least two populations containing primarily phosphatidylcholine or lysobisphosphatidic acid as major phospholipid, arguing for the existence of significant microheterogeneity within late endosomal membranes (107, 109).

In addition to the fluid glycerophospholipid-rich regions of the bilayer, the plasma membrane also contains regions rich in cholesterol and glycosphingolipids (the raft lipids), in which GPI (glycosylphosphatidylinositol) anchored proteins and double-acylated proteins partition. Lipid rafts are believed to act as cell surface

Table 1. Endoproteases found within the endosomal system.

Name	Catalytic Group	Operating pH	Tissue Distribution	Reference(s)
Carboxypeptidase B-like	ND	4.5 - 6	Hepatocytes, Fibroblasts	Renfrew *et al.* ,1991 Lipperheide *et al.*, 1986
Cathepsin B	Cys	5.0 - 6.0	Ubiquitous	Felbor *et al.*, 2002 Guagliardi *et al.*, 1990 Blum *et al.*, 1991
Cathepsin C	Cys	5.0 - 7	Ubiquitous	Benenson *et al.*, 1971
Cathepsin D	Asp	2.8 - 7	Ubiquitous	Diment *et al.*, 1985 Blum *et al.*, 1991
Cathepsin E	Asp	3.0 - 3.5	Restricted	Bohley *et al.*, 1992, Ikuzawa *et al.*, 2003
Cathepsin F	Asp	4.5 - 6.0	Macrophages	Shi *et al.*, 2000
Cathepsin G	Ser	7.5	Neutrophils	Bohley *et al.*,1992
Cathepsin H	Cys	6.8	Ubiquitous	Claus *et al.*,1998 Haraguchi *et al.*, 2003
Cathepsin K	Cys	6.0 - 6.5	Osteoclasts	Bromme *et al.*, 2004
Cathepsin L	Cys	4.5 - 6.0	Ubiquitous	Zwad *et al.*, 2002
Cathepsin N	Cys	3.5	Ubiquitous	Kirschke *et al.*,1994 Cuozzo *et al.*, 1998
Cathepsin O	Cys	6	Ubiquitous	Shi *et al.*, 1995
Cathepsin S	Cys	6.0 -7.5	Macrophages/ Monocytes, Dendritic cells	Beers *et al.*, 2005 Cuozzo *et al.*, 1998 Lautwein *et al.*, 2004
Cathepsin T	Cys	6.9	Restricted	Bohley *et al.*,1992
Cathepsin V			Thymus, testis	Tolosa *et al.*, 2003
Cathepsin W	Cys	6	CD8+ T cells and NK cells	Ondr *et al.*, 2004
Cathepsin X	Cys	5 - 5.5	Ubiquitous	Klemenei *et al.*, 2000
Cathepsin Z	Cys	5.5	Ubiquitous	Santamaria *et al.*, 1998
Endosomal acidic insulinase (EAI)	Cys, Metallo	5.5	Restricted	Authier *et al.*,1994
Legumain	Cys,	4 - 6	Ubiquitous	Kirschke *et al.*,1994
Trypsin-like	ND	ND	Hepatocytes, Fibroblasts	Renfrew *et al.*,1991

ND – Not detected

platforms involved in non-clathrin mediated internalization and play important role in protein sorting along the endocytic pathway (93, 127). Furthermore, phospholipids like PtdIns (phosphatidylinositol) in their phosphorylated states (phosphoinositides) can bind to specific protein domains like Fab 1, YOT B, Vac 1, EEA 1 (FYVE) domain, the PX (phox-homology) domain, the PH (pleckstrin-homology) domain and the ENTH (epsin-N-terminal-homology) domain.

In doing so, they target these phosphoinositide-binding proteins to specific endocytic organelles like early endosomes, multivesicular bodies and late endosomes. Hence, phosphoinositides play an essential role in both phagocytic and pinocytic processes (117).

1.2.2 Mechanism of endocytosis

1.2.2.1 Types of endocytosis and endocytic vesicles

The unique nature of cellular uptake and processing of extracellular materials that range from inorganic molecules to large microbes has historically generated considerable interest in endocytosis. The plasma membrane of eukaryotic cells maintains a tightly regulated barrier that allows very limited access to cytoplasm from the extracellular environment. Accordingly, cells have evolved complex set of strategies to internalize particles and solutes. Such strategies are broadly viewed in two functional categories: *'pinocytosis'* ('pina' or cell drinking) that describes the uptake of fluid and solutes and *'phagocytosis'* ('phago' or cell eating) that describes the uptake of large particles (44).

1.2.2.1.1 Phagocytosis

Phagocytosis is the process of engulfment of large extracellular particles > 0.5 μm in diameter via vesicles called as phagosomes, generally 0.3 - 0.5 μm in diameter. The process initiates when particles bind specific 'pattern-recognition receptors' (PRRs) on the cell surface and stimulates engulfment by the host cell plasma membrane. For internalization, distal regions of plasma membrane surrounding the target particle

eventually meet and fuse, by a complex actin-dependent mechanism that is usually independent of clathrin (3, 207). After internalization actin is shed from the phagosome and the phagosome matures by a series of fusion and fission events with components of the endocytic pathway. This process transforms the early phagosome into an acidic, protease-rich phagolysosome. It has been hypothesized that maturation pathway of early phagosomes mimic the mechanism proposed for endosome maturation and acquire hydrolytic enzymes, endosomal protein, membrane and functions similar to endosomes and lysosomes. Since endosome-lysosome trafficking occurs primarily in association with microtubules, phagosome maturation requires coordinated interaction of actin and tubulin based cytoskeletons. However, several components distinguish phagosomes from endosomes which include: membrane induced respiratory burst and storage of antimicrobial peptides like defensins. Phagocytosis is prominent in specialized cells of the immune system including leukocytes (particularly neutrophils), macrophages, and dendritic cells that can neutralize whole bacteria or related antigens. The process therefore plays a major role in host defense, antigen presentation, secondary immunological responses and signal transduction (131). Phagocytosis is also exploited by a number of intracellular pathogens which thrives within phagosomes and infects a large proportion of the world's population (57). Besides mammalian cells, phagocytosis also plays major role(s) in nutrition, tissue remodelling and apoptotic clearance in *Dictyostelium discoideum,* nematodes and insect haemocytes (58).

1.2.2.1.2 Pinocytosis

In comparison, pinocytosis or fluid-phase uptake is prevalent in all eukaryotic cells, and occurs in all cells by at least four basic mechanisms: macropinocytosis, CME (clathrin-mediated endocytosis), caveolae-mediated endocytosis as well as clathrin- and caveolae-independent endocytosis. Adsorptive pinocytosis (nonspecific binding of solutes to the cell membrane) offers greater endocytic efficiency while receptor mediated endocytosis (uptake of dilute solutes by specific high-affinity receptors concentrated into specialized endocytic transport vesicles) confers most efficient uptake. The degree of internalization of fluid-phase markers is directly proportional to

their concentration in the medium and the volume encased by the transport vesicles. Pinocytosis can be measured by the intracellular accumulation of tracer molecules (for example, an enzyme or labelled compound) present in the medium (44).

(i) Macropinocytosis: Macropinocytosis is actin-dependent, clathrin-independent endocytic process that results in the non selective internalization of extracellular solutes and nutrients by the formation of fluid-filled macropinosomes. The resulting macropinosomes are non-clathrin coated, irregular, large endocytic vesicles (0.5 - 5.0 μm). They are formed when plasma membrane ruffles fuse together. Ruffling alone is not sufficient for macropinocytosis as other signaling factors including F-actin, integrins, AP 1 adaptor complex, phosphatidylinositol 3-kinase and the Rho and Rab family GTPase, Rah/Rab 34 have also been implicated in the formation of macropinosomes. The macropinosomes finally migrate towards the cell centre and are modified by fission and fusion reactions with the endo-lysosomal pathway that eventually transform these vacuoles into a lysosomal environment. However, little is known about the molecular mechanisms that regulate the formation or maturation of macropinosomes. It is interesting to know that, despite the differences in the regulation of macropinocytosis and phagocytosis, the morphology, protein composition and dynamics of phagocytosis as well as macropinocytosis are similar indicating that they share common structural properties and contractile mechanisms (44, 208). Macropinocytosis occurs in amoeba and mammalian cells such as macrophages, dendritic cells, neutrophils, fibroblasts and epithelial cells. The process is an essential aspect of normal cell function contributing to both growth and motility of cells (44). Macropinocytosis can be constitutive as in dendritic cells. Immature dendritic cells use this process to continually sample the extracellular medium capturing exogenous antigens and storing lysosomally derived peptides in complex with MHC (major histocompatibility complex) class II molecules. Activation of immature dendritic cells leads to a signalling switch that shuts off macropinocytosis and triggers delivery of antigen-MHC II complexes to the cell surface where they are presented to T-cells (131). Macropinocytosis is also the main route for fluid-phase uptake in *Dictyostelium discoideum* (58). The process can also be induced in different

cells by PMA (Phorbol Myristate Acetate), growth factors and modified LDLs (low density lipoproteins) (98). Furthermore, naked plasmid DNA enters human keratinocytes mainly by macropinocytosis (208). Since the ruffling that leads to macropinocytosis is regulated, it has also been exploited by some pathogenic bacteria like *Salmonella, Shigella, Neisseria* and viruses like HIV as a novel route for entry into cells (91, 183, 232).

(ii) Clathrin Mediated Endocytosis: Clathrin-mediated endocytosis (CME) is one of the major mechanisms employed by cells for the import of cargo from the extracellular environment. The clathrin system performs both absorptive and receptor mediated internalization, primarily by formation of endocytic CCV's (clathrin-coated vesicles). These vesicles develop through the recruitment of soluble clathrin from the cytoplasm to the plasma membrane by protein-protein and protein-lipid interactions. Phosphorylation and dephosphorylation are emerging as key regulators of these interactions. Internalization involves three steps, each distinguishable by morphologically and mechanistically distinct phases. First assembly of clathrin into a polygonal lattice is followed by formation of coated pits. The latter and related coat proteins polymerize around the cytosolic face of the budding vesicle, which helps the vesicle to bud off from the membrane. This is followed by recruitment of GTP-binding proteins (Rabs) and related effector proteins to the surface of the coated membrane which regulates the rate of vesicle formation. Finally invagination of coated pits leads to fragmentation of the membrane to release the coated vesicles (117).

Current views suggest that the formation of coated pits appears to start at specific assembly sites on the plasma membrane called 'coated pit zones'. Coated pits cover some 0.5 - 2 % of the cell surface. Their formation occurs through a two-step process in which endocytic adaptors first specify the location of clathrin assembly on the plasma membrane and then recruit and promote clathrin polymerization. The 2 hinge of AP 2 can interact with the N- terminal region of clathrin heavy chain and promote lattice assembly. The clathrin assembly functions as a stabilizing coat for the components of the sorting machinery that concentrates plasma membrane proteins

into the clathrin-coated pit. In addition, phospholipids such as phosphatidylinositol-4,5-bisphosphate found in coated pits bind to clathrin adaptors like epsins and facilitate vesicle formation and budding. After its formation, the clathrin-coated pit invaginates and then pinches off to form a CCV (117, 125). Numerous proteins including amphiphysin, synaptojanin, endophilin, epsin and dynamin are involved in this process (27, 125, 172). In particular, dynamin is thought to be involved in the pinching-off process (211) but whether it is acting as a mechanoenzyme (203, 210) or a signaling molecule (188) is still not clear. After pinching off, a CCV is uncoated rapidly in a process that requires the ATP-binding molecular chaperone of Hsc 70 and the J- domain protein auxilin. Hsc 70 and auxillin are not only required for uncoating but are also involved in returning clathrin and APs to the plasma membrane to form new clathrin-coated pits. The uncoating process may also involve hydrolysis of phosphatidylinositol 4,5-biphosphate by synaptojanin. After loosing their coats the vesicles become unstable and in turn fuse with other endocytic vesicles to form the early endosome (Rab5-GTP is instrumental in this homotypic fusion of the endocytic vesicles to form endosomes). The fusion events also use tethering and docking proteins called EEA 1 and Rabaptin 5 which help align the vesicles and bring them close enough together to effect the fusion. A pair of SNAREs (v-snares and t-snares) also function in this process. The SNARE (SNAP receptor; where SNAP is 'soluble*N*-ethylmaleimide sensitive factor attachment protein') help vesicles to tether to or dock with the target membrane before finally fusing with it. CCVs mediate endocytic transport from the cell's limiting membrane to the lysosomes and between the TGN (*trans*-Golgi network) and the endosome. Most protein internalization from the cell's limiting membrane occurs by the process of clathrin-mediated endocytosis. Protein and lipid which have been packaged into CCVs are internalized and delivered to the early endosome, the first part of the endosomal system. From the early endosome (pH 6.5), the cargo can follow various pathways. It may recycle directly to the cell surface for reuse. This may occur either immediately (as is the case for nutrient receptors) or (as in the case of synaptic vesicle fusion proteins) after waiting in a 'pool' until the proper stimulus is received. Cargo may also move on to the Golgi apparatus (for example, Golgi marker proteins such as TGN 38) or continue through

the endocytic pathway to the 'late' endosome (pH 5) and ultimately to the lysosome (pH 4) where it may end up being degraded by hydrolytic enzymes as is the case for activated growth factor receptors. Endocytic transport of cargo is accompanied by a sequential drop in the pH of the corresponding endosome due to the active pumping of protons into the vesicle lumen.

The protein cargo carried in endocytic vesicles include signaling receptors such as peptide growth factor, chemokine receptors, nutrient receptors, gated channels, pores and pumps, markers of membrane identity and transmembrane proteins involved in cell-cell and cell-substratum contacts. CME therefore plays a key role in cell signalling, division and differentiation, homoeostasis as well as the interaction of cell with its surroundings (44, 143).

(iii) Caveolae-mediated endocytosis: Caveolae are 50 - 100 nm dynamic omega or flask-shaped invaginations of the plasma membrane. They cycle between an 'open' state in which they are accessible to the extracellular media and a 'closed' state or vesicular conformation when they can be pinched off from the plasma membrane. Caveolae can be easily distinguished from clathrin-coated pits by their size and the lack of the basket-like clathrin coat. The shape and structural organization of caveolae are conferred by cholesterol, sphingolipids and caveolin, a dimeric protein that binds cholesterol. Of the three isoforms (caveolins 1, caveolins 2 and caveolins 3) of caveolin, caveolin/VIP 21 (an integral membrane protein) has been found to be the major component of the caveolar coat (44). Furthermore, important molecules like GPI-linked receptors and proteins, inositol 1,4,5-triphosphate receptor, lipid second messenger molecules like sphingomyelin and gangliosides, G protein-coupled membrane receptors, multiple heterotrimeric GTP-binding proteins, non-receptor tyrosine kinases and ATP-dependent Ca^{2+} pumps have also been identified in caveolae (117, 157). Caveolae mediated endocytosis are highly regulated and like phagocytosis and macropinocytosis are driven by the cargo molecules themselves. Caveolin inserts as loops into the inner leaflet of the plasma membrane and self-associates to form a striated caveolin coat on the surface of the membrane invaginations. Tyrosine-phosphorylation of caveolae constituents and increased

protein kinase C activity results in internalization and pinching off of caveolae and forces them to take part in fluid phase and receptor mediated endocytosis (117, 157). Caveolae seem to be present in most cell types. Apart from the endothelial cells in which they were first observed, caveolae are abundantly present in adipocytes, simple squamous epithelial cells, smooth muscle cells, fibroblasts, astroglial cells, macrophages, neurons and leukocytes. They are apparently absent in lymphocytes (103).

The exact function of caveolae in various cell types is still unclear. They mediate intracellular cholesterol trafficking, homeostasis and the transcellular shuttling of serum proteins from the bloodstream into tissues across the endothelial cell layer. Although not exclusively endocytic vesicles, under special circumstances such as elicitation whereby the rate of endocytosis is greatly increased, caveolae can function as alternative carriers. Importantly, caveolae and caveolins have been shown to play prominent roles in various human patho-biological conditions like atherosclerosis, cardiac hypertrophy, cardiomyopathy, pulmonary hypertension, cancer, diabetes, bladder dysfunction, and muscular dystrophy (103, 230).

(iv) Clathrin and Caveolin- independent endocytosis: The mechanisms of clathrin and caveolin independent endocytosis are poorly understood. The processes starting from cargo uptake, vesicle formation to intracellular destination are different in different cells. The processes are dynamin independent. Hence, these unique pathways might have evolved to facilitate pinocytic uptake in more complex aspects of cell physiology like signal transduction, development and modulation of cellular behavior in response to extracellular stimuli. They occur in neurons, neuroendocrine cells, HeLa cells etc (44, 117).

1.2.3 Models for Endocytosis

Current debate about the mechanisms by which endocytosed materials are transported through the endocytic pathways centers around two models primarily suggested by Helenius *et al.* (92) and corroborated by others (76, 139).

1.2.3.1 Vesicle Shuttle Model

This model describes early endosomes, late endosomes and lysosomes as stable, functionally and morphologically distinct organelles. Internalized components are processed through each type of endosome sequentially. Thus, newly formed endocytic vesicles fuse with and deliver their contents into the early endosomes. Next, vesicles budding from the early endosomes recycle to the cell surface or deliver part of that compartments content, into the late endosomes. Movement from late endosomes to the lysosomes occurs in a similar fashion. The vesicular shuttle mechanism in this model resembles the formation of early endosomes from the internalized plasma membrane (76).

1.2.3.2 The Maturation Model

This model describes endosome biogenesis as *de novo* process that involves formation of endosomes at the periphery of the cytoplasm by the fusion of incoming primary endocytic vesicles (freshly formed by the invaginations in the plasma membrane). The gradual maturation of early endosomes through condensations, reciprocal fusions and micro-invaginations into their membranes lead to multi-vesicular organelles that collect various ligands and move into the perinuclear area. Finally mature endosomes fuse with primary or secondary lysosomes. This completes the degradation of endosomal contents. Maturation model thereby defines individual endocytic compartment(s) as transient structures that mature and fuse with lysosomes (139).

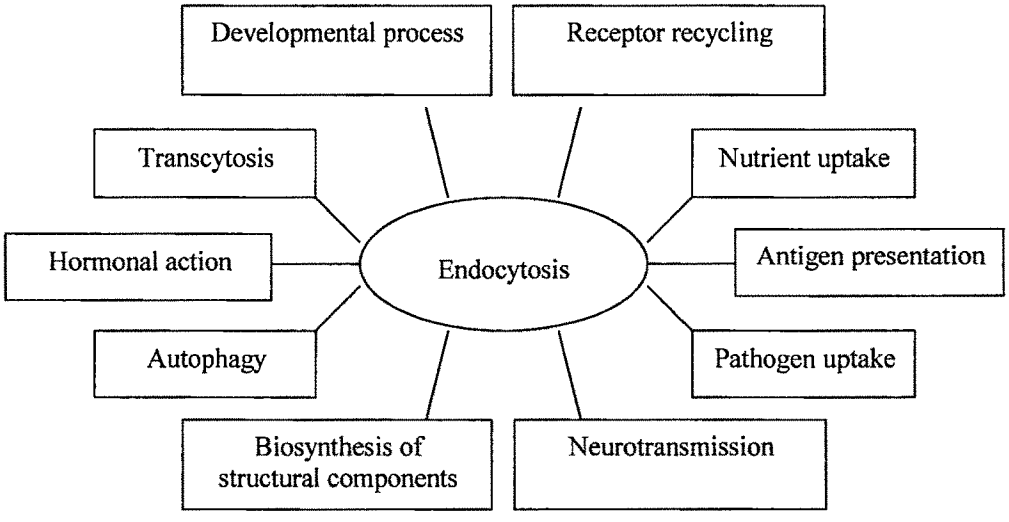
Although the two models seem distinct, they have in common the interaction between stable and transient populations of vesicles. In the vesicle shuttle model, the transport vesicles are transient and the compartments are stable. In the maturation model, the compartment identity is transient, but the vesicles they interact with and that modify them can be considered stable. Viewed in this way, intermediate models become conceivable.

1.3 Significance of endocytosis in cell biology

1.3.1 Functions of endocytic vesicles

Significant research efforts have unveiled the critical importance of endocytosis in cellular biology. The mechanistically diverse and highly regulated endocytic pathways function to control complex physiological processes like nutrient uptake, pathogen uptake, transcytosis, receptor recycling, hormonal action, autophagy, development, immune response, neurotransmission, intercellular communication, signal transduction, cellular and organismal homeostasis and antigen presentation (30, 57, 81, 106, 131, 133, 136, 161). Within the cells, endosomes act as a sorting station for a multiple of receptor ligands. This has particular significance for a variety of receptors which discharge their ligands at low pH and return back to the plasma membrane without entering the perilious environment of the acidic lysosomes (30).

In addition to endosomal (partial) proteolysis of lysosomal enzymes, prelysosomal hydrolysis of other types of substrates is also reported. Early endosomes have been reported to function in insulin degradation. Proteases attenuate the signal of this hormone and this may terminate the hormone signal. Endosome proteolysis has also been demonstrated for parathyroid hormone and epidermal growth factor (7). Besides, an important physiological function of endosomal/prelysosomal proteolysis is processing of antigens that may bind to MHC II in the late endosomal compartments (18, 65).



1.3.2 Significance in pathophysiology

1.3.2.1 Microbial pathogenesis

The mammalian endocytic apparatus presents a highly selective mechanism of internalization that allows restricted entry of extracellular components. Extracellular materials confined in highly structured vesicle(s) are sorted and/or undergo multi-enzyme mediated processing along the endocytic pathway that efficiently neutralizes potential threat offered by internalized microbes and related agents. However, certain pathogens have evolved strategies to utilize endocytic machinery to gain entry into host cells and thereby thrive within the vesicular niche offered by endosomes. For example, *Leishmania donovani* is a protozoa; the amastigote form of this pathogen is the etiological agent of visceral leishmaniasis. *Leishmania* is an obligate intracellular parasite residing in membrane-bound compartments of macrophages called PV (parasitophorous vacuoles) where they evade the microbicidal atmosphere. Infectious *Leishmania* promastigote initially prevents phagosome-lysosome fusion (47). However, concomitant with their transformation into the replicative amastigote stage, the phagosomes merge with lysosomes, where the protozoa replicate ultimately killing the host macrophage (47). Other endocytosed pathogens use various strategies to ensure survival within the intravacuolar environment (57). For example, *Mycobacterium tuberculosis*, *Legionella pneumophilia*, and *Toxoplasma gondii* survive and proliferate in the vacuolar compartments that do not mature into phagolysosomes (209) whereas *Trypanosoma cruzi*, *Shigella flexneri* or *Listeria monocytogenes* lyse the phagosomal membranes to reside in the cytoplasm (4, 57, 140).

Among the best characterized system of endocytosis mediated pathogensis is *Salmonella* sp that induces typhoid fever and gastroenteritis in mammalian hosts. *Salmonella* pathogenesis utilizes multiple strategies that include introduction of virulence factors into the host cell through a type III secretion system. The factors promote membrane ruffling and reorganization of the host actin-cytoskeleton that allows internalization of the pathogen in macrophages and epithelial cells (57). Once

inside the early phagosome, *Salmonella* selectively recruits Rab 5-GTP complex and promotes fusion with the early endosomal compartments. *Salmonella* containing early endosomes retain Rab 5 and Rab 18 and do not acquire late endosome characteristics (Rab 7 and Rab 9). This is hypothesized to allow the pathogen to avoid transport to lysosomes through late endosomes. Besides, *Salmonella* selectively deplete the vacuolar H^+-ATPases from the membrane of the host phagosome/early endosome to prevent endosomal acidification (88).

1.3.2.2 Genetically pre-disposed disorders

A group of approximately 40 different diseases, often loosely called as mammalian lysosomal storage diseases are attributed to genetic abnormalities manifested by malfunctions in the endosome/lysosome biology in the host (222). These diseases result from *(i) deficiencies of endosomal/lysosomal enzymes*, for example Tay-Sachs disease which is caused by a defect in hexosaminidase A (145), *(ii) lack of signal sequences or modifications* required for the delivery of enzymes to the lysosomes (I-cell disease), *(iii) lack of or defective protein(s) factors* required for endosome/lysosome biogenesis/function which is represented by Chediak-Higashi syndrome (192).

At least in one case, endosome lipid constituents have been directly implicated in genetically predisposed pathophysiology. Kobayashi *et al.* determined that LBPA (lysobisphosphatidic acid), a major acidic phospholipid of late endosome is the target for human antiphospholipid syndrome - a genetically predisposed autoimmune disorder (108). Similarly in Niemann-Pick C disease, Kobayashi *et al.* (108) showed that cholesterol accumulation is principally in late endosomes rather than lysosomes. Recent studies by Williams *et al.* have uncovered the roles of caveolae and caveolins in the pathogenesis of many human diseases like atherosclerosis, cardiac hypertrophy, cardiomyopathy, pulmonary hypertension, cancer, diabetes, muscular dystrophy etc (230).

1.3.3 Role of endocytosis in drug delivery

In the past few years, the endocytic processes like receptor mediated uptake has been considerably exploited to design efficient site specific and target oriented drug delivery systems. Small radiolabelled peptides containing endosomal escape signals target drugs in cancer, cardiology, neurology, inflammation/infection, atherosclerosis and thrombosis. Of particular importance are the Syn B vectors, which deliver hydrophilic molecules across the blood-brain barriers (202). Significantly, ligand mediated active targeting has emerged as a novel paradigm to target DNA to the desired cells. DNA has been complexed with receptor-ligands through covalently linked polycationic adjuncts and efficiently targeted through the endocytic pathway (82). One of the most preferred receptor systems is the 'transferrin system'. The unique internalization and recycling functions of transferrin receptor have made it an attractive choice for drug targeting and delivery of large protein-based therapeutics, toxins, anti cancer drugs, CNS drugs and therapeutic genes into proliferating malignant cells that overexpress the transferrin receptors (119). Similar to the transferrin receptors, folate receptors too are upregulated in many human cancers, including malignancies of the ovary, brain, kidney, breast, myeloid cells and lungs. Folate receptor density appears to increase as the stage/grade of the cancer worsens. Thus, cancers that are most difficult to treat by classical methods have been easily targeted with folate-linked therapeutics. Some of the important macromolecules targeted by folate receptors are protein toxins, low-molecular-weight chemotherapeutic agents, radioimaging agents, radiotherapeutic agents, liposomes with entrapped drugs, genes, etc (168). Furthermore, in human leukemic cells, antineoplastic lipophilic drugs like aminoglycosides, anionic liposomes (coupled to LDL) have been targeted via LDL receptors (38). Apart from the above mentioned, other ligand-receptor systems that have been investigated to date are blood carbohydrate (lectin) receptors, Fc receptors, complement receptors, interleukin receptors, insulin receptors, scavenger receptors, receptors/epitopes expressed on tumor cells and cell adhesion receptors (225). Recently the caveolar/lipid raft proteins are being targeted for efficient delivery of the respective ligand-bound drugs to non lysosomal compartments (12).

1.4 Tools for analysis of endocytosis

The unique structure function relationships of endocytic vesicles have necessitated the development of unique set of techniques for purification and characterization of endocytic vesicles. Some of the key tools in this context are discussed here.

1.4.1 Tools for endosome isolation/purification

Three basic techniques using independent principles have been used for endosome isolation.

1.4.1.1 Centrifugation methods

For years, density gradient centrifugation (using percoll, sucrose or iodinated density gradient materials) was the sole method employed for the isolation of endosomes/lysosomes and related organelles (84). In 1955, de Duve introduced a relatively faster technique-'differential centrifugation' and the latter soon became the method of choice (50). Later in 1969, Bohley *et al.* used hypotonic lysis in combination with differential centrifugation to isolate lysosomes (23). This permitted the isolation of huge quantities of endosomes and lysosomes or of any other particular subcellular fraction. However such traditional techniques separated the organelles on the basis of size, shape and density differences. Subcellular organelles like endosomes, lysosomes, golgi, mitochondria and other plasma membrane derived vesicles differ little in respect to these parameters. Hence, the purity of the preparations dependent on centrifugation methods was questionable. In alternate approaches, the density of endosomes/lysosomes was experimentally decreased or increased by feeding Triton-WR-1339, iron-sorbitol citric acid complex, colloidal gold to separate them from other organelles (143, 196). This afforded better separation but the isolated organelles were modified. Modified approaches like immunoaffinity based partition have also been tried to isolate endosomes (5, 218).

1.4.1.2 Free flow electrophoresis

Henning *et al.* and Harms *et al.* introduced FFE (preparative free-flow electrophoresis) to purify lysosomes (87, 94). The latter is a charge based separation procedure that exploits the difference in electrophoretic mobility between endosomes and other subcellular structures. FFE has also been used for separation of early and late endosomes (126). Though FFE offers a rapid, partially automated technique for endosome purification, reports have indicated that purified fractions from FFE were often contaminated with mitochondria and plasma membrane fragments. In an effort to overcome this, FFE was used on samples partially purified by density (percoll) gradient centrifugation: however the inherent limitations of both techniques have resulted in only limited success (121, 179). Morre *et al.* have reported a two step partition technique involving phase partitioning followed by FFE that yielded relatively pure endosomal fractions (137).

1.4.1.3 Magnetic affinity chromatography

An innovative technique called 'electromagnetic affinity chromatography' was used by Rodgriguez-Paris *et al.* to isolate high quality lysosomes from *Dictyostelium discoideum* (174). The purification system exploits the unique properties of endocytic vesicles that allow them to internalize ferromagnetic particles like iron-dextran. Subsequently, the induced ferromagnetic properties of such vesicles help them to selectively bind to the electro-magnetic matrix, usually prepared by a high-grade steel wire mesh within a chromatography column system surrounded by a strong electromagnetic field. Samples are applied under strong magnetic field, unbound material is washed and iron-laden vesicles are eluted by removing the magnetic field. The process offers a relatively quick, single step isolation of high quality endocytic vesicles. Furthermore, it allows some degree of selectivity in purification of specific classes of endocytic vesicles by controlling the time of uptake (pulse). Other than *Dictyostelium discoideum* (150, 151), magnetic purification has been successfully reported in *Entamobia histolytica* (213) and *Xenopus* oocytes (13, 171). In at least two cases, magnetic purification of endosomes have been reported in mammalian cell lines (15, 160).

1.4.2 Tools for endosome characterization to study endocytic pathways

Within the vast gamut of tools to study cellular and molecular biology of subcellular organelles, some key methodologies have found significant applications to study and characterize endocytic processes.

1.4.2.1 Kinetic studies

One of the key functions of endocytic vesicles being ligand uptake, a major part of endosome characterisation has focused on the kinetics of ligand uptake and determinants that control the latter. While radioactively labelled ligands and fluorescent tagged ligands have found use in studying kinetics of endocytosis and exocytosis, various tagged enzyme substrates have been used to study both uptake and subsequent processing kinetics. The studies have allowed characterisation of determinants like temperature, ionic strength and other physical parameters on endocytic functions and served as a general tool to analyse endocytic function in health and disease (217). Further, the use of specific size restricted probes, have allowed characterization of phagocytosis, macropinocytosis, clathrin dependent and independent endocytosis (19).

1.4.2.2 *In vitro* assays

In vitro reconstitution in cell free systems has proven to be a powerful tool for identifying the proteins/factors that mediate the complex processes of transport and sorting, vesicle fusion and its regulation along endocytic pathway. These assays have helped to study the mechanisms of virulence of specific pathogens that use evolutionary conserved strategies to overcome the harsh endocytic milieu and replicate within endosomes. *In vitro* fusion assays have been extensively used to study phagosome-endosome and endosome-endosome fusion mechanisms in different mammalian cell lines. Such processes require salt, energy, cytosol, and membrane-associated factors (128, 193). *In vitro* assays have also characterized the major endosomal enzymatic machinery ie the vacuolar H^+-ATPases, hydrolases and GTPases (40, 160) and several other proteins mediating several steps of endocytic process.

1.4.2.3 Immunological tools

The endocytic pathways of different cell systems have been extensively characterized by pulse feeding of fluorescent ligands, or immunostaining for the respective proteins Furthermore, Western blots, immunoassays and immunoprecipitation techniques have also extensively characterized endosomes and related organelles. Recently, 1D and 2D protein electrophoresis techniques along with MALDI have thrown substantial light on endosomal proteomics (40, 117).

1.4.2.4 Genetic tools and mutant analysis

Considerable information on endosome function and biogenesis have been derived from studying mutant cell lines that exhibit altered endocytic functions owing to mutation of specific endosome related components. Transfection of cultured cells with genes encoding mutant endosome proteins have played a major role in elucidating the roles of the major components like the Rab group of endosomal GTPase, SNARE proteins as well as several other proteins (173, 193). The role of dynamin was revealed by studying dominant negative dynK 44A mutant cell lines (51). Very recently, Paleotti *et al.* used GTP locked mutants to demonstrate the major role of the GTP-binding protein Arf 6 (ADP-ribosylation factor 6) in clathrin-mediated endocytosis (156). In a significant finding, Kobiyashi *et al.* showed mutation defects in the endosomal lipid LBPA results in antiphospholipid syndrome (109).

1.4.2.5 Microscopic tools

Microscopic analysis has played a significant role in describing endocytic vesicles and their functions. The unique advantage of such studies is that it allows analysis of both purified vesicles and endosomal organelles in intact cells. Such analysis has used transmission electron microscopy (TEM) as well as epifluorescence or confocal microscopy. All systems have used the unique capacity of endocytic vesicles that allow incorporation of specific ligands like colloidal gold or iron (for TEM analysis)

or fluorescent probes like FITC-dextran or immunological tools that are visualised by epifluorescence or confocal microscopy (128, 130, 167).

Considering the importance of endocytosis in both normal physiological and pathological conditions in eukaryotic cells it becomes imperative to isolate, study and characterize endocytic vesicles.

1.5 Project aims

Hence, the present investigation aims to isolate mammalian pinocytic vesicles by 'electromagnetic affinity chromatography'.

1.5.1 Objectives

Analysis of rat (*Rattus novergicus*) macrophages as a system to study mammalian endosomes, particularly those involved in pinocytosis.

Establishment of a novel (electromagnetic column chromatography) purification technique to isolate (peritoneal macrophage) endosomes of high purity.

Identification of specific endosomal populations of different maturation states and study their functions by immunodetection techniques using specific antibodies against known endosomal markers by Western blotting.

Characterization of sequential endosomal populations for lipids, proteins and acid proteases like cathepsins.