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Study progress of cell endocytosis*

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Abstract Endocytosis is a process through which extracellular materials are transported into cell through membrane deformation. This process is not a simple step-by-step process in which a series of proteins function according to the chronological order, but rather a complex process comprising many members which are regulated precisely. The role of endocytosis is broadly divided into two categories, phagocytosis and pinocytosis, the latter is divided into four species in accordance with the size of endocytosis substances: clathrin dependent endocytosis, the diameter of clathrin-coated vesicle is 100–150 nm; caveolin dependent endocytosis, the diameter of caveolin protein-coated vesicle is 50–100 nm; macropinocytosis, the diameter of macropinocytosis is generally 0.5–2 μm, sometimes up to 5 μm; clathrin and caveolin independent endocytosis. Many proteins including endophilin A1, A2, A3, and endocytotic proteins B, B1a, and B1b as well as dynamin, actin and Rab protein families are involved in endocytosis and play an important role in different stages. The abnormal endocytosis may be involved in the development of certain diseases.

Key words endocytosis; clathrin; caveolin; endophilin; signalling pathway

In the process of cell metabolism, various kinds of materials including some ions, small molecules, macromolecular materials and some granular matters keep in and out of cells. The macromolecular substances and granular materials can not go through the membrane. They complete the transfer across the cell membrane in another way, that is, materials cross in and out of cell membrane in the form of vesicle which is fused with membrane and then sciss into the cell, which is currently recognized as the main pathway of intaking biological macromolecules-endocytosis. Endocytosis is broadly divided into two categories, phagocytosis and pinocytosis; the latter is divided into four species according to their different mechanisms: clathrin dependent endocytosis, caveolin dependent endocytosis, macropinocytosis, and clathrin and caveolin independent endocytosis.

The recent study reported that the abnormal expression of endocytosis may be involved in the mechanism of certain diseases, such as diabetes and neurological diseases and also closely related to the malignant transformation of cells. The role of endocytosis was paid increasing attention. Further research in this area will help us understand these diseases, thereby found new treatments. Here we introduce about pathway of cell endocytosis, mechanisms of regulation and endocytotic proteins.

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Endocytosis pathway

Phagocytosis

Phagocytosis refers to endocytosing large granular matters (> 250 nm), and it provides the host a direct way digesting exogenous substances, which is one of the most important immune protecting mechanisms [1]. In mammalian, phagocytosis can only be completed by specific cells, such as macrophages and neutrophils which are called phagocytic cells. Phagocytic cells recognize and combine with IgFc and complement packing pathogens through IgFc receptor and complement receptor respectively. When the cell surface receptors combined with the corresponding ligands, downstream signal transduction was activated, which caused actin polymerization under plasma membrane of intaking site, actin contraction makes phagocytic cell membrane form pseudo-foot fusing into vesicle to pack pathogens. In cytoplasm, dynamin assembled into ring at the neck of vesicle, and hydrolysed the binding GTP, dynamin contraction forced vesicle to sciss from membrane at the neck and form phagosome which integrated with the lysosome, the acid hydrolysis enzyme of lysosomal digested pathogens. In addition to phagocytosing pathogens, macrophages of phagocytic cells can

swallow foreign bodies through ligand-receptor binding pattern. Identify and kill tumor cells, identify and remove degenerative plasma protein, lipids, and other macromolecules. Remove aging and damaged cells and cell debris.

Pinocytosis

Pinocytosis refers to the intake process that endocytoses extracellular liquid and the dissolving materials.

Clathrin dependent endocytosis

Clathrin plays an important role in the regulation of composition of plasma membrane proteins, and research on clathrin can help us understand how cells interact with the surrounding environment, signal transduction of mitogenic, nutrition intake of the cell, establishment of extracellular environment cell identity including the interaction with the immune system, keep a balance in the stability of the environment of cell.

(1) Clathrin and adaptor protein (AP)

Clathrin is the skeleton protein outside the vesicle, clathrin-coated vesicle is 100–150 nm in diameter and exists in all eukaryotic cells and it mediates the way of transportation from the plasma membrane to intracellular of proteins, lipids, nutrients, antibodies and growth factors and also the vector through which proteins and lipids transport from trans Golgi net work (TGN) to endosome. Clathrin is spider-like and polymerized by three chains at the top, which is known as triskelion [1]. Adapter lies inside the clathrin-coated vesicle, which mediates membrane binding, localization, sorting signals, identification and inositol phosphate. It not only serves to combine cargo and clathrin, but also connect with polyphosphatidylinositol headgroup. It is now found four kinds of adapters (AP1–4), all of which comprise a pair of 100–130 kD subunit. These subunits are able to identify 6-phosphate mannose receptor, transferrin, low-density lipoprotein and epidermal growth factor receptor, protease and de-sialic acid receptor.

(2) Mechanism

The process from recruitment to disassemble is very short. It comprises as follows:

1) Recruitment of adapter and clathrin. Recruitment of AP2 complex to high activity, saturated, easy enzymolysis site, activate formation of clathrin-coated vesicle in the plasma membrane under the effect of sorting signal and docking protein [2].

2) Invagination, scission and budding of clathrin-coated vesicle. The planar clathrin protein can be transformed into curved one without nucleic acid and cytoplasm *in vitro*; *In vivo*, clathrin-coated vesicle curves to some degree. Invagination may be due to structure changes or rearrangement of clathrin assembling in the crystal lattice or between clathrin. The budding of clathrin-coated vesicle is involved with GTPase dynamin, actin tubulizes or forms ring-shape *in vitro*, it is the trigger that vesicle dissociated from the membrane.

3) Decapsulation of clathrin-coated vesicle. This is a wasting process that needs hsc 70, auxilin and ATP. The large chain of clathrin has two sites which interact with the adapter and also with hsc 70. The interaction between clathrin and hsc 70 destroyed the interaction between clathrin and adapter. Over-expression of hsc 70 mutant blocked the cycle of transferrin receptor so that the "assembly – dismantle" balance moved to the assembly direction. Hsc 70 dissociated clathrin from the vesicle *in vitro*, but could not dissociate adapter. Auxilin plays an important role in decapsulation. Auxilin can not only recruit hsc 70 to clathrin-coated vesicle, but also stimulate activity of hsc 70 ATP enzyme.

Caveolin dependent endocytosis

Caveolae/caveolins mediate endocytosis of many substances and is the main form of clathrin-independent endocytosis.

(1) As early as 1950, Japanese scholar Yamada used transmission electron microscopy to observe some small caveolae which was about 50–100 nm in diameter for the first time. These vesicles appeared alone or string-like, in the form of invagination of the cytoplasmic membrane, it had typical lipid bilayer structure [2]. Caveolae is currently considered to be the signal transduction center, and many signal transduction receptors, protein kinase and binding proteins are highly enriched in caveolae region. In the regulation of caveolae endocytosis, activation of tyrosine kinase-dependent signal is an important step. After the treatment of phosphatase inhibitor, the endocytosis of cell by caveolae had increased notably.

(2) Caveolin is the main surface marker of a caveolae, a kind of membrane integrin family modified in the inner surface of caveolae, 21–25 kD. It consists of N-terminal region, transmembrane region and C-terminal region, N-terminal and C-terminal cytoplasmic moves inward into the cytoplasm and its peptide chains like hairpin structure. Caveolin plays a critical role in the assembly process of caveolae and cholesterol and is the signaling molecule of the scaffold proteins and negative regulatory proteins and belongs to a highly conserved integrity membrane protein family [3]. Rajjayabun [4] confirmed that caveolin-1 was the key factor of caveolae endocytosis. If caveolin gene was knockout, caveolae can not be formed. So far, four kinds of caveolin isomers have been found in mammals: caveolin-1α, 1β, 2 and 3, which are products of different genes, most cells expressed caveolin-1 and caveolin-2, the two form a stable heterologous oligomers complexes, particularly rich in terminal differentiated cells, such as fat cells, endothelial cells and fibroblasts, and caveolin-3 mainly exists in various muscle cells (such as myocardial cells, rhabdomyosarcoma cells and skeletal muscle cells) and is closely related to the synthesis of muscle cells [5]. Neither caveolin protein nor caveolae structure appeared

in peripheral blood cells and nerve cells.

(3) The biological feature of caveolin. Caveolae as a signaling molecule plays a role as a platform in signal transduction. Caveolin-1 is in the center of signaling pathways at all these platforms. Caveolin, as a signaling molecule's scaffold protein and negative regulatory protein, inhibits kinase activities of signaling molecules in the normal signal transduction pathways. Caveolin-2's skeleton region has no inhibitory activities, and other signaling molecules inhibitory regions may exit in it. Caveolin-3 is involved in energy metabolisms in muscle cells. Caveolin-1 has strong affinity to cholesterol, thus the cholesterol levels of caveolae is far higher than other biofilm. The study showed that the absence of caveolae eventually resulted in formation of foam cells, suggesting that caveolae and caveolin-1 can maintain the cholesterol balance by removing the excessive lipoprotein cholesterol out of cells [6].

The correlation between caveolin and tumors have become one of the hot spots in tumor biology, among which, the relationship between caveolin-1 and tumor occurrence and metastasis has been studied a lot. Gene coding for caveolin-1 (CAV) locates in the suspicious tumor suppressive site (D7S522; 7q31.1). This site depletes or fractures in a variety of tumors (such as liver cancer, ovarian cancer, breast cancer, uterine fibroids, gastric adenocarcinoma, etc. [7]). The normal NIH 3T3 cell which was introduced by antisense caveolin-1 was transplanted into nude mice to observe the formation of tumor, suggesting caveolin-1 has tumor suppressive function. In many tumors and activated oncogene transfected cells, the expression of mRNA and its protein of caveolin-1 decreased or lost [8]. The experiments *in vivo* demonstrated mutation or deletion of caveolin-1 could lead to the excessive proliferation of breast epithelial cells, and then increase the occurrence of breast cancers [9]. Besides, the expression of caveolin uprehulated in diabetes and hypercholesterolemia and plays a significant role in Alzheimer's disease [10], degenerative muscle disease [11], heart and lung diseases.

Macropinocytosis

Large and irregular original endocytosis vesicles are formed by folding membrane on the verge of extending cell under stimulation by certain factors, and they are known as macropinosome whose size varies with diameter generally 0. 5–2 μm. Macropinocytosis plays a major role in macrophages and dendritic cells, also in many tumor cells [11].

Macropinosome has no clathrin or caveolins coating; it is closely related to actin at the early stages of formation, which provides an effective way for non-selective endocytosis of extracellular nutrients and liquid phase macromolecules. Macropinocytosis and phagocytosis, regulated by a variety of proteins, such as actin, Scar protein, Ap21 complexes and RabB. Different membrane wrinkle results in different rates of developing macropinocytosis.

The formation of macropinosomes can be significantly inhibited by cytochalasin D and colchicine, suggesting that microtubules and microfilaments play an important role in this process. The mechanism may be the stimulus by a variety of factors that activate corresponding receptor tyrosine kinase and then self-phosphorylate quickly. Phosphorylated residues recruit PI3 kinase and activate it, the activated PI3 kinase activates Rac1 which may cause microfilament reconstruction through two ways. Firstly, the activated Rac1 activates PAK1 which regulates phosphorylation of myosin light chain. The interaction between myosin and actin is regulated by the phosphorylated myosin light chain and the interaction promotes reconstruction of microfilaments, development of the cell membrane ruffles and formation of macropinosomes; Secondly, the activated Rac1 binds with the amino-terminal of its target protein IRSp53, SH3 region at the carboxyl end of IRSp53 combines with WAVE to form three molecular complexes which could activate WAVE [12]. The latter further activates actin related protein Arp2/3 complexes which stimulate microfilament nucleation, promote microfilament reconstruction, development of cell membrane ruffles and formation of macropinosomes [13]. In-depth regulation mechanism needs further study.

Microdomain

Microdomain has similar lipid composition with caveolae but not function through caveolin. It functions through dynamin and Rho A-dependent mechanism, or through clathrin-independent, dynamin-2-independent, Cdc4 mediated pathway to endocytose glycosylated phosphatidylinositol-hexanol anchored proteins [13]. The pathway plays a role in endocytosis of IL-2.

Endophilin

The biological features of endophilin

The main function of endophilin is related to the endocytosis of neurotransmitter. Endophilin has a common special structure, whose C-terminal has a unique SH3 domain with the unique binding ability [14], which can interact with a number of special proteins such as dynamin, thus affecting the functions of other proteins. While N-terminal participates in membrane invagination of vesicles. They were named endophilin A1, A2, A3 in 2002. Huntingtin belongs to binding protein of endophilin A1, the complex composed of it and the huntingtin binding protein 40 (HAP40) inhibited early microtubule-dependent endosome movement, increased connection between early endosome and actin [15]. Then a new family was discovered, which was named endophilin B.

Endophilin and its role in signal transduction

Endophilin can interact with various proteins through which involved in different signal transduction pathway

Endophilins which interact with cell membrane receptors are mainly distributed in the cytoplasm and cell membrane, it can interact with cytoplasmic membrane receptor and conduct signals, such as beta 1-adrenergic receptor. Endophilin is also involved in phospholipid signal transduction. Many endophilins contain the binding sites of inositol 4,5-diphosphate (PIP2). The degradation of PIP2 is necessary for the completion of the endocytosis. Mark PIP2 connected domain by immunohistochemical fluorescent protein and track endocytosis movement of living cells, and PIP2 was found present in the cell membrane surface in the form of many small plaques, these plaques entered gradually into the cell, which confirmed that the shift was the endocytosis. The disappearance of PIP2 matched the recruitment of phosphoinositide phosphatase enzyme, and we can propose that the latter can degraded the former. When PIP2 degradation was blocked, abnormal invagination can be observed, shearing machine of endocytosis gathered around the plaque on the membrane and failed to complete endocytosis. Therefore, PIP2 degradation is the necessary step of vesicle scission [16].

Recycling of the neurotransmitter

Neurotransmitter recycle is essentially a process of cell endocytosis, clathrin interact with AP2 or AP180 (adapter protein) and membrane lipid to form clathrin-coated vesicles. The process of its formation is just that endophilin A1 drove the rupture of membrane vesicles from the donor membrane at the neck of vesicles, LPAAT at N-terminal of endophilin A1 can catalyze lysophosphatidic acid (LPA) and arachidonic ene-CoA to form lysophosphatidic acid (PA), LPA is a three-dimensional structure like inverted cone-type and PA is cone-type, the former structure is beneficial for positive membrane deformation, the later is conducive to negative membrane deformation. It is the transformation of lysophosphatidic acid that drive rupture between vesicle and the donor vesicle membrane. After endophilin mutation, it will severely affect recycle mechanism of synaptic particle [17].

Function besides endocytosis

Endophilin A2 is actually involved in absorbing nutrients and growth factors and endocytosis of pathogens and receptors, participating in a variety of membrane transport mechanism . While endophilin B1 locates in the membrane of the cell and its main function is related to cell membrane dynamics. Apoptosis receptor Fas/CD95 can not correctly located in the membrane without endophilin, resulting in inhibition of the mechanism of apoptosis [18].

Endocytosis related proteins

Dynamin

Dynamin is a 100 kD GTPase, and its GTPase region at N-terminal can bind and hydrolyse GTP, PH (pleckstrin homology) region can bind with membrane and mediate polymerization between dynamins, PRD (praline arginine rich) region at C-terminal mediate the interaction between other proteins. In addition, dynamin has a small GED (GTPase effector) region which is necessary in hydrolysis of GTP [18], and also plays an important role in some clathrin independent endocytosis. Dynamin is necessary in pinching and formation of vesicles. After its binding with ligand amphiphysin-1, dynamin-1 plays a key role in dynamin dependent endocytosis under regulation of cycle-dependent kinase 5 (Cdk5). Dynamin-1-dependent endocytosis occurs quickly, usually only a few seconds, while dynamin-2 mediated endocytosis is slow, usually ten minutes.

Dynamin uses mechanochemical activity – specifically a twisting action – to pinch off endocytic vesicles [19]. Dynamin was, early on, localized to the collar around the neck of forming endocytic vesicles. This suggested that dynamin may use the energy of GTP hydrolysis to directly pinch a membranous neck. Indeed, dynamin could tubulate lipids and break apart the tubules *in vitro*, although later it seemed that the breaking apart was happening as the samples dried on EM grids. Meanwhile, Schmid had come up with a "regulatory GTPase" mode: that dynamin was active not as it hydrolyzed GTP but in its GTPbound form, which recruited other proteins to do the pinching. The Yale group has more evidence for the earlier "pinchase" model. They used light microscopy rather than EM to follow tubulation and fission directed by dynamin *in vitro*. Longitudinal tension was needed with constriction to achieve fission. In mammalian, the dynamin collars are relatively short, so cortical actin is the most likely source of tension that would help the dynamin to wrench an endocytic vesicle free [20–23].

Dynamin has intrinsic activity of GTP and is also the cerebellum Dyrk1A kinase substrate, with its own aggregation feature of the endometrium can help new processes from the cell membrane vesicles isolated. Dynamin is involved in tubulation by PH epsin and ENTH (epsin NH2-terminal homology) [21].

Wild-type dynamin mutant block the tube, which can be restricted to speculate dynamin of these membrane protein of the ability to control grid protein coated vesicle endocytosis and other media invagination degree.

Actin

Actin is the main component of microfilament. Microfilament is involved in cell shape and polarity of the maintenance of endocytosis, intracellular transport,

cell shrinkage and movement, cell division, and many other functions. Actin has two kinds: G-actin and F-actin. The importance of F-actin in endocytosis was clearly illustrated by the effect of addition of the actin-monomersequestering drug latrunculin A. Within 5 minutes of its addition, actin patches were no longer visible and endocytosis was completely abrogated. This was the point at which the yeast WASP orthologue Las17p and the type I myosins (Myo3p and Myo5p) began to nucleate actin filaments through activation of the Arp2/3 complex [24]. This group of proteins has been called the "actin network growth machinery". Some researchers combined epifluorescence with total internal reflection microscopy (TIRF) to follow the internalisation of individual coated pits in living cells expressing a fluorescent tagged form of clathrin light chain (DsRed clathrin). They showed that transient recruitment of actin coincides with the inward movement of vesicles, they also observed recruitment of the Arp2/3 complex to clathrin-coated pits. PH-sensitive probes have been developed that allow internalisation of cargo into individual clathrin-coated vesicles to be visualized, allowing us to follow the time course of invagination and identify the point of scission. The recruitment of actin during invagination is thought to provide the force that drives the invagination of the coated pit [25]. It is currently identified that the vesicle scission module contains the two yeast amphiphysin proteins Rvs161p and Rvs167p. These proteins contain BAR (Bin-Amphiphysin-Rvs) domains that bind to and tabulate, actin and associated proteins recruit amphiphysins and thus mark the site at which membrane tubulation should occur. Tubulation is then suggested to facilitate the scission process. Following scission, the vesicle is uncoated and moves away from the membrane until it fuses with an endosome. There are two possible roles for actin at this final stage: vesicles could move along actin cables; alternatively actin could be nucleated at the vesicle surface to facilitate their movement within the cell. Then the actin depolymerized from the endocytosis site. The most representative depolymerizing factor is cofilin. Cofilin is necessary for endocytosis in mamamals, but its mechanism is still unknown [26]. In the process of invagination, Sac6p/microfilament binding protein/fimbrin is also required, invagination induced by actin failed without Sac6p [27].

PCH (Pombe Cdc15 homology) / F-BAR

PCH (Pombe Cdc15 homology) / F-BAR is another family of proteins which plays an important role in endocytosis. The BAR region of these proteins combine with phosphoinositide. FBP17 is a member of PCH family, containing PCH domain and extended FC domain. The EFC domains show weak homology to the Bin-amphiphysin-Rvs (BAR) domain. The EFC domains bound strongly to phosphatidylserine and phosphatidylinositol 4,5-bisphosphate and deformed the plasma membrane and liposomes into narrow tubules. Most PCH proteins possess an SH3 domain that is known to bind to dynamin and that recruited and activated neural Wiskott-Aldrich syndrome protein (N-WASP) at the plasma membrane. FBP17 contributed to the formation of the protein complex, including N-WASP and dynamin-2, in the early stage of endocytosis. Furthermore, knockdown of endogenous FBP17 impaired endocytosis, suggesting FBP17 is necessary for dynamin-dependent endocytosis [28].

Rab proteins

Rab protein which is a small GTP enzyme is an important regulator of endocytosis. Rab5 is involved in tubulization and its role in endocytosis has been clear. Rab5 protein has three isomers, Rab5A, Rab5B and Rab5C. Rab5 protein exists primarily on the plasma membrane, clathrin-coated vesicle and early endosome, taking charge of vesicle fusion and recycling. Rab5 protein functions with ongoing GTP/GDP cycle. As molecular switch of vesicle transport, the activated state of combining with GTP is "open", the unactivated state of combining with GTP is called "close". Rab5 regulates transport of endocytotic materials between membrane and early endosome, and help vesicle movement along microtubules. HAP40 is an effective regulatory factor of Rab5, and Rab5 increase connections of endosome with actin under existence of HAP40. RAB5A protein was related to endocytosis of prostacyclin [29]. A-synuclein is the root of Parkinson's disease, Lewy body Dementia and Alzheimer's disease and other central nervous system diseases. Moreover, Young found the mutant can decrease endocytosis of A-synuclein according to RAB5A GTP mutant enzyme research while Lewy body-like decreased in cytoplasm, the cytotoxicity also decreased. Over-expression of RAB5A protein in immune system deficiency, which can slower macrophage phagocytosis of pathogens and decrease the rate of degradation of pathogens and IFN-C-mediated phagocytosis weakened.

Conclusion

We have found many molecules involved in endocytosis in the past few decades, and had a preliminary understanding about its process and metabolism. We now know that the process of endocytosis is not a simple step-by-step process in which a series of proteins function according to the chronological order, but rather a complex process comprising many members which are regulated precisely. Although the mechanism of regulation of endocytosis is still unknown, it can be predicted that as more and more new research techniques applied in this area, we will be able to understand the mechanism of cell endocytosis more comprehensively. Whether tumor cells

endocytose more nutrients than normal cells and whether tumor cells increase sizes through endocytosing more growth factors? Can we inhibit tumor growth through inhibiting cell endocytosis or can we cure tumor by inducing specific drug endocytosis of tumor cells? Whether the endocytosis of 6-phosphate mannose receptor (MPR) which plays a very important role in cell death signal transduction is restrained in tumor cells? The mechanism of cell endocytosis can be further elucidated by solving all of the above problems.

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