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Review Article

Macropinocytosis and SARS-CoV-2 cell entry

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ABSTRACT

Macropinocytosis is a type of large-scale endocytosis that is triggered by the interaction of receptor proteins and ligands, such as growth factors, cytokines, chemokines, and lipopolysaccharide (LPS). Macropinocytosis ingests the extracellular fluid solutes and conveys them into the lysosome in the context of cell growth and differentiation. Aside from its physiological functions, macropinocytosis has been observed in viral infections. While the infectious mechanism of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is still unknown, recent studies suggest the involvement of macropinocytosis in its cell entry. In this review, we discuss the roles of endocytosis in SARS-CoV/SARS-CoV-2 cell entries and propose a hypothetical role of macropinocytosis in SARS-CoV-2 cell entry.

Keywords: SARS-CoV-2, macropinocytosis, endocytosis, neuropilin-1, viral cell entry

INTRODUCTION

Cell membrane dynamics are controlled precisely by signal transduction and cytoskeletal mechanisms[1−3]. Endocytosis is a cellular mechanism by which cells ingest extracellular components by modulating the plasma membrane. Based on their functions and mechanisms, endocytosis can be categorized into at least four different types[4−6]: clathrin-mediated endocytosis, caveolae-dependent

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endocytosis, phagocytosis, and macropinocytosis. While the first two types of endocytosis are relatively small-scale ingestion processes (less than 200 nm at the diameter of the resulting vesicle), the last two evoke large-scale uptake (more than 200 nm in scale). Each endocytosis has a critical role in cell metabolism. The functions can be utilized by pathogens for their infection strategies[7].

Macropinocytosis is induced by growth factors, cytokines, chemokines, LPS, and chemical compounds[8–10]. During the process, target fluid solutes are surrounded by an elongated and waved plasma membrane, then isolated from the extracellular environment *via* the fusion of the evoked membrane structure, which becomes a vesicle called a macropinosome (macropinosome formation). The macropinosome is pinched out from the plasma membrane and moves to the center of the cell using the microtubule network until it encounters the lysosome and/or other organelles (macropinosome maturation).

It has been shown that some pathogens can "hijack" macropinocytosis and invade the host cell[7,11]. For instance, the pathogenic bacteria *Salmonella typhimurium* (2.0 –5.0 mm in length and 0.5 –1.5 mm in diameter) and *Shigella flexneri* (1.0–3.0 mm in length and 0.4–0.6 mm in diameter) inject their own proteins into the host cells to induce macropinocytosis as a means of cell entry[7,12–13]. The vaccinia (300 nm in length and 200 nm in diameter) and Ebola (1 000 nm in length and 80 nm in diameter) viruses also take advantage of macropinocytosis[11,14–15]. Likewise, although the molecular function is not yet clear, the involvement of macropinocytosis in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) cell entry has been suggested[16]. Inhibitor treatment experiments show that Abl and PIKfyve, both of which regulate macropinocytosis[17–19], have some roles in SARS-CoV-2 cell entry[20–21]. More directly, the macropinocytosis inhibitor ethylisopropylamiloride (EIPA) blocks cell entry[22].

In this review, we will discuss the role of macropinocytosis in SARS-CoV-2 cell entry. Since the genome sequence reveals high similarity between severe acute respiratory syndrome coronavirus (SARS-CoV) and SARS-CoV-2[23–24], knowledge from SARS-CoV study can be used as a framework to determine research directions for investigating SARS-CoV-2 cell entry[25]. Thus, firstly, we present an overview of studies on endocytosis and SARS-CoV cell entry as the background information. We then describe the molecular mechanism of SARS-CoV-2 cell entry, focusing on macropinocytosis.

ENDOCYTOSIS

Background of endocytosis

One of the critical differences between small and large endocytosis is the membrane dynamics morphology. While membrane invagination is the precursor of small size endocytosis, the membrane ruffle is the first step of large-scale endocytosis (***Fig. 1A***). Although endocytosis is an infection strategy for pathogens to invade the host cells, phagocytosis is a rarely observed viral entry strategy due to the ingestion process[7,26]. Phagocytosis is regulated by the "zipper mechanism" for the uptake of solid objects[6] (***Fig. 1A***). In this mechanism, membrane proteins function as sensors to identify objects such as bacteria and dead cells. The interaction between these objects and sensor proteins trigger membrane ruffles. The plasma membrane elongates and expands according to the shape of the objects and forms a cup-like structure; then, the structure gradually fuses to become a sphere-like structure, called a phagosome. The virus is not large enough to be engulfed by phagocytosis. The mechanisms of SARS-CoV/SARS-CoV-2 cell entries, clathrin-mediated endocytosis, caveolae-dependent endocytosis, and macropinocytosis have previously been proposed[16,25]. There are several established inhibitors for each endocytosis, although in some cases their activities are unspecific[27].

Clathrin-mediated endocytosis

Clathrin-mediated endocytosis occurs *via* the formation of small membrane invaginations called clathrin-coated pits (CCPs)[4,28] (***Fig. 1A***). CCPs are composed of clathrin triskelion, which consists of three clathrin heavy chains and three clathrin light chains, and adaptor proteins. Due to the uniqueness of their structure, clathrin triskelia are assembled as a cup-like shape, and then interact with the plasma membrane *via* adaptor proteins. Construction of the cup-like shape assembly beneath the plasma membrane produces a force, which generates a small membrane invagination formation known as a CCP. Once the CCP matures, the shape changes to a vesicle-like structure with a diameter of less than 200 nm. Large GTPase dynamin is recruited to the nascent CCP for fission from the plasma membrane, and the resulting 100 nm size vesicle, called a clathrin-coated vesicle (CCV), is released into the cytosol. Because of its critical role in CCV formation, the inhibition/depletion of clathrin used to block clathrin-mediated endocytosis has become an established method.

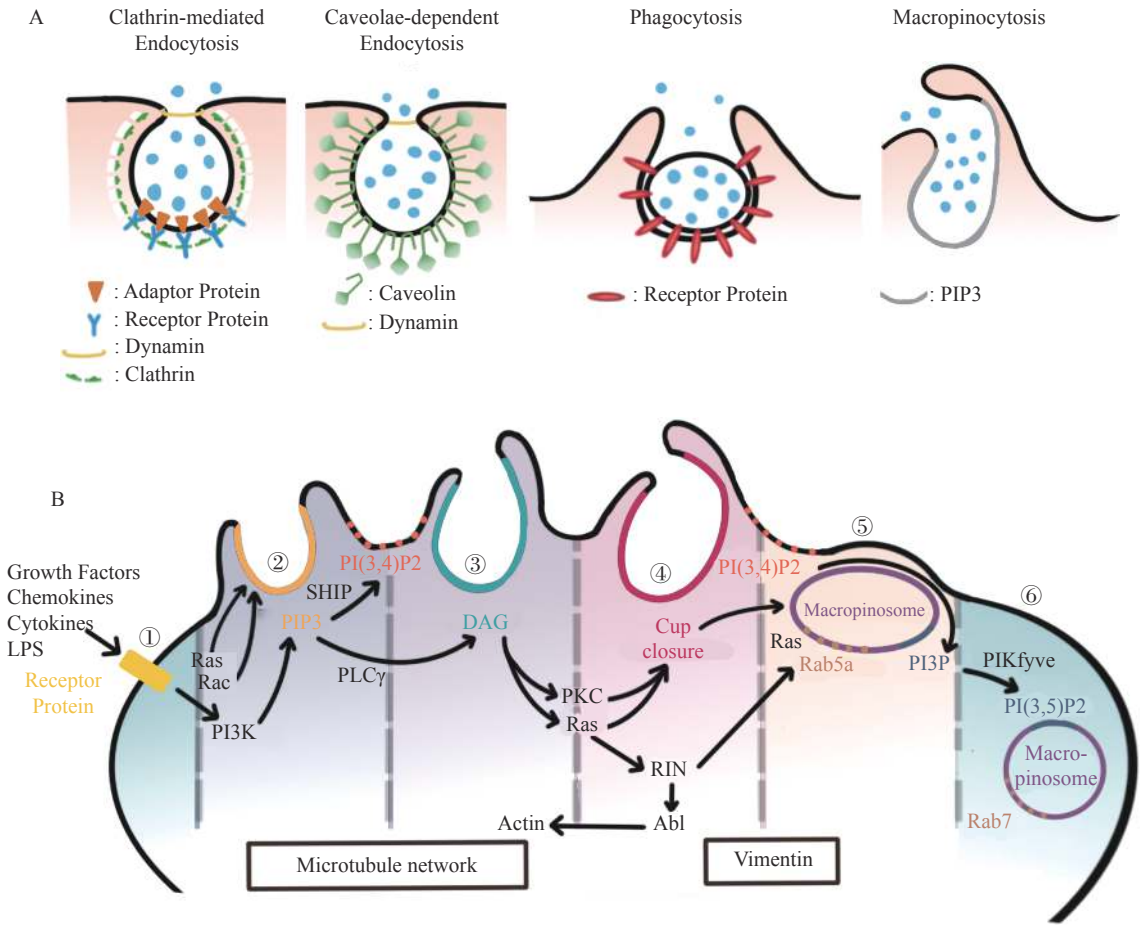

Fig. 1 **Summary of different types of endocytosis.** A: Comparison of different types of endocytosis. Membrane invagination is the precursor of clathrin-mediated endocytosis and caveolae-dependent endocytosis. They are relatively small in size (less than 200 nm of the diameter of the resulted vesicles). Membrane ruffle is induced as the first step of phagocytosis and macropinocytosis, and the resulting vesicles are larger than 200 nm. Details of the molecular mechanisms are described in the main text. B: Molecular mechanism of macropinocytosis, which consists of macropinosome formation (STEPs 1-4) and macropinosome maturation (STEPs 5-6). STEPs 1-2: Extracellular stimuli induce membrane ruffles *via* Ras, Rac, and PI3K, followed by the formation of macropinocytic cups. Phosphatidylinositol (3,4,5)-triphosphate (PIP3) is generated/accumulated in the cups by an unknown mechanism. STEPs 3-4: PIP3 is converted to diacylglycerol (DAG) by PLCg and phosphatidylinositol 3,4-biphosphate (PI(3,4)P2) by SHIP. DAG activates PKC and Ras, which trigger cup closure. Ras also activates RIN-Abl pathway to regulate actin cytoskeleton. STEPs 5-6: The cups are closed, forming vesicles, known as macropinosomes. PI(3,4)P2 is converted to phosphatidylinositol 3-phosphate (PI3P) at macropinosomes, where Rab5a is recruited. Ras is also recruited to macropinosomes at the early stage. Once Rab5a is dissociated from macropinosomes, Rab7 is recruited at the late stage. PI3P is converted to phosphatidylinositol 3,5-biphosphate (PI(3,5)P2) by PIKfyve. Microtubule and vimentin are also involved in the process.

Chloroquine[29], chlorpromazine[29], dynasore[30], and pitstop 2[31] are often used as the inhibitors of clathrin-mediated endocytosis[27,32]. Clathrin-mediated endocytosis is the most prominent and abundant viral entry pathway among mammalian viruses[7,26].

Caveolae-dependent endocytosis

The precise molecular mechanism of caveolae-dependent endocytosis is not clear[5,33] (***Fig. 1A***). Caveolae are small plasma membrane invaginations (approximately 70 nm in diameter) formed by caveolins, which consist of hairpin-like integral membrane proteins, together with associated proteins. Caveolins cluster with specific lipids at the plasma membrane, then form a small invagination, known as a caveola, through the interaction between the caveolins and adapter proteins. Caveolae-dependent endocytosis occurs once the caveolae are pinched off from the plasma membrane by dynamin, which localizes at the neck of caveolae. Methyl-β-cyclodextrin (MβCD)[34], filipin[35], and nystatin[35] are used as caveolin-mediated endocytosis inhibitors[27,32].

Although the physiological role of caveolae-dependent endocytosis is indeterminate, the involvement of caveolae in cell entry by viruses has been proposed[7]. For instance, simian virus 40 (SV40) utilizes caveolae for cell entry[36].

Macropinocytosis

While phagocytosis is called a "cell-eating" process, because it ingests solid targets such as bacteria and dead cells, macropinocytosis is called a "cell-drinking" process; a large-scale extracellular solute uptake process (***Fig. 1B***)[6,8–10,37]. Historically, "pinocytosis" was found by Warren Lewis in the 1930s[38]. After the discovery, the application of electron microscopy enabled researchers to observe nano scale cellular events, such as clathrin-mediated endocytosis and caveolae-dependent endocytosis, in other words "micro"-pinocytosis. Thus, the original "pinocytosis" was renamed as "macropinocytosis", which is a clathrin/caveolae-independent cell drinking mechanism. Macropinocytosis consists of two major processes: macropinosome formation and macropinosome maturation[8]. After ligand stimulation, membrane ruffles are induced and changed into cup-like structures to engulf extracellular solutes by closing the cup. Interaction between the ligand and receptor protein activates at least three signaling molecules, small GTPases Ras and Rac and phosphatidylinositol 3-kinase (PI3K)[6,37]. Activated Ras and Rac induce membrane ruffles, which gradually change to cup-like structures. Inside the cups, phosphatidylinositol (3,4,5)-triphosphate (PIP3) is generated by activated PI3K and then converted to diacylglycerol (DAG) by PLCγ1[37,39–40]. DAG activates protein kinase C (PKC) to close the open area of the cups following the pinching off the resulting vesicles as macropinosomes from the plasma membrane. Cytoskeletal mechanisms, such as actin, vimentin, and microtubules, are also involved in the formation of macropinosome[6,41–42]. Ras effector RIN functions as a guanine nucleotide exchange factor (GEF) for small GTPase Rab5a, which is recruited to macropinosomes[18,37,43]. RIN also activates the tyrosine kinase Abl, which regulates actin dynamics during macropinosome formation[17–18]. Ethyl-isopropyl Amiloride (EIPA) prevents the activation of Rac1 by decreasing submembranous pH, and is used as a macropinocytosis inhibitor[27,44].

Macropinosome maturation is defined as a process from the end of macropinosome formation to lysosomal fusion[8]. Nascent macropinosomes depart from the plasma membrane and migrate into the cytosol along to the microtubule network. Phosphoinositide (PI) signaling pathways and small GTPases have critical roles in the maturation process[37,45–46]. PIP3 in the cup structure is also converted to phosphatidylinositol 3,4-bisphosphate (PI (3,4) P2) by lipid phosphatase SHIP2 when the cup is closed[45]. PI (3,4) P2 is converted to phosphatidylinositol 3-phosphate (PI3P) by inositol polyphosphate-4-phosphatase type Ⅱ (INPP4B) after nascent macropinosomes start to migrate[45]. Phosphoinositide kinase PIKfyve phosphorylates PI3P to phosphatidylinositol 3,5-biphosphate (PI (3,5) P2) to regulate the maturation process[19]. RIN activates small GTPase and Rab5a in early macropinosomes[18]. Once Rab5a is dissociated from the macropinosomes, another small GTPase Rab7 is recruited[37]. Recruitment of H-Ras to early macropinosomes has also been observed[47]. The role of macropinocytosis in virus entry is well studied, and there are several outstanding reviews[7,11].

SARS-CoV cell entry

SARS-CoV spike protein

Although the precise mechanism is still unknown, SARS-CoV has been shown to induce endocytosis to invade the host cells[48–49]. SARS-CoV is a spherical virus with a diameter of 80 –120 nm. The viral envelope consists of a lipid bilayer and three proteins: the membrane protein, envelope protein, and spike protein. The Spike protein (CoV S protein) forms trimers as protrusion-like structures on the viral surface. The CoV S protein is a 180-200 kDa type I transmembrane glycoprotein protein[48,50–51](***Fig. 2A***). It has a cleavage site in the ectodomain and the CoV S protein can be divided into two functional domains, S1 and S2[52]. The receptor binding domain (RBD) exists as a 190-amino acid fragment in the S1 domain as a critical site for interaction with ACE2[50,53–54]. More specifically, structural analysis revealed that RBD interacts with ACE2 *via* the receptor-binding motif (RBM) as a 70-amino acid fragment[55]. While the cleavage site at the boundary of S1 and S2 is identified (S1/S2 cleavage site), there is another cleavage site in S2 known as the S2 cleavage site[56]. Several host proteases have been identified to recognize cleavage sites for the priming of CoV-S[48–49]. For instance, trypsin cleaves at the SLLR/S sequence[57] and cathepsin cleaves at the VAYT/M sequence[58] in the S1/S2 cleavage site. Among these proteases, transmembrane protease serine 2 (TMPRSS2) has been well studied in the context of cell entry[49,56,59–62].

Angiotensin converting enzyme 2 (ACE2) is

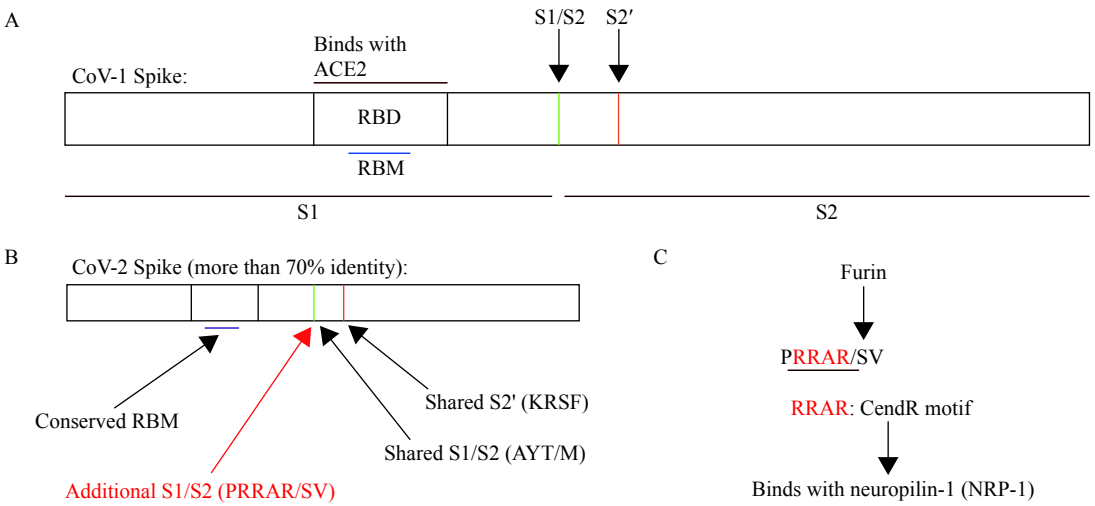

***Fig. 2* Comparison of SARS-CoV and CoV-2 Spike proteins.** A: Structure of SARS-CoV Spike protein (CoV S). CoV S is cleaved at the S1/S2 site forming two different proteins: S1 and S2. The ACE2 binding site in S1 and the Receptor Binding Motif (RBM) in the Receptor-Binding Domain (RBD) are identified. Another cleavage site, S2', is identified in S2. B: Structure of SARS CoV-2 Spike protein (CoV-2 S). Comparison with the sequence of CoV S showed that there is a unique cleavage site (PRRAR/SV) at S1/S2 of CoV-2 S. C: Scheme of the furin-induced cell entry process. The PRRAR/SV site is cleaved by the furin protease, resulting in the exposure of RRAR (CendR motif) at the c-terminal of CoV-2 S1. CendR binds to receptor protein neuropilin-1.

identified as the functional receptor of SARS-CoV cell entry[53,63−65]. ACE2 is a 110-120 kDa type-I transmembrane protein (805 amino acids)[66−67]. As a monocarboxypeptidase, ACE2 plays a critical role in the renin-angiotensin system to regulate the physiology of the cardiovascular system, such as blood pressure and fluid balance[66]. Surprisingly, excluding the case of SARS CoV cell entry, the role of ACE2 in endocytosis has not yet been shown. A previous study showed that ACE2 interacts with TMPRSS2 at the surface of SARS-CoV-infected 293T cells[51,60]. Although the role of ACE2 in viral entry has been strongly suggested, the precise mechanism by which the interaction of the CoV S protein and ACE evokes endocytosis is still unknown.

Background of SARS-CoV cell entry

Since SARS-CoV requires biosafety level 3 (BSL3) facilities, pseudotyped particles have often been used to investigate the molecular mechanisms of cell entry *in vitro*[68]. It has been shown that pseudoviruses expressing the CoV S protein efficiently enter human cell lines such as the embryonal kidney cell line 293T and the hepatoma cell lines HepG2 and Huh7[65,69−70]. Moreover, in animal studies, the pseudovirus was found to infect African green monkey kidney cell lines Vero[65] and Vero E6[69]. NH_4Cl, which is used as a lysosomotropic agent[71], inhibits the infection of the pseudovirus in 293T cells, Huh7 cells, and the monkey kidney cell line COS7 cells expressing ACE2[65,69−70]. The grivet kidney cell line BSC-1 is used for the time-course experiment[72].

Historically, the African green monkey kidney cell line Vero E6 has been used as the standard model to investigate the molecular mechanism of cell entry because ACE2 was identified as the CoV-S target protein using Vero E6 lysates[63] and its expression level is robust[73]. Given that lung epithelial cells are the initial target of the viral infection[49], human lung epithelial cell line Calu-3 cell is characterized as an alternative *in vitro* model[74]. This paper showed that ACE2 expression in the apical domain of polarized Calu-3 was observed, and that SARS-CoV (Urbani strain) was co-localized with ACE2 in infected cells. ACE2 expression in Calu-3 has also been confirmed by other researchers[73,75]. It is also shown that SARS-CoV (Frankfurt 1 strain) infects Calu-3 cells[75].

Clathrin-mediated endocytosis and caveolae-dependent endocytosis in SARS-CoV cell entry

The role of clathrin-mediated endocytosis in viral entry has been proposed[70]. In this study, the human hepatocyte cell line HepG2 was mainly used. The authors showed that inhibition of clathrin-mediated endocytosis by chlorpromazine attenuated the entry of pseudovirus into the cells. Treatment with methyl-β-cyclodextrin (MβCD) partially inhibited entry. Thus, they concluded that the pseudovirus internalizes into HepG2 cells *via* clathrin-mediated endocytosis but not caveolae-dependent endocytosis. Although another study argues against this conclusion by insisting that HepG2 is not an appropriate cell culture system to

investigate ACE2-dependent cell entry because the expression level of ACE2 is not robust[76], several reports also suggest the role of clathrin-mediated endocytosis in the viral entry. Sequence analysis revealed that ACE2 has a calmodulin binding domain; biochemical methods confirmed the interaction of calmodulin and ACE2[77–78]. It has been shown that calmodulin regulates clathrin-mediated endocytosis[79]. Therefore, it is hypothesized that stimulation of ACE2 by the CoV S protein triggers clathrin-mediated endocytosis.

The role of caveolae-dependent endocytosis in cell entry has been discussed. Cell entry by SARS-CoV (Frankfurt-1 strain)[80] or pseudovirus[81] into Vero E6 cells was inhibited by MβCD treatment. MβCD also partially blocks the entry efficiency of the pseudovirus into HepG2 cells[70]. However, other caveolae-dependent endocytosis inhibitors, filipin, and nystatin, do not block cell entry of the pseudovirus into Vero E6 cells[81]. As another approach to determining the role of caveolae in cell entry, the localization of ACE2 in the plasma membrane has also been studied. Sucrose fractionation assay using Vero E6 cells and Chinese hamster ovary (CHO) cells showed that ACE2 is not located in the fraction containing lipid-raft related proteins[80,82], suggesting that ACE2 is not localized in lipid rafts[80]. However, biochemical data using Vero E6 cell lysates from other groups show that ACE2 is localized in the caveolin-1-positive fractions[83] or in the fractions of raft marker floitin[84]. This discrepancy could be explained by the different buffer conditions as well as the difference between CHO and Vero E6 cells[84], or could be interpreted as the contribution of an ACE2-independent mechanism.

Clathrin/caveolae-independent endocytosis and macropinocytosis in SARS-CoV cell entry

The role of clathrin/caveolae-independent endocytosis in SARS-CoV cell entry was proposed by Wang *et al*[76,81]. The recombinant fusion protein of the CoV S protein and human IgG Fc fragment (named S1190-Fc) was added to human embryonic kidney 293E cells expressing GFP-ACE2 (HEK293E-ACE2-GFP), the co-localization of S1190-Fc and GFP in the cells was observed 3h later. Pseudovirus infection in Vero E6 was barely inhibited by treatment with the clathrin-mediated endocytosis inhibitor chlorpromazine, knockdown of the clathrin heavy chain, and overexpression of the dominant negative form of Eps15, which is required for clathrin-mediated endocytosis. Meanwhile, colocalization of caveolin-1 and the pseudovirus was not observed and caveolae-dependent endocytosis inhibitors nystatin and filipin did not block cell entry of the pseudovirus into Vero E6 cells.

These data indicate that the interaction of CoV S protein and ACE2 is sufficient to induce the ingestion process and that the process is clathrin/caveolae-independent endocytosis, presumably macropinocytosis. However, as the authors mentioned in the discussion section[76], direct evidences are needed to conclude if the virus takes advantage of macropinocytosis to invade into the host cells. Interestingly, besides these studies, there are several reports suggesting the role of macropinocytosis in SARS-CoV cell entry[85]. It was shown that SARS-CoV (Urbani strain) induces membrane ruffles in ACE2-expressing murine delayed brain tumor (DBT) astrocytoma cells[86]. Macropinosome formation and SARS-CoV-induced cell entry share several signaling molecules, such as PI3K, vimentin, Abl, and Ras (***Fig. 1B***). PI3K inhibitors LY294002 and wortmannin block the entry of pseudovirus cells into Vero E6 cells[87]. Biochemical methods show a direct interaction of vimentin and the CoV S protein; pseudovirus entry into Vero E6 cells is partially blocked by the knockdown of vimentin[88]. Abl inhibitor imatinib mesylate blocks SARA-CoV (MA15 strain)-induced cytopathic effects in Vero E6 cells[89], and attenuates the replication of SARS-CoV (MA15 strain: a mouse-adapted SARS-CoV)[89–90] in Vero E6 and Calu3 cells. Although, the drug did not affect the colocalization of LAMP1 and the pseudovirus in Huh7 cells, suggesting that it blocks the cell entry process after endocytosis[90]. Treatment with purified CoV-S induces ERK phosphorylation in human Ⅱ pneumocyte A549 within 15 min *via* ACE2, which is blocked by the Ras inhibitor, suggesting that an interaction between CoV-S and ACE2 activates Ras[91].

SARS-CoV-2 cell entry

Background of SARS-CoV-2 cell entry

Sequence analysis of SARS-CoV-2 revealed that the CoV-2 S protein and CoV S protein share more than 70% amino acid identity[23–24] (***Fig. 2B***). It was also shown that SARS-CoV-2 invades the human epithelial-like cell line HeLa expressing human ACE2, but not the control cells, suggesting that ACE2 is the receptor of SARS-CoV-2 cell entry[24]. Since then, the study of SARS-CoV-2 has been carried out according to the hypothesis that SARS-CoV and SARS-CoV-2 share molecular mechanisms in cell entry[20,92].

Inhibitor treatment experiments have been

performed to investigate the molecular mechanism of SARS-CoV-2 cell entry[92–95]. Lysosomal function inhibitors NH_4Cl and bafilomycin A block viral entry into 293T cells expressing ACE2[20]. In addition, hydroxychloroquine and chloroquine, which also inhibit lysosomal function, block cytotoxicity[93]. These results suggest the involvement of lysosomal mechanisms in cell entry[16,25]. To date, the role of clathrin-mediated endocytosis is controversial. Clathrin-mediated endocytosis inhibitors, amantadine[96] and chlorpromazine, barely block SARS-CoV-2 induced cellular cytotoxicity in Vero E6 cells[95]. Purified-CoV-2 S protein induces endocytosis in 293T cells expressing ACE2, which is blocked by clathrin-mediated endocytosis inhibitors dynasore and Pitstop2[97]. Because the experimental systems and drugs used were not the same, it would be difficult to determine the accuracy of each set of data. Pseudovirus entry into 293T cells expressing ACE2 was blocked by cathepsin inhibitor E64D and cathepsin L-specific inhibitor SID 26681509[20], suggesting that protease activity is necessary for cell entry.

Vero E6 cells expressing TMPRSS2 are more susceptible to SARS CoV-2 infection than other control cell lines[92,98]. The TMPRSS2 inhibitor camostat mesylate blocks pseudovirus entry into a variety of cell lines, such as Calu-3, HeLa expressing ACE2, Caco-2 (human epithelial cell), and MRC-5 (human lung fibroblasts)[92,99]. The role of CD-147 in SARS CoV-2 cell entry has been shown[94,100–101]. As other receptor candidates, CD209L (L-SIGN) and CD209 (DC-SIGN), have been suggested[102]. Similar to SARS-CoV, the Abl inhibitor imatinib blocks SARS-CoV-2 cell entry into Vero cells[21].

Macropinocytosis and SARS-CoV-2 cell entry

The macropinocytosis inhibitor EIPA substantially decreased the concentration of viral RNAs in the culture supernatant of SARS-CoV-2-infected Vero E6 cells[22]. Several airborne viral pathogens, such as influenza A virus[103] and human adenovirus serotype 35 (HAdV-35)[104], enter into the host cells *via* macropinocytosis. The expression levels of proteins related to macropinocytosis are relatively high in pneumocytes[16]. Based on these reports, the role of macropinocytosis in SARS CoV-2 infection has been proposed[16].

Genome sequencing revealed that there are at least three cleavage sites in the CoV-2 S protein[105]. While the CoV-2 S protein shares the S1/S2 cleavage site (AYT/M sequence) and S2' cleavage site (KR/SF sequence) with CoV S protein, there is an additional S1/S2 cleavage site (PRRAR/SV sequence), which is recognized by furin protease[99,105–108] (***Fig. 2C***). In fact, biochemical methods using furin-targeting siRNA treatment and furin inhibitor decanoyl-RVKR-CMK[109] showed that the S1/S2 cleavage site of the CoV-2 S protein is dependent on furin function[99,108]. The cell entry efficiency of pseudovirus, which express the furin-cleavage-site mutant CoV-2 S protein, into Calu-3 cells, MRC-5 cells, and HeLa cells expressing ACE2 were lower than that of the control pseudovirus[99]. These results suggest that furin-dependent cleavage at the S1/S2 site is critical for SARS CoV-2 cell entry but not for SARS CoV and that this site has a role in determining the tropism of SARS CoV-2[105,110]. It has been proposed that TMPRSS2 and furin coordinately work with the priming of the CoV-2 S protein[99,108].

After furin cleavage, the RRAR sequence is exposed at the C-terminal end of the S1 subdomain[105] (***Fig. 2C***). The RRAR sequence at the end of the C-terminus is called CendR, which binds and activates the receptor protein neuropilin-1 (NRP1)[111]. Accordingly, NRP-1 is identified as a novel receptor for SARS CoV-2 cell entry in at least three different studies[110,112–113]. Expression of NRP1 enhances pseudoviral entry into 293T cells and Caco-2 cells; the infection efficiency is attenuated by the addition of anti-NRP1 antibody[110], SARS CoV-2 (SARS-CoV-2/human/Liverpool/REMRQ001/2020 isolate) cell entry into HeLa cells expressing ACE2 hindered by knockout of NRP1[112]. Biochemical methods suggest the interaction between NRP1 and CoV-2 S protein[112]. These results suggest that NRP1 is another key receptor for SARS-CoV-2 cell entry and the interaction between CoV-2 S protein and NRP1 induces endocytosis. Interestingly, it has been shown that stimulation of NRP1 induces macropinocytosis-like endocytosis: nanoparticle beads with CendR peptide induce membrane ruffles which are engulfed into large-scale vesicles in the prostate tumor cell line (PPC1) and HeLa cells expressing NRP1[114]. As macropinocytosis is negatively regulated by the mTORC1 pathway[115], CendR-induced endocytosis is enhanced by the inhibition of mTORC1[114]. Additionally, the Epstein-Barr virus (EBV) has been shown to induce macropinocytosis by the interaction of EBV glycoprotein B (EBV gB) and NRP1 for cell entry[116–117]. Furin cleaves EBV gB at the host cell surface, exposing CendR. CendR interacts with NRP1 to form a complex with epidermal growth factor receptor (EGFR), resulting in the induction of macropinocytosis[117]. These results strongly suggest that SARS-CoV-2 modulates macropinocytosis *via* the priming of the CoV-2 S protein by furin protease.

The role of PIKfyve in SARS-CoV-2 cell entry has been shown. PIKfyve inhibitors apilimod and YM201636 partially block the entry of pseudovirus cells into 293T cells expressing ACE2[20]. Apilimod also inhibits SARS-CoV-2 (strain 2019-nCoV/USA-WA1/2020) cell entry into Vero E6 cells[22,118] and pseudovirus entry into African green monkey kidney epithelial MA104 cells[118]. Given that the involvement of PIKfyve in macropinosome maturation has been shown[19], these results suggest that macropinosome maturation is involved in the SARS-CoV-2 cell entry process.

Proposed model of SARS-CoV-2 induced macropinocytosis

As discussed above, recent research suggests a role for macropinocytosis in SARS-CoV-2 cell entry. Here, we propose that SARS-CoV-2 uses two different receptor proteins, ACE2 and NRP1 for cell entry, in which macropinocytosis is utilized as the main pathway (***Fig. 3***). There are two cleavage sites (site 1 and 2) in S1/S2 of the CoV-2 S protein[105] (***Fig. 2B***). While site 2 is shared with CoV S protein and can be identified by cathepsin L, site 1 is CoV-2 S protein-specific and recognized by furin protease. At the surface of host cells, the CoV-2 S protein is primed by cleavage at site 1 by furin, it then binds to ACE2 *via* the RBM[110,112−113]. The cleavage by furin also expose CendR motif, which binds to NRP1 (***Fig. 2C***), inducing macropinocytosis, presumably by stimulating growth factor signaling (***Fig. 3***)[114]. Therefore, we presume that ACE2 binds to the cell surface following NRP1 stimulation (***Fig. 3***). If this is the case, Abl participates in macropinosome formation[21], and the resulting macropinosomes containing the viruses are regulated by PIKfyve for the maturation process[118]. Meanwhile, given that PI3K, Abl, vimentin, and Ras have been observed in SARS-CoV cell entry[87−88,90−91], it is likely that SARS-CoV also modulates macropinocytosis. While the priming process of the CoV S protein is still not clear, TMPRSS2 can cleave the protein at different sites[62]. In addition, the role of TMPRSS2 in the cell entries of both SARS-CoV and SARS-CoV2 has been reported[99,108]. Thus, cleavage by TMPRSS2 in the S2 domain would expose novel sequences, which might trigger macropinocytosis.

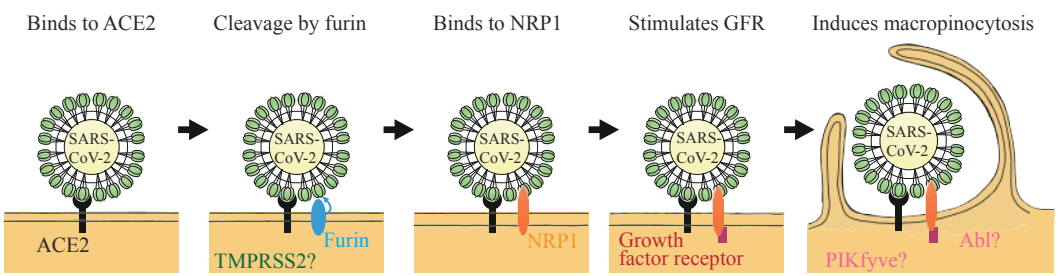

Fig. 3 **Proposed model of SARS CoV-2 -induced macropinocytosis.** SARS CoV-2 Spike protein (CoV-2 S) binds to ACE2 *via* the RBD. Furin cleaves CoV-2 S at the S1/S2 site for priming. Involvement of TMPRSS2 in the priming process is suggested. The CendR motif is exposed at the C-terminal of CoV-2 S1 and interacts with NRP1. This interaction somehow activates growth factor signaling and induces macropinocytosis. Abl and PIKfyve could be involved in the process. Macropinocytosis is used as a means for the virus to internalize into host cells.

CONCLUSION

The role of macropinocytosis as a pathogen infection strategy has been studied in recent decades. In this review, we discuss the involvement of macropinocytosis in SARS-CoV-2 cell entry and propose its molecular mechanisms. Owing to the limitations of *in vivo* experiments, revealing the molecular mechanism that the virus utilizes for cell entry could be a good strategy to investigate viral entry as well as for developing pharmaceutical treatment methods. Outputs from the cell biology in terms of the mechanisms/functions of macropinocytosis should give us clues regarding SARS-CoV 2 cell entry.

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