A Novel Implication of the Physiological Importance of Local Protons at the Membrane Surface of Organellar Vesicles in Eukaryote Cells: Organellar Type Na\(^+/\)H\(^+\) Exchanger Nhx1p from *Saccharomyces cerevisiae* Plays an Important Role in the Formation of Multivesicular Bodies

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Intracellular pH homeostasis is the essential element for living cells. Optimal pH has to be provided for proteins to function properly within the cells. pH is kept at around 7.3 in most cells from bacterial to mammalian. In contrast, for various endosomes in the cytoplasm their own weak acidic pH is maintained. For the homeostatic pH regulation in the compartments like cytoplasm and endosomal lumen are performed by H\(^+\) transporting membrane integral proteins. One of these is the Na\(^+/\)H\(^+\) exchanger (NHE or Nha). Here the roles of Na\(^+/\)H\(^+\) exchangers in the cells are summarized based on our recent findings. One of the most important findings is that the acidic pH regulated by NHE in the yeast endosomes contributes to recruitment of various factors required for membrane protein trafficking on the membrane surface of the endosomes. This acidic pH leads to multivesicular body (MVB) formation. These observations raise a new important role for NHE, not only in the pH regulation of the endosome lumen but also for pH regulation on the cytoplasmic surface of the endosomes. Based on this new physiological role of organelar NHE from yeast (Nh1) we propose a new conceptual idea of the importance of local protons regulated by the NHEs in cell physiology.

**Key Words:** pH regulation, Na\(^+/\)H\(^+\) exchangers, ion transporter, multivesicular body, membrane trafficking

**Homeostasis of pH within the cells**

Intracellular ionic condition is an essential factor for living cells to survive. In particular, H\(^+\) is the most important element to be kept at a certain value, the concentration because it provides the best environment for proteins to perform their roles. It is well established that cytoplasmic pH is kept at around 7.2–7.4 in the cells from most bacteria to humans [1, 2]. For eukaryotic cells, intracellular vesicular organelles are found in which pH shows an acidic value for the luminal condition (Fig. 1) [1]. For instance, lysosomes, one of these organelles, in which pH is set at around less than 5.5 [1], provide the best pH condition for the lysosomal enzymes to play a role.

The cytoplasmic pH becomes acidic as a result of whole metabolism in the cells. To maintain the neutral pH in the cytoplasm, excess protons should
be removed from the cytoplasm. For this, various H⁺ transporters and pumps (H⁺ transporting ATPases) have been provided in the plasma membranes of living cells from bacteria to humans [1]. H⁺ is exported from inside the cells to the outside by H⁺ transporting ATPase for bacterial and yeast cells. H⁺ extrusion is also performed by the respiratory chain for bacterial cells. This extrusion leads to the formation of a proton electrochemical gradient across the plasma membranes [3, 4]. The proton gradient drives various transporters for the nutrients and ions in bacterial and yeast cells. The H⁺ extrusion is also performed by Na⁺/H⁺ exchangers (NHE) for mammalian cells. To drive H⁺ extrusion by NHEs a Na⁺ gradient is used that is established by Na⁺/K⁺ ATPase. Excess H⁺ in the cytoplasm is also removed by uptake of H⁺ into organelar lumen by V-type H⁺ transporting ATPase [1, 2].

An extensively acidic condition outside of the cells eventually leads to acidification of cytoplasm and inhibits the growth of cells [5]. For a NHE1-deficient cultured cell line under an acidic condition in a culture medium cell growth was inhibited, supporting the idea of functional importance of NHE1 in the pH regulation. Thus pH regulation inside cells is accomplished by total functional regulation of various H⁺ transporters such as NHEs together with H⁺ or Na⁺ transporting ATPases. The precise molecular mechanisms of this coordinated control of various H⁺ transporters are still unclear.

**Na⁺/H⁺ exchangers (NHE)**

One of the most important H⁺ transporters is the Na⁺/H⁺ exchanger (NHE) also named Na⁺/H⁺ antiporter (Nha) found in bacterial to human cells. NHE and Nha form a family of several isoforms. Na⁺ and H⁺ are transported in the opposite direction. The transport direction of the ions is determined primarily by the ion gradient formed by the ion transporting ATPases. For bacteria and yeast Na⁺ is exported to the outside of cells to maintain the optimal osmotic pressure of the cells by NHEs [3]. This extrusion of Na⁺ is driven by the H⁺ gradient formed by H⁺-transporting ATPase. Thus Nha plays an important role for bacterial and yeast cells to survive under high saline environments [3].

For mammalian cells, NHE1 to NHE5 are found in the plasma membranes [2, 6]. For organelar membranes, NHE6 to NHE9 are found [7] (Fig. 2). For yeast cells, one organelar type antiporter (Nhx1p) and one plasma membrane type Nha (Nha1p) are known.

**Organellar type NHEs**

In eukaryotic cells various types of vesicular structures are found as organelles. These organelar vesicular structures include lysosomes, peroxisomes, and other endosomes. The internal acidic pH in these organelles (Fig. 1) is established as described earlier here by V type–ATPase transporting H⁺ from the cytoplasmic space to the inside of the vesicles (lumen) (Fig. 3). The acidic value of a type of organelar lumen is specific for each type of organelles as shown in Fig. 3. The organelar type NHEs can export H⁺ to the cytoplasmic space driven by a K⁺ gradient across the membranes instead of Na⁺ because high K⁺ and low Na⁺ are established by Na⁺/K⁺ ATPase [7, 8]. For mammalian cells, NHE6 is localized to the early and recycling endosomes [8, 9]. NHE 9 is found at the late endosomes. NHE 7 is localized at the Trans-Golgi network, while NHE8 is found at the mid-Golgi. Thus the organelar type NHEs are localized to different organelles and play a role to keep the weak acidic pH value in the lumen specific to each organelle together with V-ATPase [7, 8, 10].

Although acidic pH for various organelar lumen seems to be regulated by both V-ATPase and NHEs,
the physiological significance of this pH regulation is not fully known in detail. NHE6 is localized at the early and recycling endosomes. However, about 20 percent of NHE6 in HeLa cells is also found in the plasma membranes [11]. Knockdown of NHE6 expression in HeLa cells caused reduction of transferrin uptake from the outside of cells, especially at early stage of endocytosis of transferrin receptor (Fig. 4). NHE6 may affect clathrin recruitment to the endosome surface [12]. NHE6 binds RACK1 that had been found as a cytoplasmic scaffold protein for a protein kinase and other transporters [11]. Knockdown of RACK1 expression leads to a decrease of NHE6 molecules at the plasma membrane and accumulation at the early endosomes. NHE6 shuttles between the plasma membrane and endosomes which is regulated by RACK1 [11]. NHE6 is also found at endosomes in HepG2 cells, a hepatoma cell line, in which formation of cell-polarity as observed for the formation of distinct apical and basolateral structures in a cell is observed during in vitro cell culture [13]. This polarity formation involves pH regulation of endosomes by NHE6 that is supported by knockdown experiments of NHE6. This knockdown caused a decrease of bile canalicular structure observed for the apical area of HepG2 cells [13].

NHE6 is identified as a causative gene for human mental retardation syndrome such as Angelman syndrome like syndrome [14]. We have found a
Japanese family lacking the normal NHE6 gene [15]. Because of the defect in the primary sequence in the NHE6 gene found as a nucleotide deletion at position 441, a termination codon and resultant truncation of the NHE6 protein sequence was taken place. This mutation causes a malfunction of NHE6. Detailed studies to clarify the correlation between the disease and gene defect will be required. Interestingly our preliminary experimental results suggest that NHE6 is required for neurite growth for neuronal cell model PC12 cells [16]. NHE9 is found at the late endosomes and has been found as the causative gene in case of the autism [17]. Mechanistic studies to reveal the physiological role of NHE9 in neuronal cells are needed.

**Organellar type Nhx1p from yeast and its physiological role**

As found for mammalian NHE6 and NHE9, organellar type NHEs are an important pH regulator in cell physiology, probably through membrane protein trafficking in the cells [7]. However, the mechanisms that involve NHEs including NHE6 are largely unknown. The yeast counterpart of NHE6 is Nhx1p [18] (Fig. 5). Knockout of Nhx1 affects protein trafficking of CPY (carboxy peptidase Y) that is localized to the vacuole [19]. For the knockout cells CPY is excreted to the outside of the cells.

Further accumulation of prevacuolar compartments was observed [19]. Based on these observations we analyzed Nhx knockout on the trafficking of another protease CPS (carboxy peptidase S) localized at the vacuole (Fig. 5) because we thought that the role of Nhx1 in protein trafficking might give a clue to the mechanism involving NHE6 and other organellar type NHEs [20]. Knockout of Nhx1
caused the accumulation of CPS in the prevacuolar compartment which blocks the destination of CPS to the vacuole. It has been described that trafficking of CPS from prevacuoles to the vacuole, multivesicular body structures (MVBs) [20] occurs (Fig. 5). In this process a series of proteins named VPS (vacuolar protein sorting) are involved [19, 21]. The first step involves Vps27. Vps27 knockout also caused accumulation of CPS in the prevacuolar compartments [20]. These results suggested that Nhxl1p might affect the process of Vps27p recruitment to the early endosomes by pH regulation of the endosomes. We successfully established an in vitro system of MVB formation and found that Nhxl1 knockout affects MVB formation in vitro [20] (Fig. 6). Vps27p recruitment on the early endosomes was also affected for the Nhxl1 knockout cells [20]. The most important finding was that for the in vitro MVB formation, acidic pH is better than neutral pH (Fig. 7). We further analyzed pH on the endosome surface facing to the cytoplasm by using chimeric protein of Vps27p and pHluorin, a pH indicator GFP variant. The results indicated that pH on the endosome surface is significantly more acidic than the cytoplasm detected by free pHluorin (Fig. 8). Based on these observations we concluded that acidic pH environment on the endosome surface provided by Nhxl1p is required for recruitment of Vps27p and acts as the trigger for MVB formation. This acidic pH may be also required for fusion of MBV to the vacuole. It was reported that Nhxl1p is required for the fusion of MVB to the vacuole although it is not shown how pH regulation by Nhxl1p is involved in the process [22]. It has been shown that Vps27p has an amino acid sequence called FYVE required for binding to phosphatidylinositol phosphate (PIP) [23]. We found that Vps27p binds to liposomes with PIP more efficiently in acidic pH conditions [24].

**Conclusion**

We found that acidic pH on the early endosome surface formed by Nhxl1p is an important factor for the recruitment of Vps27p, the first element of MVB formation. This finding reveals a new physiological role for Nhxl1p because pH regulation by Nhxl1 is
thought to be important for the luminal pH regulation. This observation also raises a new possibility of the importance of H\(^+\) localized in a certain area of the cells. In this connection two similar observations should be noted. Firstly, it is shown that local pH regulation by NHE5 in neuronal cells is important in the feedback regulation of NMDA (N-methyl-D-aspartate) receptors [25]. NHE5 shuttles between the endosomes and plasma membrane in nerve cells. NMDA induces accumulation of NHE5 molecules at the plasma membrane of the dendritic spine and causes decrease in the activity of receptors in the synapse. Thus the pH based negative feedback mechanism of NMDA receptor is proposed. Secondly, it has been described that plasma membrane type NHE1 provides a local microdomain with higher pH on the plasma membrane surface that induces recruitment of carcineurin B and subsequent enhancement of NFkB [26]. These two instances support the importance of local pH regulation in the cell physiology.

The precise molecular mechanisms regulated by NHE6 are still open to future studies. However, the new physiological role of Nhxi1 proposed here will give a clue for future studies to elucidate the roles of pH on the surface of the plasma membrane or endosome membrane by NHE6 in mammalian cells.

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