GENERAL INTRODUCTION

Eukaryotic cells are characterized by the presence of numerous functionally distinct membrane-enclosed organelles (Fig.1). The functional identity of the intracellular organelles is defined in large part by the composition of proteins. Therefore, the accurate and efficient delivery of proteins to a given organelle is of fundamental importance in establishing and maintaining its structure and function. In the biosynthetic pathway, proteins newly synthesized in the endoplasmic reticulum (ER) are transported through the Golgi apparatus to the *trans*-Golgi network (TGN) where they are sorted and delivered to their appropriate destinations, such as the plasma membrane, secretory granules and the endosomal system. On the other hand, in the endocytic pathway, extracellular and plasma membrane proteins, such as nutrients, growth factors and their receptors, are endocytosed and delivered to early endosomes. At these compartments, proteins are sorted and transported through late endosomes/multivesicular bodies (MVB) to lysosomes for their degradation, or transported to recycling endosomes to be recycled back to the plasma membrane. The TGN and endosomes also communicate with each other, and mechanisms must exist at the TGN or endosomes that recognize and divert proteins to distinct subcellular locations.

Transport between organelles of the endocytic or biosynthetic pathway mediated by transport vesicles. Carrier vesicles that capture cargo molecules bud from a donor compartment and deliver the contents by fusing with an acceptor compartment. The vesicle budding requires assembly of specific coat protein complexes onto the donor membrane. Clathrin, the first coat protein to be identified, is required for several types of vesicle formation at the TGN, endosomes, and the plasma membrane (for review, see Refs. 1, 2). A variety of adaptor protein (AP) complex is used for each type of vesicle. Transport vesicle formation is a multi-step process (Fig.2). In the case of clathrin/AP-1-coated vesicle, TGN membranes need to be 'primed' with the small GTPase,

ADP-ribosylation factor (ARF), before AP-1 can bind. ARF has a low affinity for membranes when bound to GDP, but when this is exchanged for GTP the membrane-binding conformation of the protein is stabilized. Specific exchange factors associated peripherally with Golgi membranes create a pool of ARF-GTP. Next, the cytosolic AP-1 complexes are assembled to the TGN membranes. Adaptor complexes interact directly or indirectly with sorting signals on cargo molecules, and ensuring the efficient and selective packaging of the cargo into the newly emerging transport vesicle. Clathrin also coassembled to the bud site through the interaction with adaptor complexes. Given the ability of clathrin to spontaneously self-assemble into cages and coats, it is generally assumed to play a major role in deforming the underlying membranes to form the budding vesicle. A fully sealed coated vesicle is formed when the emerging bud detaches from the TGN. Adaptor complexes and clathrin are removed from the vesicle (uncoating), and a transport vesicle is released for fusion with endosomes. Several factors that bind to adaptor complexes have been identified, and it is assumed that these factors regulate vesicular transport.

Four AP complexes have been identified so far and called as AP-1, AP-2, AP-3 and AP-4 (for review, see Refs. 3-6) (Fig.3). The AP-1 complex is found at the TGN and is responsible for delivery of lysosomal proteins to endosomes, although there have been lines of evidence indicating that AP-1 is also associated with endosomes (7-10) and with immature secretory granules in neuroendocrine cells (11, 12). The AP-2 complex is found at the plasma membrane and is involved in endocytosis of cell surface receptors. The AP-3 complex localizes at endosomes, and is involved in delivery to specialized organelles such as lysosomes and related organelles; melanosomes and platelet dense bodies (13). The AP-4 complex is involved in transport from the TGN to basolateral plasma membrane in polarized cells (14).

Each AP complex is a heterotetramer composed of two large subunits

(more than 100 kDa) often called as adaptins, one medium subunit (approximately 50 kDa) and one small subunit (approximately 20 kDa); γ- and β1-adaptins, μ1 and σ1 in AP-1: α- and β2-adaptins, μ2 and σ2 in AP-2: δ- and β3-adaptins, μ3 and σ3 in AP-3: and ε- and β4-adaptins, μ4 and σ4 in AP-4 (3-6). In addition, our group and others identified a γ-adaptin homologue, designated as γ2-adaptin (the original γ-adaptin was renamed as γ1-adaptin in consequence) (15, 16), which may constitute an AP-1 subtype in place of γ1-adaptin. On the basis of electron microscopic visualization of the AP-2 complex (17), adaptin polypeptides can be divided into two major domains; the NH_2-terminal head or trunk domain and the COOH-terminal ear or appendage domain, which are connected to each other by a proline-rich hinge region (Fig.3). Each AP subunit is thought to play specific roles. The μ subunits are responsible for recognition of tyrosine-based sorting signals within the cytoplasmic domains of transmembrane cargo proteins (18, 19; reviewed in Refs. 6, 20). β-adaptins are able to interact with clathrin heavy chain through the clathrin-binding motifs within their hinge regions and promote clathrin assembly (21-23). α-Adaptin, through its ear domain, is capable of interacting with a wide variety of cytosolic accessory molecules, which regulate the endocytic processes (24, 25). γ1-adaptin has recently been shown to interact with γ-synergin, the function of which is currently unknown (26).

To explore the regulatory mechanism underlying the transport from the TGN to endosomes, I searched for binding proteins of AP-1. In a yeast two-hybrid screening with γ1-adaptin as bait, I found that it is able to interact with Rabaptin-5. In CHAPTER I, I provide the first evidence for a direct interaction between γ-adaptins and Rabaptin-5 and address the physiological relevance of the interaction. The results show that the ear domain of γ1-adaptin binds Rabaptin-5. Subsequently, I reveal the structure of the ear domain of γ1-adaptin by X-ray crystallography by collaborating with Wakatsuki and

colleague, and define its binding site for Rabaptin-5 by two-hybrid and biochemical analyses (CHAPTER II). In the course of these studies, I also found that AP-1 localizes in recycling endosomes. The result raises a possibility that AP-1 can function not only on the TGN but also on recycling endosomes.

For a long time, clathrin/AP-1-coated vesicles were thought to form at the TGN and transport mannose-6-phosphate receptors (MPRs), the sorting receptors for lysosomal hydrolases that contain mannose-6-phosphate residues, to late endosomes (27). Several observations supported this idea. Firstly, AP-1 colocalizes with MPRs on the TGN in clathrin-coated areas (28). Secondly, by time-lapse imaging, AP-1 containing vesicles and tubules depart from the TGN and move to cell periphery (29, 30). Finally, KIF13A, which is plus-end directed microtubule-dependent motor protein and binds β1-adaptin, is involved in MPR transport from the TGN (31).

However, the study of AP-1-knockout mice implicated AP-1 in retrograde transport from endosomes to the TGN as well as anterograde transport (32). If AP-1 mediates anterograde transport from the TGN to endosomes, one would expect that in AP-1 knockouts the MPRs would get stuck in the TGN. However, this was not the case. The MPRs exit the Golgi, get transported to the plasma membrane and are re-endocytosed from there, accumulating in early endosomes. This indicates that AP-1 might mediate not anterograde, but retrograde transport. In fact, AP-1 has previously been shown to function in the removal of MPRs from immature secretory granules after their budding from the TGN (12). Endosomal localization of AP-1 also supports this idea. Clathrin/AP-1-coated vesicles might bud from early or recycling endosomes, and transport endosomal proteins back to the TGN. Are there any other molecules that are essential for transport from the TGN to endosomes?

In 2000, a novel family of monomeric clathrin adaptor proteins named GGAs (Golgi-localizing, γ-adaptin ear domain homology, ARF-binding

proteins), was identified (33-37) (Fig.4). There are three GGAs in humans, GGA1-GGA3. Among them, GGA3 has two splicing variants. GGAs are composed of four functional domains; The N-terminal VHS (Vps27p/Hrs/Stam) domain recognizes the acidic amino acid cluster-dileucine (ACLL) motifs found in the cytoplasmic domains of TGN sorting receptors such as MPRs (40-42); The GAT (GGA and Tom1) domain interacts with a GTP-bound form of the small GTPase ARF and is responsible for association of GGAs with TGN membranes (34,36,43); the proline-rich hinge region mediates recruitment of clathrin (38); and the C-terminal GAE (γ-adaptin ear homology) domain interacts with various accessory proteins (Ref. 44, and references therein). One isoform of GGA3 (GGA3-short) is unable to recognize ACLL sorting signal (42), for a deletion in its VHS domain. GGA3-short is expressed ubiquitously (45). Another isoform of GGA3 (GGA3-long), which has no deletion, is able to recognize MPRs (39). GGA3-long is expressed mainly in brain although its cDNA was originally cloned from the human kidney library (45). GGA proteins are localized at the TGN and binding partners of each domain implicated its functional similarities to AP-1.

In 2001, Bonifacino and colleagues showed that the VHS+GAT domain of GGA1 dominant negatively inhibits exit of the MPRs from the TGN (38, 39). I also obtained similar results using the VHS+GAT domains of GGA2 and GGA3-long. These results indicate that GGAs are indeed essential factors in clathrin-mediated trafficking from the TGN to endosomes of MPRs, which have long been assumed to be cargo proteins of AP-1 at the TGN. In contrast, now AP-1 is assumed to be essential for retrograde transport from endosomes to the TGN. However, these studies do not totally exclude the possibility that AP-1 functions in the TGN. For the present, it is not clear whether GGAs and AP-1 mediate transport in different directions, act in parallel pathways, or cooperate in the same transport steps. To explore the transport from the TGN, I also focused on GGAs.

In CHAPTER III, using pull-down and two-hybrid assays, I showed that the GAT domains of GGAs bind to ubiquitin. I delineate the molecular basis for the interaction between ubiquitin and GAT by collaborating with Wakatsuki and colleagues. By biochemical and immunocytochemical analyses, I address the issue whether GGA-mediated trafficking is regulated by the ubiquitin system as endosomal trafficking mediated by other ubiquitin-binding proteins. Furthermore, I report that GGA itself is ubiquitinated in a manner dependent on the GAT-ubiquitin interaction

FIGURES

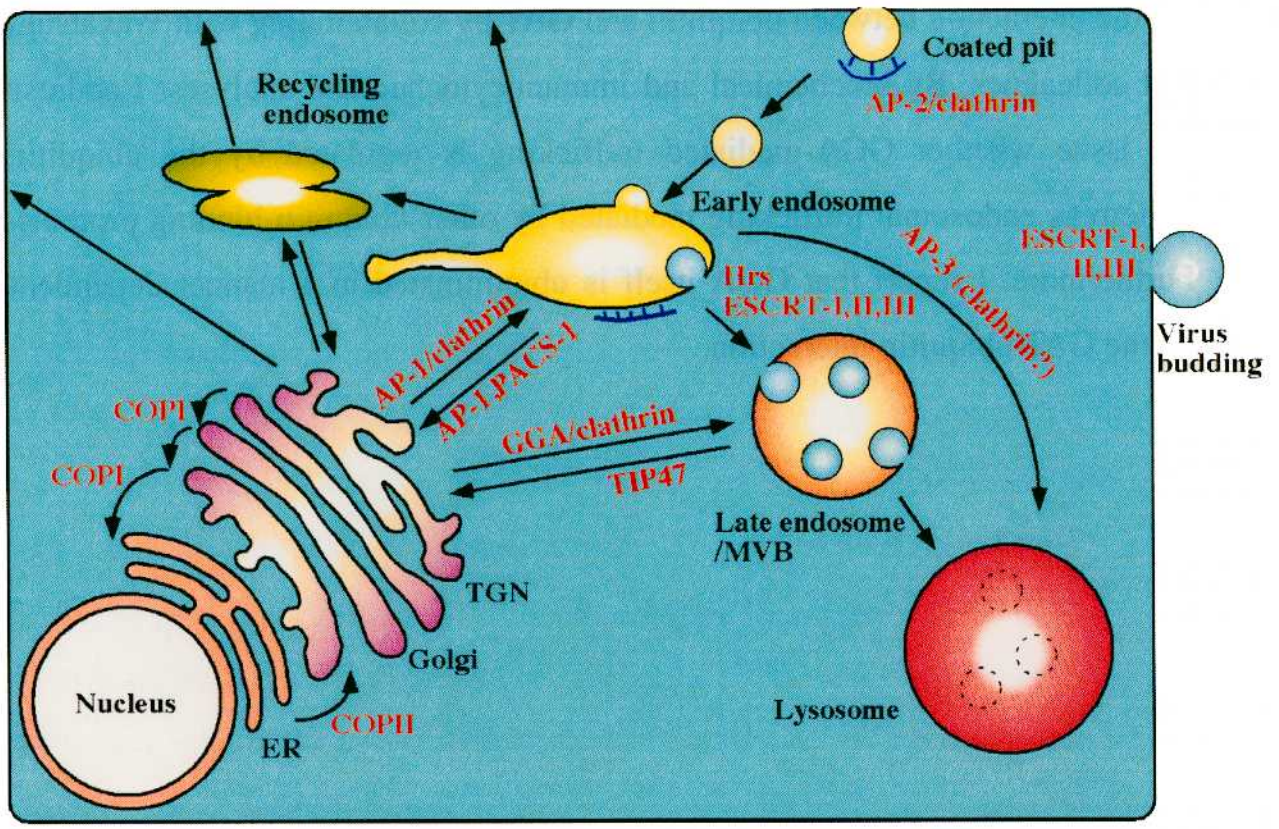

Fig. 1. Intracellular membrane traffic in mammalian cells. In the biosynthetic pathway, newly synthesized proteins are transported from the ER through the Golgi complex to the TGN, where the proteins are sorted to be delivered to the plasma membrane, endosomes, lysosomes, and secretory storage granules. In the endocytic pathway, macromolecules are internalized at the plasma membrane and delivered to early endosomes, from where they are forwarded to late endosomes/MVBs and lysosomes for degradation, or recycling endosomes to be recycled to the plasma membrane. COPI- and COPII-coated vesicles transport proteins between the ER and the Golgi complex. The clathrin pathway has two major routes, from the plasma membrane to early endosomes, and from the TGN to endosomes. Black letters describe the names of intracellular compartments, and red describe the names of proteins involved in trafficking along the pathways indicated by arrows.

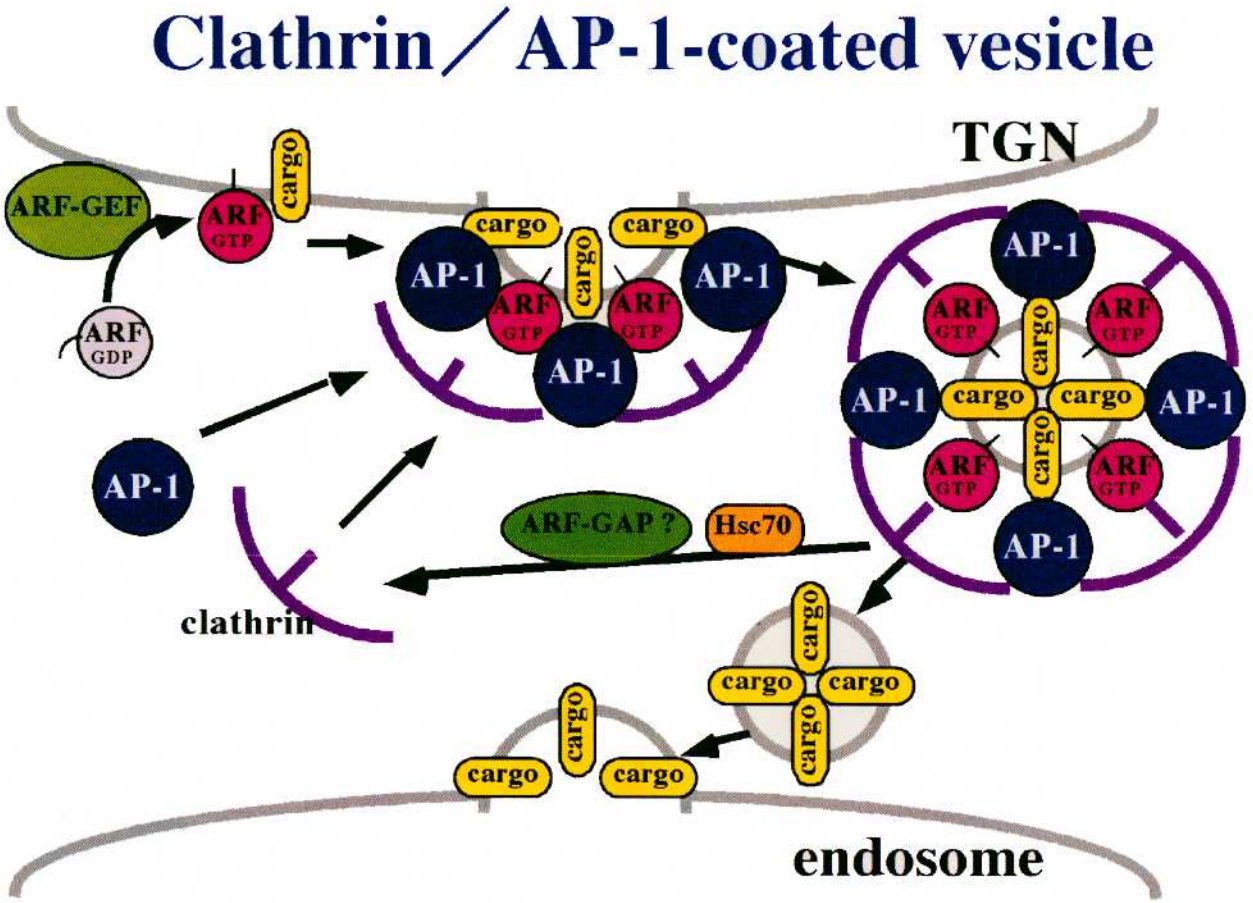

Fig.2. The key steps in the formation of clathrin/AP–1-coated vesicles. At the TGN, coat assembly is triggered by the recruitment of ARF1 onto the membranes. The AP-1 complex simultaneously binds to cargo molecules and coat components including clathrin. Clathrin triskelions polymerize into hexagons and pentagons, forming a cage, which leads to membrane deformation. When the coat almost completes, dynamin pinches off the vesicle. Uncoating requires ATP hydrolysis by the aid of Hsc70 and auxilin.

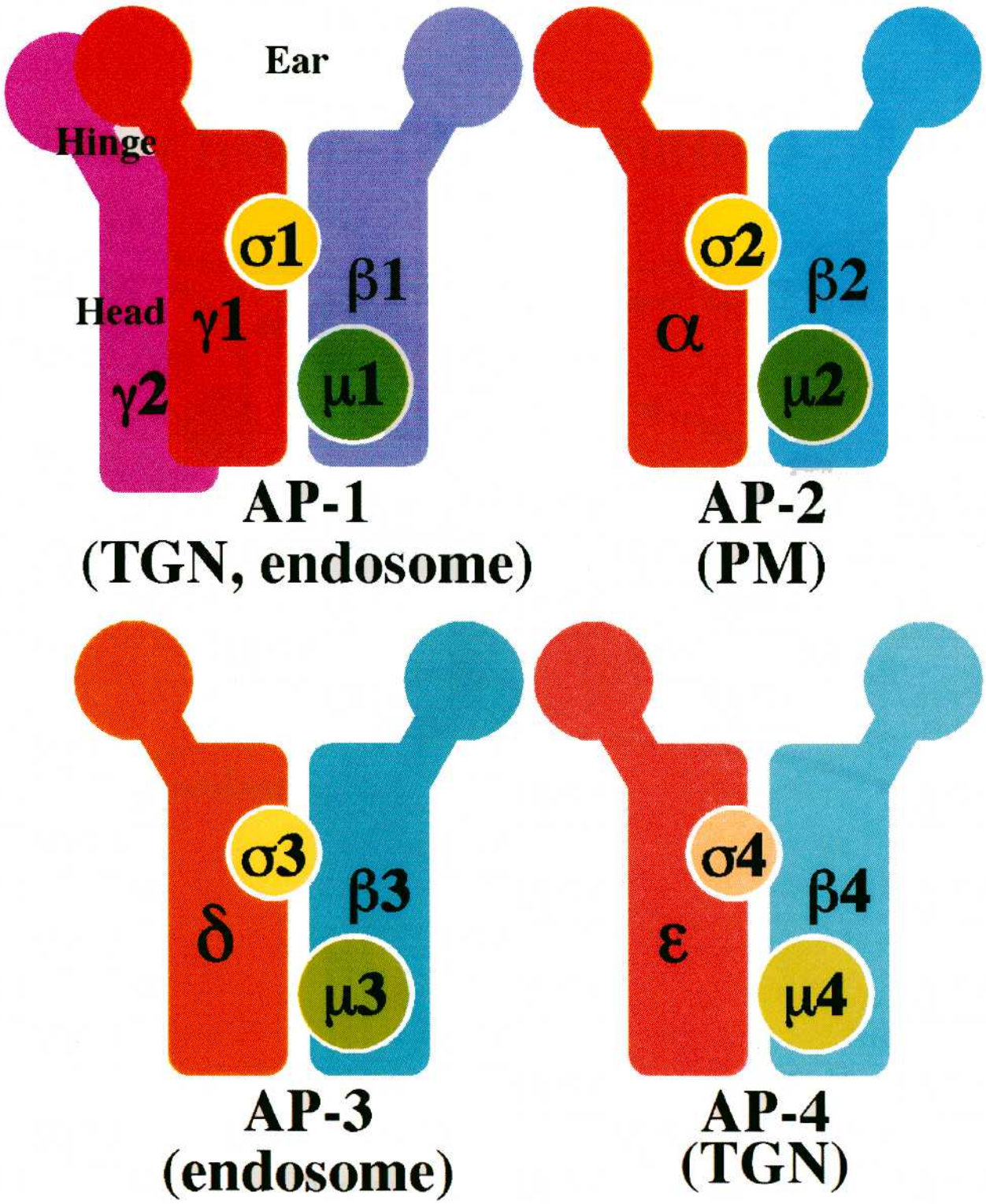

Fig. 3. Schematic diagrams of the adaptor and adaptor related complexes. Heterotetrameric adaptors that localize at different regions of the cell have a similar composition and apparent structure.

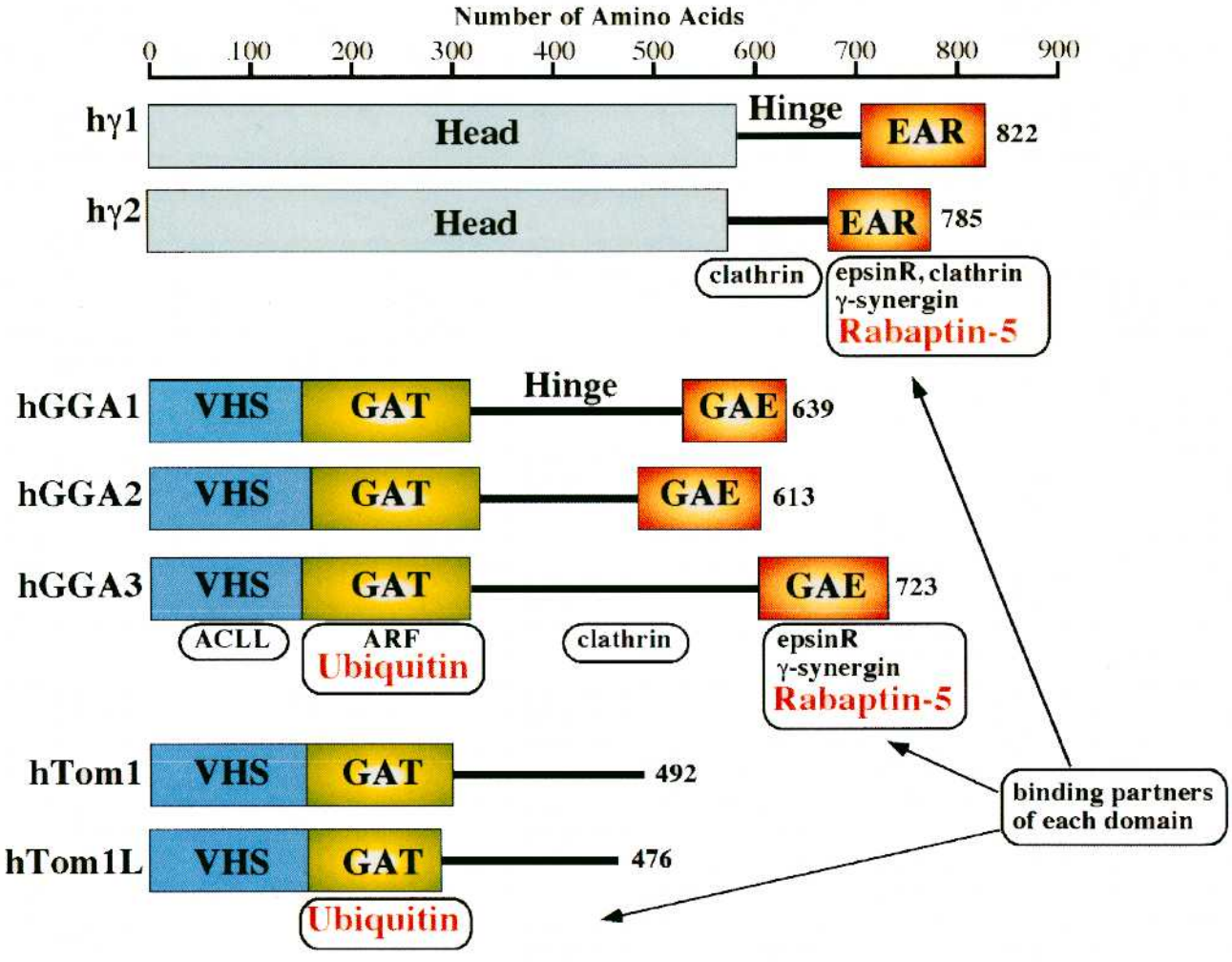

Fig. 4. Domain organization of GGAs and related proteins in humans. The total number of the amino acid residue of each protein is noted on the right. Specific domains or regions of homology are color coded. Black lines denote variable regions with no significant homology. Binding partners indicated by red letters were identified in this study.