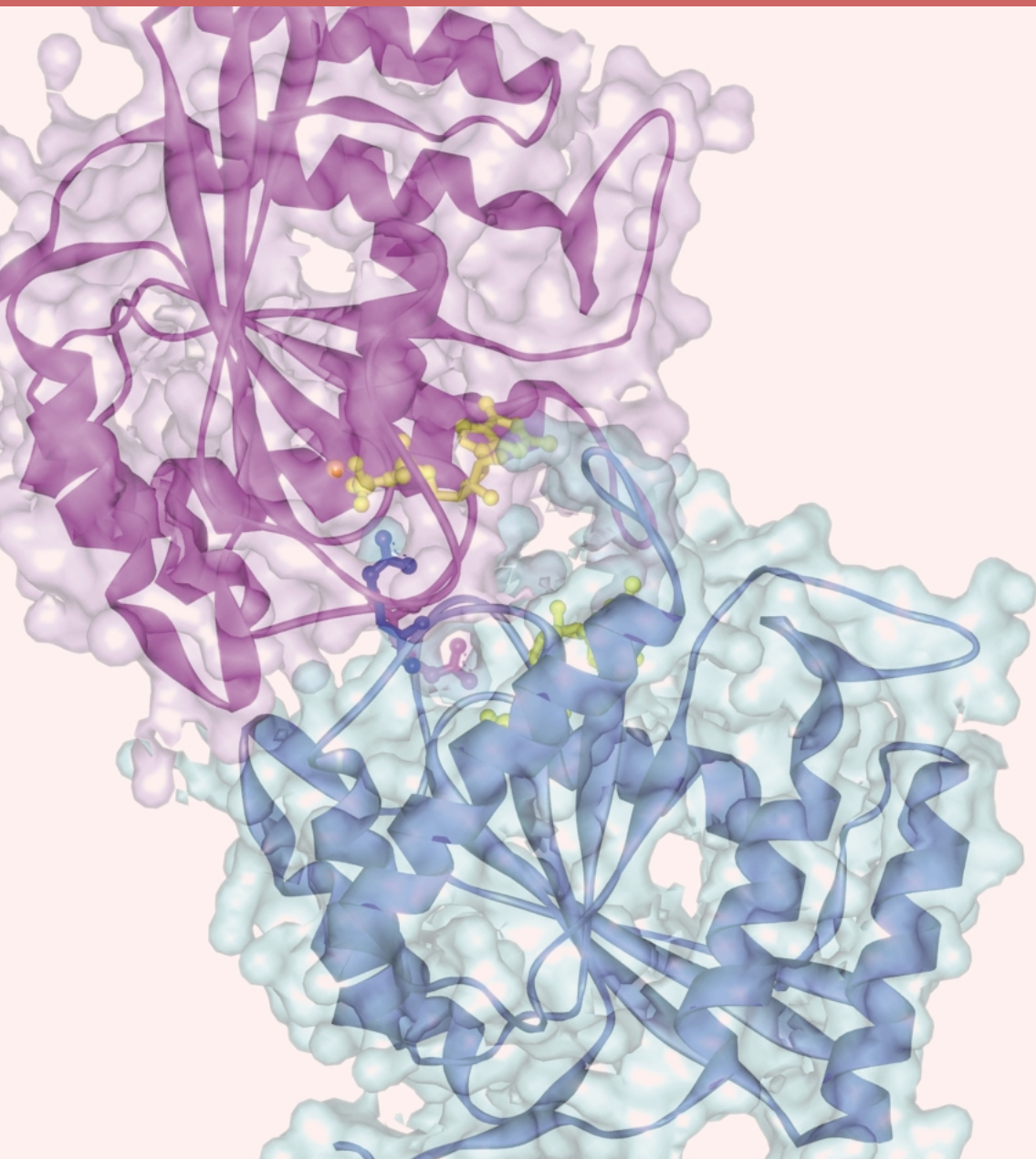*Highlights*

8-1 Structure of Toc34, a Novel GTPase of the Chloroplast Translocon Complex

Protein transport is vital to well-being of all living organisms. Newly synthesized proteins have to be modified and transported the correct destinations such as outside of the cell, nucleus, plasma membranes or various compartment knows as organelles. If proteins are mislocalised, they cannot function properly and thus it will lead to malfunction of organelles and diseases. Mitochondria and chloroplasts are examples of essential organelles; mitochondria produce ATPs which are used as chemical fuel of the cell while chloroplasts is the venue for photosynthesis. Both have their own DNA to synthesize the core proteins, but there are many important proteins that are coded by the cell's nuclear DNA, synthesized and incorporated into the organelles. Protein import machineries of the two organelles have many aspects in common, although that of mitochondria are better characterized.

Toc34, Toc159, and Toc75 are the most character-ized components of the Toc (translocon at the outer membrane of chloroplast) complex that associates with precursor proteins during protein transport across the chloroplast outer membrane. Toc34 and Toc159 are two homologous GTPases [1]. Substantial biochemical data suggest that there is a direct link between the function of these GTPases and the import of proteins into the main channel of the Toc complex, Toc75 [2]. The key receptor of the complex, Toc159, is believed to interact, via its highly acidic N-terminal domain, with the predominantly positively-charged transit region of a protein destined to be transported into chloroplasts. The role of Toc34, however, in the protein import is unclear. It may either regulate the function of Toc75, by its GTPase molecular switching or function by interacting with the G-domain of Toc159.

The crystal structure of Toc34 [3] (Fig. 1) has revealed a set of unique GTP binding (G) motifs (Fig. 2) that have not been observed in other GTPase structures reported to date. Excluding G1 (P-loop) of Toc34, which can be easily superimposed with that of the canonical
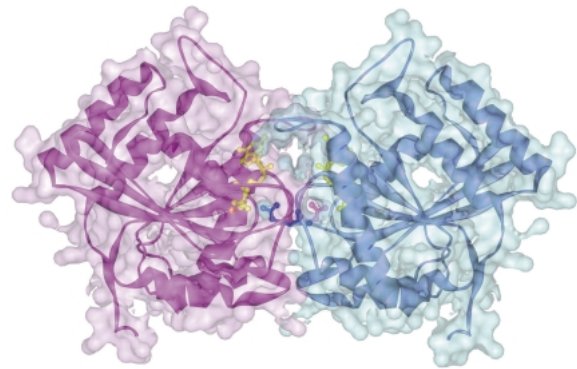

Figure 1
Ribbon diagram of the Toc34 dimer. The two GDP molecules are depicted as yellow ball-and-stick models. Side chains of the two Arg133s are shown in ball-and-stick models.
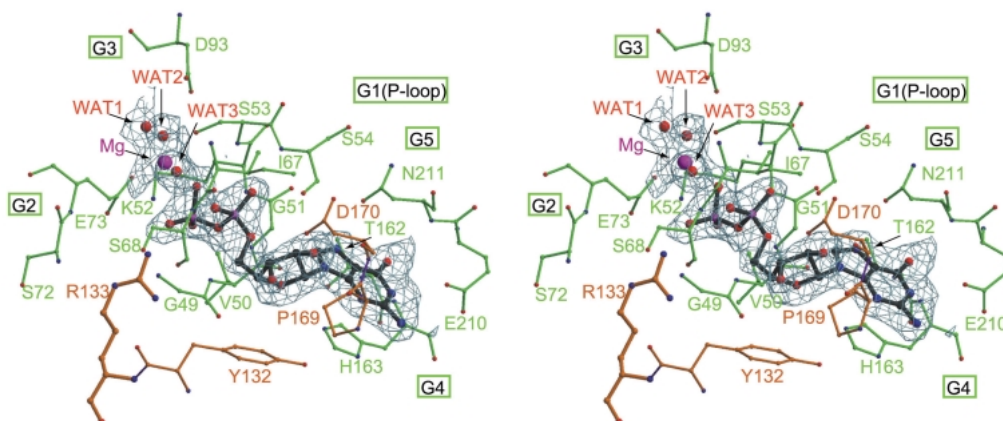

Figure 2
Stereo view of the GTP-binding site of Toc34. Ball-and-stick models of the residues involved in binding GDP are shown in green. Residues in orange are from the other interacting monomer. GDP is represented as a dark-grey ball-and-stick model, and an electron density map, after omitting GDP, Mg^{2+}, and three water molecules, is shown in blue.

GTPase, oncogenic p21 (Ras), the Toc34 and Ras G motifs do not share structural homology. For instance, the two strictly conserved residues of Ras, Thr35 and Asp119, whose counterparts have been observed in all other GTPases, are absent in Toc34. Although, Glu210 of Toc34, by making two hydrogen bonds with the exocyclic guanine ring, achieves a similar role to that of Ras Asp119, no obvious candidate is found to act as Thr35 of Ras in Toc34, mainly due to unavailability of the GTP-bound structure of Toc34. More strikingly, the presumed catalytic Gln61 of Ras, which directs a water molecule for nucleophilic attack on the oxygen of γ-phosphate, is replaced by Leu97 in Toc34, suggestive of a different mechanism of the GTP hydrolysis in Toc34 than that of Ras.

More important, the 28 kDa of Toc34 molecules, which lacks the C-terminal 52 residues including the trans-membrane domain (residues 266-284), were found as dimers, having a large surface area buried at the interface, in the crystal. The gel-filtration experiments also revealed that a third of the recombinant protein population existed as dimers in the solution at the physiological condition, implying a specific role for the dimerization. A close inspection of the dimer interface demonstrates that Arg133 of each interacting monomer hydrogen bonds to the β-phosphate oxygen of the GDP residing on the other monomer (Figs. 1&2). When Arg128, one of the key residues involved in the dimerization was mutated to Ala, the population of the Toc34 dimer decreased to undetectable number. The subsequent GTPase assay of the Arg128 mutant also showed a significantly reduced GTPase activity compared to that of the wild type. This suggests that the dimerization stimulates the two interacting GTPases. A similar result was obtained from the mutation of Arg133 to Lys, which resulted in a negligible reduction of the dimer population, but the mutant showed a pronounced reduction of the GTPase activity, comparable to that of the Arg128 mutant. The mutation of two arginines thus suggests that each interacting Toc34 monomer acts as a GTPase activating protein on the other one; and Arg133 is the "arginine finger".

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8-2 Molecular Functional Analysis of Regulatory Transcription Factor, CIB, Based on its X-ray Crystal Structure

In eukaryotes, transcription is regulated essentially in three distinct steps. The first step is the alteration of chromatin structure through modification and remodeling of chromatin components. The nucleosome, a fundamental repeating unit of chromatin consisting of a precise stoichiometry of histones and DNA, is a major target of structural changes in chromatin. The second step is the binding of regulatory transcription factors to promoter/enhancer element. The third step is the regulation of RNA polymerization by RNA polymerase II and several general transcription initiation factors.

General transcription initiation factor TFIID is a multi-protein complex composed of a TATA box-binding protein (TBP) and TBP-associated factors (TAFs), and plays a central role in both basal and regulatory transcription from naked DNA templates. TFIID binds to the TATA box of the core promoter and initiates transcription by forming a preinitiation complex with other general transcription initiation factors and RNA polymerase II. It also interacts with various kinds of regulatory transcription factors as well as cofactors and activates transcription through cooperation with them. In addition, further studies support an idea in which TFIID also plays a key role in transcriptional regulation from chromatin DNA templates.

Considering the characteristics of TFIID described above, we assumed that interactors of TFIID should also be chromatin factors and/or transcriptional regulators/co-factors. Thus, we isolated a number of proteins that interact with TFIID and showed that one of the TAF
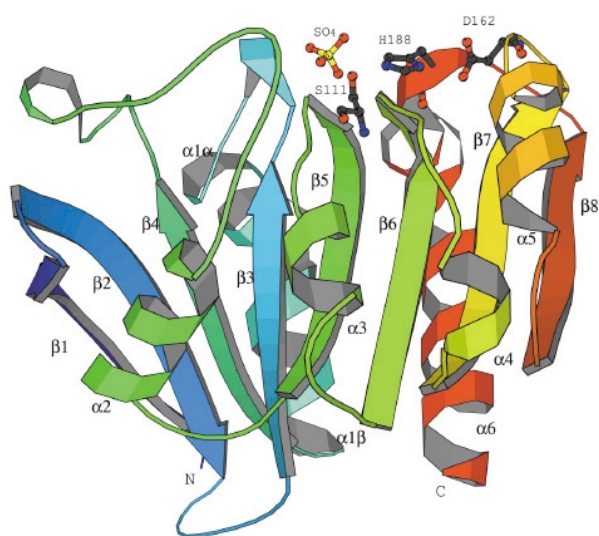

Figure 3
X-ray crystal structure of CIB. The overall fold is similar to that of prokayotic α/β-hydrolase family proteins.
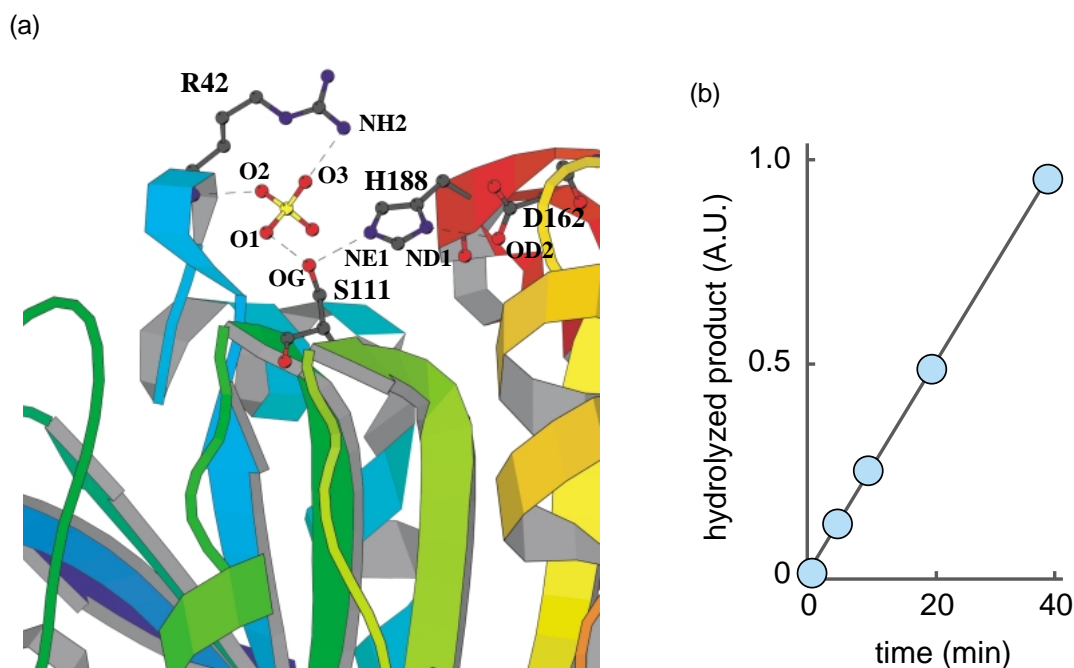
(a)

(b)

Figure 4
(a) X-ray structure of CIB is expanded near the active site. Side chains of active site amino acid residues and a sulfate ion are shown by ball-and-stick model. Interactions between the atoms are indicated by dotted lines.
(b) Time course of hydrolase activity of CIB was measured by using a *p*-nitrophenyl butyrate as a substrate.

interactors is a MYST-type histone acetyltransferase Tip60, and one of the proteins that interact with CCG1/TAF$_{II}$250 is a histone chaperone CIA. They are highly conserved among species and regulate several nuclear events including DNA replication, DNA repair, transcription, silencing and apoptosis, indicating their important roles in chromatin.

CIB (CCG1/TAF$_{II}$250 interacting factor B) is a novel factor that we have identified through screening of proteins that interact with the conserved histone acetyltransferase domain of CCG1/TAF$_{II}$250 using the yeast two-hybrid system. The primary sequence of this 22 kDa protein does not show any similarity with those of any transcription/chromatin factors known to date. To understand the functional role of CIB, we have determined the crystal structure of CIB refined at 2.2 Å resolution. The fold of the presented structure is similar to that of prokaryotic α/β-hydrolase family proteins, even though less sequence homology has been observed between them (Fig. 3).

Based on comparative studies of the primary and tertiary structures of CIB, we tested its hydrolase activity using *p*-nitrophenyl butyrate, a typical substrate for hydrolases. As shown in Fig. 4, the product obtained upon hydrolysis by CIB increased in a time-dependent manner. It indicates that CIB possesses hydrolase activity, as we predicted. To our best knowledge based on the data base search, CIB is the first protein that possesses α/β-hydrolase fold in eukaryotic transcription/chromatin factors. Our mutagenesis studies indicate that the catalytic triad in CIB is not essential for transcriptional activation. Therefore CIB has two functional surfaces

in the α/β-hydrolase fold; one is a catalytic triad for hydrolase activity and the other is a transcriptional activation domain.

Therefore, transcriptional activity at a certain level might be modified and regulated through the involvement of the α/β-hydrolase function of CIB. Identification of substrate(s) for CIB and the analysis of complex crystal structures of CIB with its substrate(s) and/or with CCG1/TAF$_{II}$250 will illuminate the novel transcriptional regulatory mechanism involving the nuclear hydrolase enzyme.

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8-3 Structural Basis for Molecular Mechanisms of Selective Transport of Cargo between Cellular Organelles

In living cells of higher organisms, lipids and proteins are transported between various cellular compartments called organelles via vesicles and tubulo-vesicular carriers. The process proceeds through a complicated series of events such as the controlled formation of transport vesicles that bud from the donor membrane, and targeting and fusion of the vesicles with the acceptor membrane. In analogy to zip codes used in postal services, correct recognition of sorting signals at the beginning of the process is essential for correct delivery of cargo molecules [1].
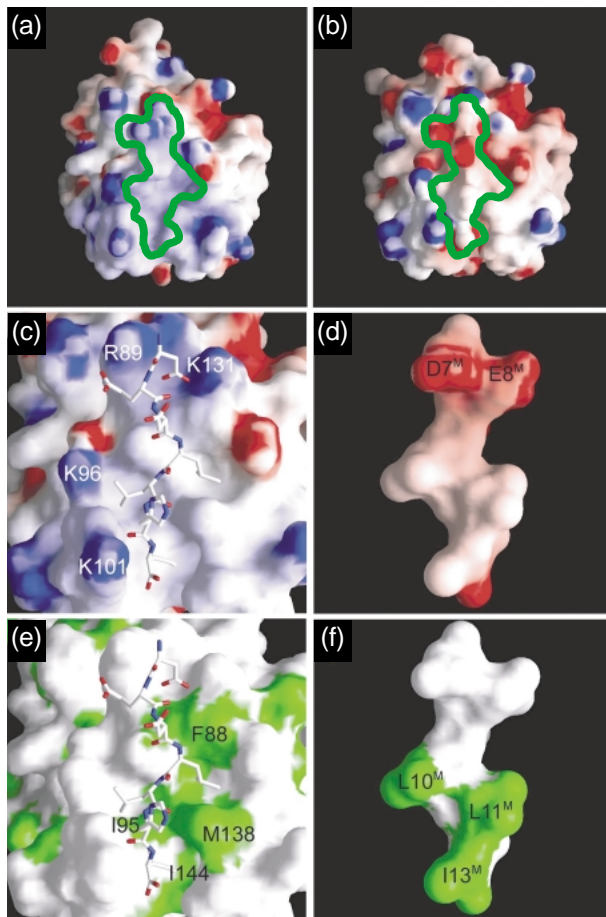
Mannose 6-phosphate receptors (MPRs) recognize

the mannose 6-phosphate groups on lysosomal hydrolases and transport them from trans-Golgi network (TGN) to endosomes [1,2]. Traditionally an adaptor protein called AP-1 was thought to recognize cargo-loaded MPRs and transport them to early lysosome with the help of clathrin protein complexes. In 2000, a family of transport proteins, GGAs, were identified and later in 2001 it was shown by several groups simultaneously that GGAs, instead of AP-1, facilitate this transport process [3,4]. GGAs are composed of 4 regions: (i) the N-terminal VHS domain which interacts with the MPRs, (ii) the GAT domain which binds to a membrane anchored GTPase, ARFs in the GTP form, (iii) the proline rich hinge region which interacts with clathrin, and (iv) the GAE domain, a docking site for different accessory proteins.
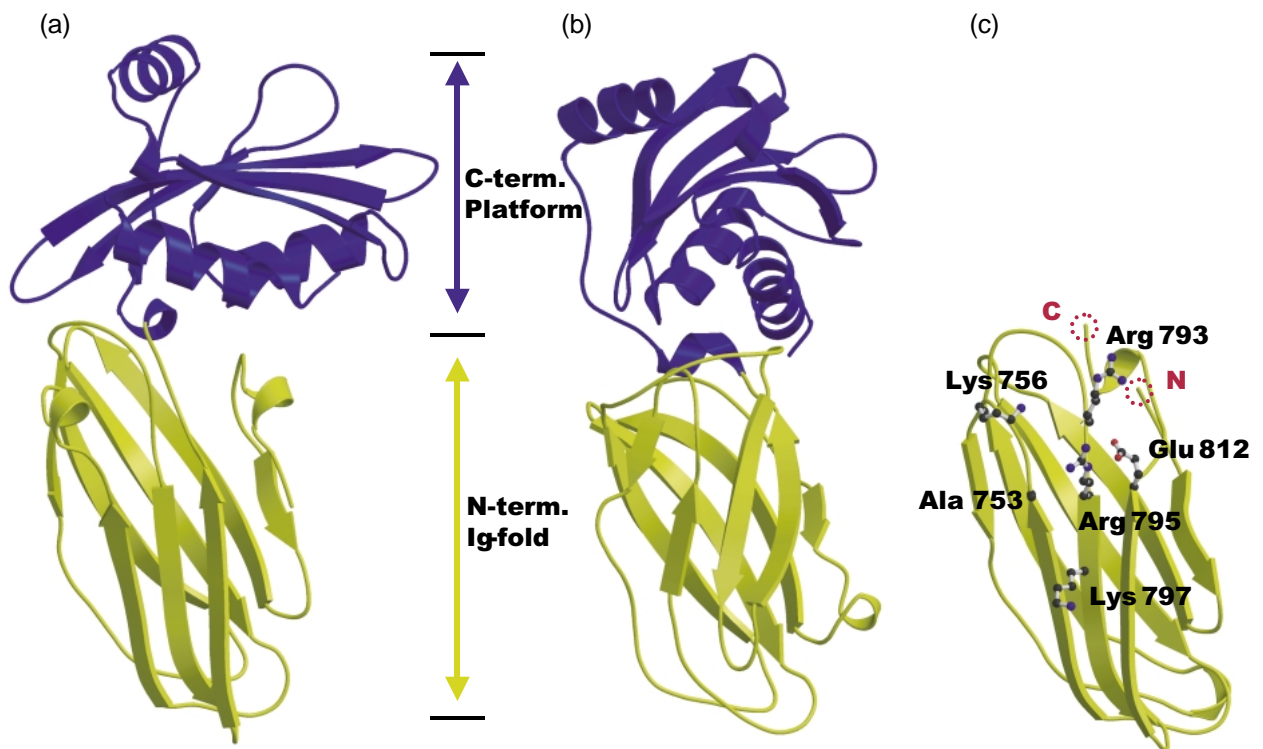

Figure 6
Structures of the α-adaptin ear, β2-adaptin ear and γ1-adaptin ear domains. (a) Ribbon diagram of the mouse α-adaptin ear domain (PDB entry 1B9K). (b) Ribbon diagram of the human β2-adaptin ear domain (PDB entry 1E42). (c) Ribbon diagram of the human γ1-adaptin ear domain (this work).

The N-terminal VHS domain recognizes the acidic-cluster dileucine (ACLL) sequence, a sorting signal in the cytoplasmic domain of MPRs. We determined the X-ray crystal structures of GGA1-VHS domain, in the apo-form and in complex with a 13-residue ACLL peptide from MPR [5]. The GGA1-VHS domain forms a right-handed super helix with eight α-helices. The hydrophobic core in the center stabilizes the super helix and there is only slight conformational change in the overall structure upon binding of the ACLL peptide. The crystal structure shows that the helices $\alpha6$ and $\alpha8$ and the loop immediately after $\alpha6$ are responsible for recognition of the ACLL sequence (Fig. 5(c)-(f)). This was confirmed by a mutant analysis [5]. The binding of the ACLL peptide to the GGA1-VHS domain changes the surface properties of the protein significantly (Fig. 5(a), (b)). This change might provide a signal to other events of membrane traffic, for instance, the interaction between the GGA-GAT domain and membrane bound ARF1, recruitment of clathrin molecules, or interaction with accessory proteins (see below) that all play important roles in the highly selective transport processes.

One of the adaptor protein complexes, AP-1, is composed of four subunits: $\gamma1$-, $\beta1$-, $\mu1$- and $\sigma1$-adaptins. The C-terminal protruding part of $\gamma1$-adaptin, referred as an ear domain, is involved in the recruitment of accessory proteins, such as γ-synergin and Rabaptin-5, which modulate the functions of AP-1 in the intracellular transport [6]. We determined the X-ray crystal structure of human $\gamma1$-adaptin ear domain [7]. The $\gamma1$ ear domain forms an immunoglobulin-like β-sandwich fold composed of eight β-strands with two α-helices (Fig. 6). The $\gamma1$ ear domain is similar to the N-terminal subdomains of the α- and $\beta2$-adaptin ear domains of the AP-2 complex, but it lacks their C-terminal platform subdomain. The crystal structure and the mutant analysis of $\gamma1$ ear clearly show that the surface basic cluster is indispensable for the recruitment of γ-synergin or Rabaptin-5. These findings indicate that $\gamma1$-adaptin ear and its homologue GGA ear domains interact with the accessory proteins in a manner different from that observed in α-adaptin of the AP-2 complex, which is predominantly hydrophobic.

T. Shiba[1], T. Nogi[1], N. Matsugaki[1], M. Kawasaki[1], N. Igarashi[1], M. Suzuki[1], R. Kato[1], H. Takatsu[2], Y. Shiba[3], K. Nakayama[3], T. Earnest[4] and S. Wakatsuki[1] ([1]KEK-PF, [2]RIKEN, [3]Univ. of Tsukuba/Kanazawa Univ., [4]Adavance Light Source, USA)

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8-4 Crystal Structure of Human Autocrine Motility Factor

Cell motility in response to extracellular signals plays a central role in diverse biological phenomena, including spermatogenesis, morphogenesis, wound healing, inflammation, angiogenesis, and metastasis. A group of cytokines that stimulate tumor cell motility in an autocrine and/or paracrine fashion has been reported. Among them, autocrine motility factor (AMF) was originally identified by its ability to stimulate directional motility (chemotaxis) and random motility (chemokinetics) of the AMF-producing tumor cells [1]. The AMF stimulates cell motility via a receptor-mediated signaling pathway. Partial amino acid sequencing of mouse AMF and full-length cDNA cloning of human AMF have identified that the AMF (558 amino acid residues) is genetically identical to extracellular cytokines neuroleukin (NLK) and maturation factor (MF) and, interestingly, to an intracellular enzyme phosphohexose isomerase (PHI) [2]. In 1999, full-length cDNA cloning for both human and mouse AMF receptor (AMFR) genes was reported and revealed that the AMFR is a novel type of seven-transmembrane helix protein [3]. Quite recently, another role of AMF has been revealed: it not only stimulates AMF-producing tumor cell motility in an autocrine manner, but it also acts as a paracrine factor to vein endothelial cells to induce angiogenesis with cell motility stimulation and may facilitate metastasis through these effects during the metastasis phase [4].

The crystal structures of the inhibitor-free open form and the inhibitor (erythrose 4-phosphate, E4P, a strong inhibitor of AMF's cytokine activity)-bound closed form of human AMF have been determined at 1.9 Å and 2.4 Å resolution, respectively, as shown in Fig. 7 [5]. Upon E4P binding, local conformational changes (open to closed) occur around the inhibitor-binding site. The E4P-bound structure shows that the location of the inhibitor (for cytokine activity) binding site of human AMF is very similar to that of PHIs (for enzymatic activity). The present study clearly shows that there is structural overlapping between the regions responsible for the cytokine and enzymatic functions of AMF/PHI. Two scenarios for the inhibition mechanism of cytokine activity of AMF by the carbohydrate phosphate could be proposed. One likely scenario is that the compound could compete for AMF binding with the carbohydrate moiety of the AMF receptor (AMFR), which is a glycosylated seven-transmembrane helix protein. The other scenario is that the local conformational changes upon inhibitor binding
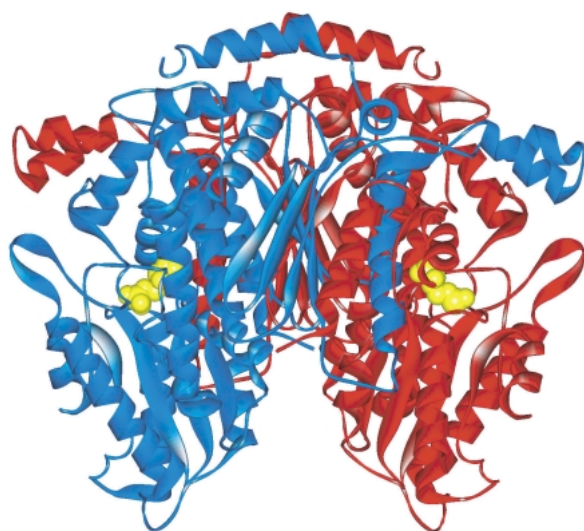
Figure 7
Dimeric structure of human AMF / erythrose 4-phosphate (E4P) complex. One subunit is colored blue, the other is colored red. The molecules of bound E4P (yellow) are shown as space-filling models for each of the two subunits.

may affect the AMF-AMFR interaction. To examine roles of the residues in the inhibitor-binding site, two mutant AMFs were prepared. Replacements of His389, which is hydrogen-bonded to the hydroxyl group of E4P, by Phe, and Thr215, which is hydrogen-bonded to the phosphate group of E4P, by Asp, lost the cytokine activity. These results suggest that these amino acids recognize a carbohydrate moiety of the AMFR. Since the E4P is one of the smallest compounds having AMF inhibitor activity, knowledge of the present crystal structure would provide an insight into the lead compound design of more effective AMF inhibitors.

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