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Rab14 is part of the early endosomal clathrin-coated TGN microdomain

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Abstract **Rab14 localizes to the Golgi/TGN and to early endosomes, but its biological function remains unclear. By structural modeling, we identified Rab14-specific residues and established a close relationship between the Rab2/Rab4/Rab14, Rab11/25 and Rab39 sub-groups within the Rab protein family. By quantitative confocal microscopy and by density centrifugation we show that Rab14 is part of the early endosomal AP-1 microdomain. Overexpression of a dominant-negative Rab14 GTP-binding mutant that solely localizes to the Golgi donor compartment accelerated EGF degradation. We suggest that the AP-1 microdomain represents the interconnecting compartment in which Rab14 vesicles cycle between early endosomes and the Golgi cisternae.**

Keywords: Rab14; AP-1; Hrs; Cluster analysis

1. Introduction

Rab GTPases represent a branch of Ras-like GTP-binding proteins and are localized at the cytoplasmic face of distinct membrane-bound organelles reviewed in [1–3]. Rab proteins have key functions in intracellular trafficking in eukaryotic cells, controlling accurate fission and fusion of transport vesicles with their target membranes during biosynthetic/secretory and endocytotic pathways [4–8]. As reflected by their compartment-specific intracellular localization, Rab proteins function in endoplasmatic reticulum (ER)-Golgi (Rab1, Rab2) or in retrograde Golgi-ER (Rab6, Rab2) traffic. Golgi/*trans*-Golgi network (TGN) transport is mediated by a large group of Rab proteins, such as Rab10, Rab12, Rab13, Rab30, and Rab33b. The secretory vesicle pathway is regulated by Rab8 and Rab26, and some Rab proteins are specialized for synaptic vesicles (Rab 3a, b, c, d) or synaptic granules (Rab26). Endocytosis is mediated by Rab5, and early endosome traffic is regulated by Rab4, Rab15, Rab18, Rab20 and Rab22. Thereby, Rab4 functions at the stage of early sorting endosomes and mediates receptor recycling to the plasma membrane [9,10]. Rab11, Rab25 and Rab17 are associated with recycling endosomes and Rab7, Rab9 and Rab24 move endosomes through the late endosome stage to lysosomal degradation (reviewed in [3]).

Rab14 is ubiquitously expressed and localizes to the Golgi/TGN and at early endosomes [11]. We show here that Rab14 decorates approximately 50% of all AP-1 vesicles, and 50% of all Hrs or EEA1 positive early endosomes. Unlike overexpression of Hrs [12], overexpression of Rab14 did not interfere with EGF degradation, but a dominant-negative GTPase binding deficient Rab14 mutant accelerated the degradation of EGF. We further present a comprehensive bioinformatic analyses of Rab14.

2. Materials and methods

2.1. Selection of Rab sequences

A converged PSI-BLAST [13] search with Rab14 (AAF00150) and an inclusion threshold ($-h$) of 0.05 was used to determine Rab sequences. Using CLANS [14], full-length sequences were extracted for all high scoring segment pairs (HSP's) up to *E*-values of 1, and clustering returned a delineated group for Rab proteins consisting of 1186 sequences. BLAST searches were performed for all 1186 representatives and full length sequences extracted for all HSP's with *E*-values better than 1e-5.

2.2. Sequence alignment, determination and visualization of relevant residues

Sequences from Rab2, Rab4, Rab14, Rab11, Rab25 and Rab39 were aligned using MUSCLE [15]. Inserts and the highly variable C-terminal region of Rab sequences were removed, as these could not be reliably aligned. Refined sequences were combined and manually aligned to one another. Using HHPred [16] and our human Rab14 cDNA isolate (AAF00150), the best hit (1UKV_Y) was selected as template to create a structural Rab14 model. To determine residues with high conservation within Rab14 but low similarity to Rab2 or Rab4, or high conservation within Rab2/4/14 and low similarity to Rab39 and Rab11/25A, sequence profiles for each group were derived and compared. Using ALNEdit and Rasmol, these residues were visualized in alignment and structural view (residue assignments for nucleotide binding, complementary determining and switch regions taken from [17–19]).

2.3. Transient transfections and quantitative confocal microscopy

According to Proikas-Cezanne [20] transient transfected HeLa cells (ATCC) were used for indirect immunofluorescence and analyzed by confocal microscopy using a LSM510 microscope (Zeiss) and a 63×1.4 DIC Plan-Apochromat oil-immersion objective. Antibodies: anti-EEA1, anti-adaptin γ (BD Biosciences), anti-Lamp2 (Santa Cruz),

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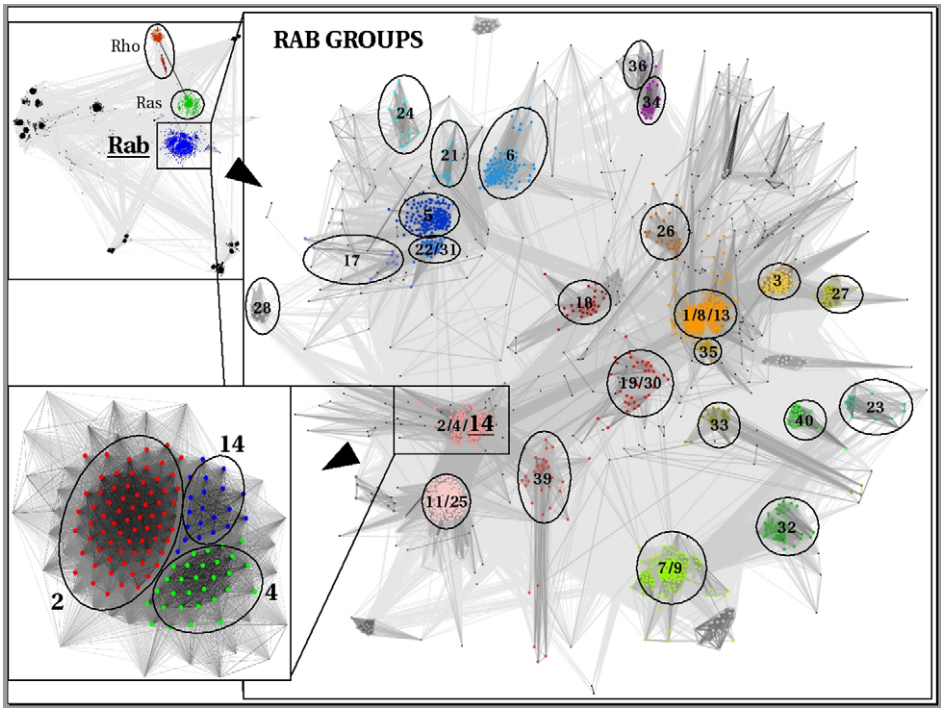

Fig. 1. Cluster analysis of human Rab14 protein and 2-dimensional representation of all pair wise sequence similarities. Sequences with high similarity are connected by dark lines and lie close together, sequences with less similarity are connected by lighter lines and lie further apart. Clusters for Ras, Rho and Rab sequence representatives (top left). Rab proteins and Rab sub-groups (right). Refined clustering of the novel Rab2/4/14 sub-group (bottom left).

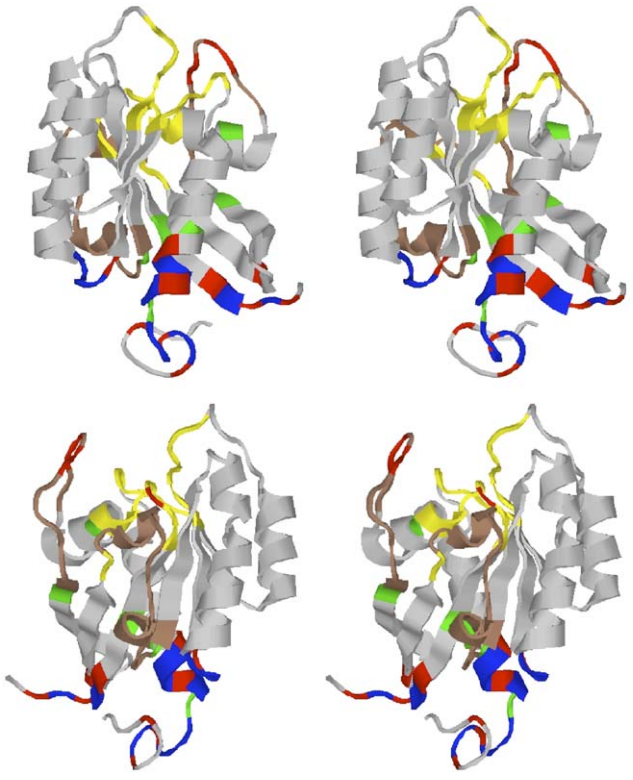

Fig. 2. Stereographic representation of a Rab14 structure model missing the hypervariable C-terminal region. Yellow: residues involved in nucleotide binding; blue: residues corresponding to the Rab3A complementary determining region; brown: residues belonging to the switch1 and switch2 regions; red: novel Rab14-specific residues; green: novel rab2/4/14 sub-group specific residues. The bottom image shows a view of the structure rotated around the *Y*-axis. The Rab2/4/14 specific residues appear buried in the structure and in close proximity of one another on three of the five beta strands (beta 1, 2, 4) and the C-terminal alpha helix. Mostly, amino acid side chains of the Rab2/4/14 specific residues are placed towards the center of the protein, indicating that these residues play a role in both protein folding ability and protein stability. This, in turn, points to these residues being slowly evolving, and the grouping of these sequences being due to features shared because of common ancestry instead of evolutionary convergence.

anti-Rab7 (Sigma), anti-GFP (Roche), Alexa Fluor 546 goat anti-mouse or anti-rabbit IgG (Molecular Probes). Respectively, Lysotracker and TO-PRO-3 (Molecular Probes) were used to stain lysosomes and cell nuclei. According to Raiborg et al. [12], endogenous Hrs was detected using anti-Hrs antibody (H. Stenmark, The Norwegian Radium Hospital, Oslo, Norway).

Using Image-Pro Plus 4.5 (MediaCybernetics) for the quantification of co-localizing vesicles, images from 3 to 4 independent experiments (10 transfected cells from each experiment) were analyzed.

2.4. EGF receptor degradation assay

Degradation of Alexa Fluor 555 labeled EGF (Molecular Probes) was assayed according to Raiborg et al. [12] and the percentage of undegraded EGF calculated from images taken from 10 pEGFP.C1 vector, 10 pEGFP.C1-Rab14 or 10 pEGFP.C1-Rab14S25N transfected cells at each time point. The mean intensity of the Alexa Fluor 555 labeled EGF was quantified using Image-Pro Plus 4.5 software (MediaCybernetics).

2.5. Subcellular fractionation

Subcellular fractionation was conducted using Optiprep (Axis-Shield) according to the manufacture's protocol S23. Using a Teflon