Characterization of the topology and functional domains of RKTG

Xiaolin LUO*, Lin FENG*, Xiaomeng JIANG*, Fei XIAO*, Zhenzhen WANG*, Gen-Sheng FENG† and Yan CHEN*†

*Key Laboratory of Nutrition and Metabolism, Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Graduate School of the Chinese Academy of Sciences, 294 Taiyuan Road, Shanghai 200031, China, and †Program in Signal Transduction, Burnham Institute for Medical Research, La Jolla, California 92037, U.S.A.

RKTG (Raf kinase trapping to Golgi) is exclusively localized at the Golgi apparatus and functions as a spatial regulator of Raf-1 kinase by sequestrating Raf-1 to the Golgi. Based on the structural similarity with adiponectin receptors, RKTG was predicted to be a seven-transmembrane protein with a cytosolic N-terminus, distinct from classical GPCRs (G-protein-coupled receptors). We analysed in detail the topology and functional domains of RKTG in this study. We determined that the N-terminus of RKTG is localized on the cytosolic side. Two short stretches of amino acid sequences at the membrane proximal to the N- and C-termini (amino acids 61–71 and 299–303 respectively) were indispensable for Golgi localization of RKTG, but were not required for the interaction with Raf-1. The three loops facing the cytosol between the transmembrane domains had different roles in Golgi localization and Raf-1 interaction. While the first cytosolic loop was only important for Golgi localization, the third cytosolic loop was necessary for both Golgi localization and Raf-1 sequestration. Taken together, these findings suggest that RKTG is a type III membrane protein with its N-terminus facing the cytosol and multiple sequences are responsible for its localization at the Golgi apparatus and Raf-1 interaction. As RKTG is the first discovered Golgi protein with seven transmembrane domains, the knowledge derived from this study would not only provide structural information about the protein, but also pave the way for future characterization of the unique functions of RKTG in the regulation of cell signalling.

Key words: Golgi apparatus, progesterone and adipoQ receptor (PAQR), Raf-1, RKTG.

INTRODUCTION

PAQR (progesterone and adipocytokine receptor) family proteins are a group of transmembrane proteins that are broadly expressed in many species, including archaebacteria, Eubacteria, Caenorhabditis elegans and mammals [1]. The high conservation of PAQR family members among different species indicates the evolutionary importance of the family [1]. In mammalian genomes, the PAQR family is composed of 11 members, PAQR1–PAQR11 [1]. All PAQR proteins are predicted to have seven TMDs (transmembrane domains) with an intracellular N-terminus and an extracellular C-terminus, distinct from classical GPCRs (G-protein-coupled receptors) in topology [1]. The functions of some members of PAQR family have been characterized previously. PAQR1 and PAQR2 (or AdipoR1 and AdipoR2) were reported to be receptors for adiponectin, which plays an important role in glucose metabolism [2]. PAQR5, PAQR7 and PAQR8 were found to be receptors for progestin [3,4].

Raf kinase relays the signals from Ras to MEK [MAPK (mitogen-activated protein kinase)/ERK (extracellular signal-regulated kinase) kinase] and ERK/MAPK [5,6]. This pathway regulates many fundamental cellular functions including cell proliferation, apoptosis, differentiation, motility and metabolism. Dysregulation of the components within the Ras/Raf/MEK/ERK pathway is implicated in many human diseases and especially in cancers [7]. Recently we demonstrated that PAQR3, another member of the PAQR family, is a spatial regulator of Raf-1 by sequestrating Raf-1 to the Golgi apparatus, blocking the signal transduction of Raf-1 to downstream targets. Because of its unique function, PAQR3 was named RKTG (Raf kinase trapping to Golgi) [8].

The Golgi apparatus has two major biological functions [9]. One of them is to serve as a major sorting machinery in the secretory pathway, and the other function is to modify exportable and lysosomal proteins. The Golgi is organized by three functionally distinct subcompartments, the cis-Golgi network, the Golgi stack and the trans-Golgi network [9,10]. In the Golgi apparatus, exportable proteins are sequentially modified by processes with N- and O-linked oligosaccharides [11], proteolytic cleavage [12] and sulfation [13]. These modifications are accomplished by enzymes localized on the luminal side of specific subcompartments of the Golgi apparatus. In addition, a variety of Golgi proteins have been found, including peripheral membrane proteins such as GM130 [14], Golgin-97 [15], Golgin-160 [16], Golgin-230/245 [17,18] and GMAP-210 [19], as well as integral membrane proteins such as Golgin-67 [20], Golgin-84 [21] and Giantin [22]. Localization of these Golgi proteins was proposed to be signal-dependent. For example, glycosyltransferase, a type II membrane protein, contains Golgi retention signals in and around its TMDs [23]. In contrast, the Golgi localization signals of type I membrane proteins are probably contained in the cytoplasmic tail, as demonstrated for TGN38 [24] and the membrane-associated endoprotease furin [25]. As RKTG is a membrane protein specifically localized to the Golgi apparatus [8], we investigated the functional domains of RKTG required for Golgi localization as well as Raf-1 interaction. As a result, we found that multiple structural motifs are involved in determining the structure/function relationship of RKTG.
EXPERIMENTAL

Cell culture and transfection

HEK-293T [HEK-293 cells (human embryonic kidney cells) expressing the large T-antigen of SV40 (simian virus 40)] and HeLa human cervical carcinoma cells were grown in DMEM (Dulbecco’s modified Eagle’s media) supplemented with 10% fetal bovine serum (GIBCO-BRL). Cells were incubated at 37°C in humidified air with 5% CO₂ and subcultured every 2 or 3 days. Transfection was performed using the PEI (polyethyleneimine) method [26].

Antibodies and plasmids

The antibodies were purchased as follows: the antibody for catalase was from Abcam (Cambridge, MA, U.S.A.); monoclonal anti-FLAG antibody was from Sigma-Aldrich (St. Louis, MO, U.S.A.); antibodies against Myc and HA (haemagglutinin) were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.); Golgin-97 monoclonal antibody, Alexa Fluor® 488 donkey anti-mouse IgG, Alexa Fluor® 546 goat anti-mouse IgG and Hoechst 33342 were from Molecular Probes (Carlsbad, CA, U.S.A.). The polyclonal RKTG antibody was generated as previously reported [8]. The HA-tagged TGN38 was kindly provided by Dr Juan S. Bonifacino (National Institute of Child Health and Human Development, Bethesda, MD, U.S.A.) [27]. FLAG-tagged Raf-1 was kindly provided by Dr Dong Xie (Chinese Academy of Sciences, Beijing, China). The full-length cDNAs of human RKTG and AdipoR1 cDNA were isolated as previously reported [8,28]. The HA-tagged TGN38 was kindly provided by Dr Juan S. Bonifacino (National Institute of Child Health and Human Development, Bethesda, MD, U.S.A.) [27]. FLAG-tagged Raf-1 was kindly provided by Dr Dong Xie (Chinese Academy of Sciences, Beijing, China). The full-length cDNAs of human RKTG and AdipoR1 cDNA were isolated as previously reported [8,28]. The wild-type human RKTG coding sequence was separately cloned into EGFP-C1 [where EGFP is enhanced GFP (green fluorescent protein); Clonetech], pRc/CMV (with a HA tag added into the N-terminus using PCR), and pCS2+. Clonetech, pRc/CMV (with a HA tag added into the N-terminus using PCR), and pCS2+ MT with six Myc tags at the N-terminus. All the RKTG mutants used in this study were generated by a PCR-based method and confirmed by DNA sequencing.

The solubility assay of RKTG

Solubility assays were carried out and analysed as previously described [14] with the following modifications. Briefly, after transfection for 24 h, HEK-293T cells were homogenized in a buffer containing 0.25 M sucrose, 10 mM Tris/HCl (pH 8.0) and 0.1 mM EDTA. The membrane component was separated by centrifugation (at 4°C, 10,000 x g, 10 min), subjected to treatment with different solvents (including 0.1 M Na₂CO₃, at pH 11.5, 1 M NaCl, 1% SDS or 1% Triton X-100), and then centrifuged once more (at 4°C, 100,000 x g, 30 min). The supernatant and pellet were analysed by Western blotting.

Protease protection assay

The protease protection assay was performed as previously described [14,29] with minor modifications. Briefly, at 24 h after transfection, HEK-293T cells were homogenized, then centrifuged at 750 g and 4°C for 10 min. The supernatant was then collected and treated with trypsin at various concentrations (0, 0.625, 1.25 or 2.5 μg/50 μl) at 4°C for 30 min, followed by Western blotting analysis.

Immunoprecipitation and immunoblotting

At 24 h after transfection, cells were washed three times in PBS, then lysed in buffer comprising 20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA and 1% Nonidet P-40 with a mixture of protease inhibitors and phosphatase inhibitors (Sigma-Aldrich). The homogenates were centrifuged for 20 min at 13,400 g at 4°C, and the supernatants were combined with 1 μg of anti-FLAG M2 antibody (Sigma, St. Louis, MO, U.S.A.) and mixed overnight at 4°C. Protein A/G plus agarose (Santa Cruz Biotechnology) was added for 4 h at 4°C. The immunoprecipitates were recovered by centrifugation for 2 min at 750 g and 4°C, and washed three times by resuspension and centrifugation for 2 min at 750 g and 4°C in cell lysis buffer, followed by Western blotting analysis with different primary antibodies and a horseradish peroxidase-conjugated secondary antibody (Amersham, Little Chalfont, Buckinghamshire, U.K.) and enhanced chemiluminescence reagents from Pierce (Rockford, IL, USA).

Selective permeabilization of plasma membranes and immunofluorescence microscopy

At 48 h after transfection, HeLa cells were washed twice with PBS. For complete permeabilization, the cells were fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.1% Triton X-100 in PBS for 10 min, blocked with 3% BSA in PBS for 1 h and then incubated with primary antibody for 1 h. After washing three times, the cells were incubated with secondary antibodies for 1 h and then washed three times. Selective permeabilization of the plasma membrane was performed as described previously with minor modifications [30]. Briefly, cells were incubated at 4°C for 45 min in sucrose buffer (1% BSA, 0.3 M sucrose, 0.1 M KCl, 2.5 mM MgCl₂, 1 mM EDTA and 10 mM Hepes, pH 7.4) containing various concentrations of digitonin, and then incubated with primary antibody in blocking solution for 1 h, followed by washing with PBS. The cells were fixed with methanol at 4°C for 6 min, incubated in blocking solution for 30 min, and then incubated with secondary antibodies for 30 min in blocking solution. The nucleus was stained with 1 μg/ml Hoechst 33342 (Sigma-Aldrich) for 10 min. Confocal images were captured with a LSM 510 confocal microscope with a 64 × 1.4 NA apochromat objective (Carl Zeiss, Thornwood, NY, U.S.A.). An argon laser was used at 488 nm for fluorescence excitation of EGFP and Alexa Fluor®-488-conjugated antibodies. A helium/neon laser (543 nm) was used for excitation of Alexa Fluor®-546-conjugated antibodies and 720 nm was used for excitation of Hoechst 33342. After data acquisition, RGB (red/green/blue) images were processed using LSM 510 software.

RESULTS

Analysis of the topology of RKTG

As previously reported by us [8], hydrophobicity analysis indicates that RKTG contains seven TMDS. Using the structure of AdipoR1 and AdipoR2 as a prototype [2], we hypothesized that RKTG is topologically different from classical GPCRs in that the N-terminus is localized in the cytosol. To confirm that RKTG is indeed a membrane-integrated protein, we analysed the solubility of RKTG in different solvents after transient expression of RKTG in HEK-293T cells. It has been shown that 1% SDS or 1% Triton X-100 can dissolve both peripheral and membrane-integrated proteins while basic solvent (such as Na₂CO₃ at pH 11.5) or high concentration salt solvent (such as 1 M NaCl) can only dissolve peripheral proteins [14]. Upon treatment with 0.1 M Na₂CO₃ (pH 11.5) or 1 M NaCl, RKTG was only detected in the insoluble fraction (Figure 1A). When 1% SDS or 1% Triton X-100 was used, RKTG was only detected in the soluble fraction (Figure 1A). Taken together, these data indicate that RKTG is indeed a membrane-integrated protein.

We next investigated the topology of RKTG as compared with the Golgi membrane-integrated protein TGN38. TGN38 is...
a type I membrane-integrated protein with the N-terminus facing the Golgi lumen [31]. When stained with an anti-TGN38 antibody under conditions in which the plasma membrane was permeabilized but the Golgi membrane was not, TGN38 had a punctated staining pattern across the plasma membrane and cytosol [24]. However, when both the plasma and Golgi membranes were permeabilized, TGN38 showed a Golgi localization pattern [24]. Therefore, the staining pattern of TGN38 can be used as a reference to determine whether or not the Golgi membrane is permeabilized. In addition, digitonin at a concentration below 3 μg/ml can specifically permeabilize the plasma membrane but not the membranes of intracellular organelles [30]. Consistent with previous reports, we found that the staining pattern of TGN38 was within the cytosol when the cells were treated with digitonin below 3 μg/ml, but had a Golgi-like pattern when the digitonin concentration was higher than 3 μg/ml (results not shown). If the N-terminus of RKTG was facing the cytosol, we should be able to detect RKTG using an antibody against the N-terminal tag when the plasma membrane is permeabilized but the Golgi membrane is not. We used different concentrations of digitonin to address this issue (Figure 1B). When the plasma membrane but not the Golgi membrane was permeabilized with 1 μg/ml of digitonin, RKTG protein with an HA tag at the N-terminus clearly had a Golgi-like pattern while TGN38 was detected across the cytosol. Meanwhile, Golgin-97, a peripheral membrane protein located at the cytoplasmic side of the Golgi membrane [15], could be detected at the Golgi. As expected, RKTG, TGN38 and Golgin-97 showed a Golgi-like pattern when 0.1 % TritonX-100 was used (Figure 1B). These results therefore indicate that the N-terminus of RKTG is on the cytosolic side.

To further support our observation, we performed a protease protection assay with RKTG that contains a Myc tag at the N-terminus. We hypothesized that if the N-terminus was facing the Golgi lumen under the condition where the Golgi membrane is not permeabilized, trypsin treatment would not cleave the N-terminus of RKTG. Meanwhile, cleavage at cytosolic loops after the first TMD would leave the N-terminus intact. In the case of the Myc-tagged RKTG used in our experiment, trypsin treatment should give rise to a 23.5 kDa protein product (based on the molecular mass of the Myc epitope plus the N-terminus) if the N-terminus of RKTG is facing the Golgi lumen under the condition where the plasma membrane is permeabilized by trypsin without a detectable band at 23.5 kDa. As expected, RKTG, TGN38 and Golgin-97 showed a Golgi-like pattern when 0.1 % TritonX-100 was used (Figure 1B). These results therefore indicate that the N-terminus of RKTG is on the cytosolic side.

Structural determinants in the N-terminus of RKTG required for Golgi localization and Raf-1 co-localization

Our previous study indicated that the N-terminus of RKTG is required for Golgi localization [8]. We further dissected the N-terminus of RKTG in this study. We constructed RKTG mutants with various lengths of truncation at the N-terminus (Figure 2A) and used them in localization studies in HeLa cells. The expression patterns of RKTG with N-terminal truncations of 60, 40 and 20 aa (amino acids) had evident Golgi localization (Figure 2B), different from the localization of the RKTG mutant with truncation of the entire 71 aa of the N-terminus (Figure 2B). On the other hand, deletion of the N-terminal 20 aa led to loss of co-localization with Raf-1 (Figure 3), similar to other truncation mutants in the N-terminus (Figure 3). These results indicate that the very distal region of the N-terminus is required for sequestration of Raf-1 to the Golgi apparatus.
Based on our observations that deletion of 71 aa but not deletion of 60 aa in the N-terminus abrogated Golgi localization of RKTG, we hypothesized that the sequence between amino acids 61 and 71 was critical for RKTG localization at the Golgi apparatus. To address this hypothesis, an RKTG mutant with deletion of amino acids 61–71 was constructed and used in the localization study.

We found that RKTGΔN61–71 was distributed in the cytoplasm without a Golgi-like pattern, and was not co-localized with the Golgi marker Golgin-97 (Figure 4A). Furthermore, we analysed the co-localization of RKTGΔN61–71 with Raf-1. As shown in Figure 4(B), RKTGΔN61–71 partially overlapped with Raf-1. In addition, RKTGΔN61–71 was able to interact with Raf-1 in
the interaction of RKTG with Raf-1. In order to analyse the functional importance of these loops and maintain the topology of RKTG at the same time, we constructed three RKTG mutants with each of the loops being swapped with the corresponding sequence of AdipoR1 (Figure 6A). AdipoR1 is a close homologue of RKTG and also contains seven TMDs. It is noteworthy that AdipoR1 is localized at the plasma membrane without Golgi localization [2].

We first investigated the cellular localization of these mutants (Figure 6B). The replacement of the first and third cytosolic loops with the corresponding sequences of AdipoR1 (Cyto-Loop1-R1 and Cyto-Loop3-R1) disrupted the Golgi localization of RKTG, while the replacement of the second cytosolic loop did not (Figure 6B), indicating that the first and last cytosolic loops are involved in Golgi localization of RKTG. We next analysed whether these mutants could affect interaction of RKTG with Raf-1. With the first cytosolic loop replaced with the corresponding sequence of AdipoR1, RKTG partially co-localized with Raf-1 in the cytoplasm (Figure 6C). Replacement of the second cytosolic loop appeared to retain partial co-localization with Raf-1 (Figure 6C), although at a reduced level in comparison with the wild type RKTG (Figure 2). However, replacement of the third cytosolic loop completely abrogated co-localization with Raf-1 (Figure 6C). To analyse further the interaction of these RKTG mutants with Raf-1, we performed a co-immunoprecipitation assay. Consistent with the co-localization studies, replacement of the third cytosolic loop but not of the first and second cytosolic loops led to loss of interaction with Raf-1 (Figure 7). Taken together, these results indicate that the first cytosolic loop is critical for the localization of RKTG to the Golgi apparatus but not interaction with Raf-1, whereas the third cytosolic loop is required for both Golgi localization and Raf-1 interaction of RKTG.

**DISCUSSION**

We extensively dissected and analysed the structure of RKTG in this study. Our results revealed that RKTG is a Golgi membrane-integrated protein with its N-terminus located on the cytosolic side of the Golgi apparatus (Figure 8). It appears that multiple structural motifs are required for Golgi targeting and Raf-1 interaction of RKTG protein. Based on our study, at least four independent segments are implicated in determination of Golgi localization of RKTG, including the membrane-proximal 10 aa at the N-terminus, the membrane-proximal 5 aa at the C-terminus and the first and third cytosolic loops between TMDs (Figure 8). In addition, at least two structural motifs are needed for the interaction of RKTG with Raf-1, the N-terminal sequence and the third cytosolic loop (Figure 8).

We determined the topological structure of RKTG in this study and our data indicate that RKTG is a Golgi membrane-integrated protein and has a Type III receptor topology with the N-terminus facing the cytosol. The topology of PAQR family members has been a controversial issue. Yamauchi et al. [2] suggested that Adirop1 and Adirop2 are plasma membrane proteins with the N-terminus facing the cytosol, distinct from classical GPCRs in which the N-terminus is located extracellularly. However, studies by Zhu et al. [3,4] predicted that the membrane progestin receptors, including PAQR5, PAQR7 and PAQR8, all have a structure similar to classical GPCRs. RKTG is different from adiponectin receptors and membrane progestin receptors in many ways. Clearly different from these two groups of membrane receptors, RKTG is a specific Golgi-targeted protein, and is not localized at the cytoplasmic membrane. In addition, the receptor-like function of RKTG has not been characterized, although it remains to be determined whether RKTG may function as an
in intracellular receptor bound and activated by intracellular signalling molecules. There are a total of 11 members of the PAQR family. On the basis of previous reports as well as the present study, it is predicted that various members in the family may have various topologies dependent on where they are localized in the cell.

Mapping of the signals for Golgi localization of a protein has been explored for years and the current consensus is that the Golgi-targeting signal is extremely complicated [23]. Studies with glycosyltransferases have revealed that the TMD of the proteins is a key determinant of Golgi localization, and in many cases this domain is sufficient to confer Golgi localization [32]. However, for some enzymes the sequences flanking the TMD, or the luminal portion of the protein, contribute to Golgi localization as well.

In addition, for some other Golgi membrane proteins, such as TGN38, short tyrosine-containing motifs in the cytoplasmic tail have been shown to be crucial for specifying Golgi localization [24]. In the present study, at least four structural determinants are required for Golgi-specific localization of RKTG (Figure 8). Our studies, in combination with numerous previous studies to analyse Golgi-resident proteins, strongly suggest that multiple structural motifs are involved in guiding newly synthesized membrane proteins to target to the Golgi apparatus. It is speculated that these motifs may be recognized by Golgi-resident proteins and/or specific lipid components in the Golgi to facilitate recruitment of these proteins into the Golgi. However, the exact molecular mechanisms underlying the processes of recognition and recruitment remain to be explored in the future.
Functional domains of RKTG

Figure 6 Analysis of the three cytosolic loops of RKTG

(A) Diagram of RKTG with the three cytosolic loops replaced by the corresponding sequences of AdipoR1. (B) Subcellular localization studies. HeLa cells were transfected with GFP-fused RKTG chimaera mutants (green) as indicated and used in confocal analysis. The Golgi apparatus was labelled with an antibody for Golgin-97 (red). (C) Co-localization studies of RKTG chimaera mutants with Raf-1. HeLa cells co-transfected with GFP-fused RKTG mutants (green) and FLAG-tagged Raf-1 (red) were used in immunofluorescence staining.

Sorting signals used within the secretory pathway to target proteins to the Golgi apparatus mainly act in two ways, either as a retention signal that anchors the protein in the Golgi or as a retrieval signal used to capture the protein when it is in the wrong place and return it to the Golgi, such as is the case for TGN38 and furin [24,33,34]. In addition, the sorting of Golgi membrane proteins involves two mechanisms. The first mechanism is an oligomerization model in which the proteins in particular cisternae interact with each other to form structures too large to enter transport vesicles, such as the case of N-acetylglucosaminyltransferase I and mannosidase II [35,36]. The other mechanism is a lipid-sorting model in which the bilayer of the Golgi cisterna is not homogeneous but contains distinct lipid domains between which the Golgi enzymes partition differentially [37]. By a co-immunoprecipitation assay, we could not find interaction of RKTG with itself (results not shown). Therefore, it is unlikely that RKTG employs the oligomerization model for Golgi localization. It has been proposed that TGN38 uses a retrieval signal to localize to the Golgi apparatus [24], consistent with our finding that TGN38 had a plasma membrane staining pattern when the cell membrane was not permeabilized (Figure 1B). However, under the same condition, RKTG could not be detected in the plasma membrane (Figure 2B). Such differences in the staining pattern between TGN38 and RKTG indicate that the retrieval model is not used by RKTG to specify Golgi localization.

Whether a retention signal is implicated in RKTG localization in the Golgi apparatus as well as the underlying molecular details remain to be determined in the future. Nevertheless, our analyses with the first identified seven-transmembrane Golgi-resident protein could pave the way for future structural and functional

Figure 7 Co-immunoprecipitation assays to analyse interaction of RKTG mutants with Raf-1

HEK-293T cells were transiently transfected with FLAG-tagged Raf-1 and Myc-tagged RKTG mutants as indicated. After transfection for 24 h, the cell lysate was used in immunoprecipitation (IP) and immunoblotting (IB) using the antibodies as indicated.

Figure 8 A model to depict functional domains of RKTG involved in Golgi localization and Raf-1 interaction

The model shows the seven transmembrane domains of RKTG inserted into the Golgi membrane. The domains involved in Golgi localization and Raf-1 interaction are indicated.
studies of RKTG, a protein that plays a critical role in regulating the central mitogenesis signalling pathway.

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