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A Novel Human Rab26 cDNA Coloning, Expression and Its Endocytosis Enhancing Function*

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Abstract Rab GTPases serve as master regulators of vesicular membrane transport on both the exo- and endocytic pathways. Though there are many reports on Rab proteins, the function of these small proteins still remain in speculation. And no report has ever clarified the character of human Rab26. Here it was reported that a novel Rab protein Rab26 is membranous organelle related and involved in endocytosis of HeLa cells. By using RT-PCR method a novel Rab26 cDNA full-length cDNA of Rab26 that is 1656 bp was identified. The cDNA sequence that at 1197 is 'A' other than 'G', while 'C' at 956 substitutes for 'T', and has 'GCC' insertion at 48 to 50 compared with published sequences. The complete open reading frame (ORF) is 771 bp in length encoding 256-residue protein with a calculated molecular mass of 27.9 ku (GenBank accession No.AY646153), rather than a shorter one with 190-amino acid residue as reported previously. GFP labeled full-length Rab26 expression showed that Rab26 was mainly sublocated in membranous organelles and could enhance endocytosis which means could took PE labeled protein as an endocytic tracer. RT-PCR analysis showed Rab26 was detected to express in several kinds of adenocarcinoma cell lines such as Acc2, AccM, SPC-A1 and HeLa cell lines, which indicated that Rab26 expression might be associated with some carcinomas.

Key words Rab26, Rab GTPases, endocytosis

Small GTP-binding proteins in Rab family have been implicated as key regulators of membrane trafficking in mammalian cell (Zhao H, 2002)[1]. Most Rab proteins are ubiquitously associated with membranous organelles in the eukaryotic cytoplasm (Lazar T, 1997[2]; Martinez O, 1998[3]). Although the details of their functional mechanisms are still open for discussion, these proteins obviously lead vesicles or granules to corresponding target organelles (Schimmöller F, 1998[4]). Rab proteins have been identified to be interconnected with CLN3, Hook1 (Luiro K, 2004[5]), G protein-coupled receptors (GPCRs) (Seachrist J L, 2003)[6], and Rab26 is reported to interact with GTP cyclohydrolase Ⅰ protein (Swick L, 2006)[7], all of which can contribute to the process of understanding Rab proteins function. Cao has recently demonstrated a novel Rab39 can facilitate endocytosis of HeLa cells (Chen T Y, 2003)[8], which shined light on function research of other Rab proteins.

Here we predicted the existence of a full length Rab26 gene by bioinformatics analysis, and obtained it from human fetal brain cDNA library, which shares 74% identity with Rab37 (Masuda E S, 2000)[9]. Confocal laser scanning microscopy assay showed that Rab26 is a membranous organelle related protein, and it could be predicted that Rab26 may involve in signal and molecular transportation between cells and environment. By using PE labeled protein as endocytosis tracer, we demonstrated that Rab26 facilitated endocytosis in HeLa cells when over expressed transiently. Results of RT-PCR showed that Rab26 expresses in several kinds of adenocarcinoma

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cell lines without being detected in epithelioma cell lines, which indict that Rab26 expression may be associated with some carcinomas.

1 Experimental procedure

1.1 Rab26 genes cloning and sequence analyzing

The primers were designed according to the new full length Rab26 cDNA reference sequence BC066913: 5′ CCGCCATGTCCAGGAAGAAG 3′& 5′ TGTTGCTGGGAGGAGGAAGG 3′ (synthesized by Sangon Shanghai, China). The PCR reaction system is as follow: In total 125 μl volume reaction solution contains 1 μl human fetal brain cDNA library (CLONTECH), 5 μl(10 μmol/L) for each primer, 5 μl dNTP(10 mmol/ L), 12.5 μl 10× PCR buffer (with Mg^{2+}), 95.5 μl water and 1 μl TaqPlus polymerase. The reaction condition is 94℃ 180 s, 94℃ 90 s, 59℃ 90 s, 72℃ 120 s, 35 cycles, 72℃ 420 s. The 937 bp product was separated and purified by 1.5% agarose electrophoresis and cloned into pGEM-T vector. The plasmid was then transformed into *E. coli* DH5α cells. The transformed clones with pGEM-Rab26 plasmid was selected on LB culture plate containing 50 mg/L ampicillin. The plasmid was then extracted and identified by PCR, restriction endonuclease digestion and DNA sequencing. The full length sequence of Rab26 was available at NCBI with Accession No. AY646153.

1.2 GFP traced Rab26 protein expression in mammalian cells

Take pGEM-rab26 as template, the whole ORF of Rab26 gene was amplified using the following primers: 5′ GGAATTCCCGCCATGTCCAGGAAG-AAG 3′and 5′ CGGGATCCCAAGGGCGGCAGCA-GGA 3′, the product was then purified and inserted into pEGFP-N1 between endonuclease site EcoR Ⅰ and BamH Ⅰ, and the plasmid was named pEGFP -Rab26. pEGFP -Rab26 was then transfected into HeLa cells using lipofectamine2000 (Invitrogen California USA) according to its manufacturer's instruction. 36 h after transfection, cells are washed with cold PBS and fixed with 4% formaldehyde, then observation are taken under laser confocal microscope (Leica TCS NT).

1.3 Assay of Rab26 expression in tumor cell lines by RT-PCR

Tumor cell lines (SMMOL/LC-7721, HepG2, K562, SPC-A21, Caco2, HeLa, Acc2, Raji, AccM all are preserved by our laboratory) were cultured in 1640 or DMEM complete medium(with 10% BCS) at 37℃, 5% CO_2 for 48 h, and the total RNA was extracted according to manufacturer's instruction (Invitrogen), briefly 1×10^7 cells was digested by pancreatin and put into Eppendorf tube, add Trizol and homogenate it, deposit proteins with chloroform, then deposit the RNA with isopropanol and dried it. Pure RNA was dissolved with 25 μl DEPC water. cDNA was synthesized by reverse transcript using olig-dT as primers, and the expression of Rab26 was detected by PCR using primers as follows: Rab26 RT+, 5′ GAGT-ACGCCCAGCACGAC 3′; Rab26 RT-, 5′ TCAAGG-GCG2GCAGCAGGA 3′ (synthesized by Sangon Shanghai China). The reaction procedure is 94℃ 60 s, 94℃ 30 s, 56℃ 45 s, 72℃ 60 s, 30 cycles, 72℃ 240 s. Take GAPDH as internal control and the primers are as follows: GAPDH+, 5′ GCAAATTCCACGGCAC-AGTCA 3′ and GAPDH-, 5′ TCACGCCACAGTTT-CCCAGAG 3′.

1.4 Overexpression of Rab26 transparently and endocytosis assay

HeLa cells were transfected according to lipofactamine2000 instructions. Cells were divided into four groups (blank, negative, exp1 and exp2). Blank cells will not be transfected by any vector, others will be transfected by pcDNA3.1, pcDNA-Rab26, pEGFP-Rab26 respectively. Three days after the transfection, cover slides were laid upside down onto a drop of fluorescence-marked tracer proteins (diluted by DMEM) for 10 min, 30 min and 60 min under 37℃, 5% CO_2. Then the cover glasses were washed with cold PBS twice and fixed by acetone. The quantity of tracer protein endocytosed by different group was observed under fluorescence microscope.

2 Results

2.1 Cloning, identification and sequence analysis of Rab26

DNA sequence obtained from human fetal brain cDNA was cloned into pGEM-T vector named pGEM-Rab26. And the results of DNA sequencing show that there is a mutation (CGG→CAG) inside the predicted functional domain, we have submitted the sequence to GenBank (No. AY646153).

Full-length cDNA of Rab26 is 1 656 bp and contains a complete open reading frame (ORF) of 771 bp encoding 256-residue protein with a calculated molecular mass of 27.9 ku. EST assembly showed

there existed a stop codon(TGA 2142) in the up flow of start codon, an 'ATG' at 152 and a 'TAA' ending at 722 site. PolyA tail locates at 3' end which indicated that the cDNA we isolated was of full-length. By performing BLAST through human genome, we found that Rab26 cDNA was highly homologous to 2 genomic sequence including NW_926018.1 (Venter JC, 2001)[10] (despite 'A' at 1197 to 'G'; 'GCC' insertion between 14 and 17) and NT_037887.4(Lowe TM, 1997)[11] (despite 'C' at 956 to 'T'; 'GCC' insertion between 48 to 50). Also Rab26 was highly homologous to several clones on GenBank including NM_014353.4[7] despite 'A' at 1197 to 'G'. By comparison with the human genome sequence, we found that Rab26 ORF region was overlapped within the Unigene cluster Hs369728, which was localized to 16p13.3 (Figure 1).

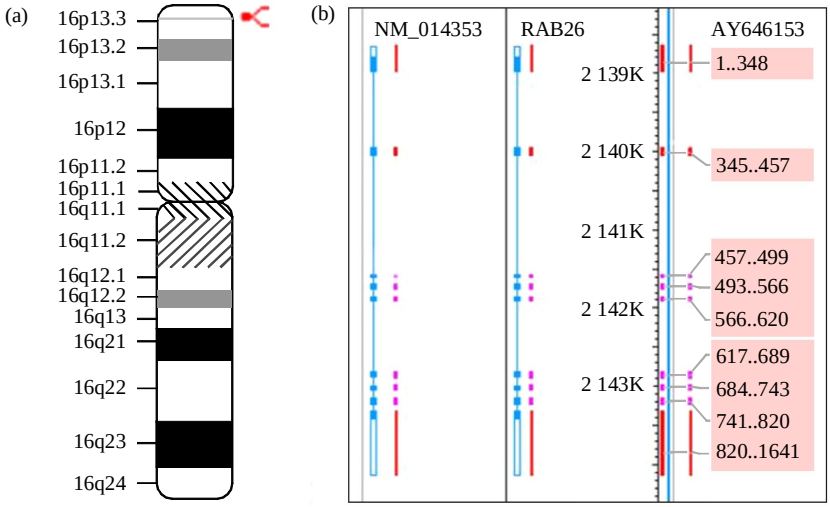

Fig. 1　Chromosome location of human Rab26 (AY646153) genes

Rab26 protein was predicted to contain functional domains as follows: a Ras GTPase domain ([64～85aa][88～104aa][106～128aa][168～181aa][203～225aa]), a RNA polymerase sigma factor 54 domain (66～79aa), Ras small GTPase (64～228aa) and small GTP-Binding Protein Domain (61～223aa). (Strausberg RL, 2002[12]; Swick L, 2006[7])

2.2　Rab26 expression assay in human tumor cell lines

RNA of 9 tumor cell lines, SMMOL/LC-7721, HepG2, K562, SPC-A21, Caco2, HeLa, Acc2, Raji and AccM, were extracted and the expression of Rab26 was analyzed, and positive results were got in 5 tumor cell lines while other 4 showed negative results as indicated in Figure 2. Rab26 mRNA expression could be detected in Acc2 and SPC-A1 which are both adenocarcinoma and also in K562 and HeLa, however it was barely expressed in other cell lines. This partly indicated that Rab26 expression level may be related to some carcinomas.

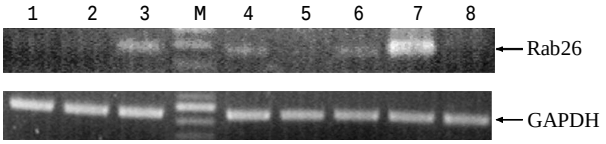

Fig. 2　RT-PCR assay of Rab26 mRNA in human tumor cells

1: SMMOL/ LC-7721 (liver cancer) ; 2: LO2 (liver); 3: K562 (chronic promyelocytic leucocythemia); M: 100 bp ladder; 4: SPC-A21 (lung adenocarcinoma); 5: Caco2(colon cancer); 6: HeLa (cervical cancer); 7: Acc2 (adenoid cystic carcinoma with low metastasis potential); 8: Raji (Burkitt's lymphoma).

2.3　Expression pattern of Rab26

pEGFPN1-Rab26 was transfected into HeLa cells and Rab26 gene was transiently expressed, observation under confocal microscope was made and the results showed that Rab26 gene was specifically expressed on membranes of organelles (Figure 3b), however the negative control plasmid (pEGFPN1) showed no location specificity (Figure 3a).

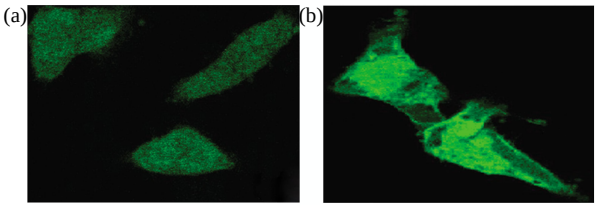

Fig. 3 Expression of Rab26 in HeLa cells transfected with pEGFPN1-Rab26 recombinant plasmid

(a) Negtive control (HeLa cells transfected with pEGFPN1). (b) HeLa cells transfected with pEGFPN1-Rab26.

2.4 Effects of Rab26 protein overexpression on endocytosis

Rab26 is speculated to involve in signal and molecular transportation between cytoplast and environment. By using red PE labeled Rat anti mouse CD4 antibody as an endocytosis tracer, we examined the effect of Rab26 over expression on endocytosis of HeLa cells (Figure 4). By observing red fluorescence density of HeLa cells at 0 min, 30 min and 60 min, we found that cells transfected with Rab26 genes performed enhanced endocytosis, in contrast cells transfected with negative control plasmids (pcDNA3.1, Figure 4a) or nothing (data not shown) didn't show any endocytosis activity, and the red fluorescence density of cells incubated with tracer for 60 min is much stronger than that of 30 min (data not shown). Experimental results showed that GFP fusion didn't block Rab protein endocytosis function, so the function domain that is related with endocytosis could be located in the N terminal of Rab26.

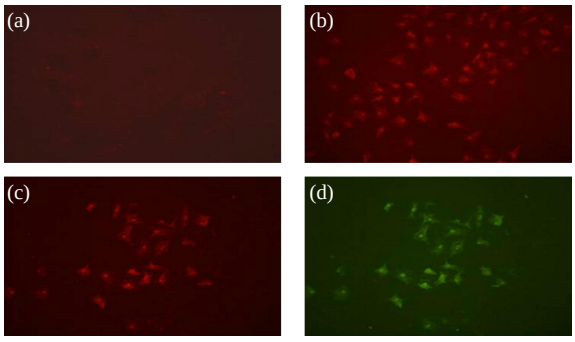

Fig. 4 Effects on endocytosis function of HeLa cells after transient transfection

The red PE labeled Rat anti mouse CD4 antibody was used as an endocytosis tracer. (a) Negative control, HeLa cells transfected with pCDNA3.1 and incubated with tracer for 1 h. (b) HeLa cells transfected with pCDNA-Rab26 and incubated with tracer for 60 min. (c) HeLa cells transfected with pEGFP-Rab26 and incubated with tracer for 60 min. (d) GFP fused Rab26 expression in HeLa cells transfected with pEGFP-Rab26.

3 Discussion

Rab proteins are a large subfamily of the Ras superfamily and contained many members, some of which have been studied in detail while leaving most of them functionally unknown (Simons K, 1993 [13]; Somsel Rodman J, 2000[14]). In this article, we isolated a novel Rab GTPase that is widely expressed. When overexpressed, it can significantly increase the endocytosis activity for PE-anti CD4 antibody by HeLa cells. And by confocal microscopy, we found that Rab26 was colocalized with membranous organelles, indicating that Rab26 may regulate endocytosis by acting as a cross membrane acceptor. Thus finding molecules that could confunction with Rab26 is a key step to clarify the mechanisms of Rab26 function.

Results of RT-PCR assay showed that Rab26 was mainly expressed in adenocarcinoma while not so significantly detected in other tumor cell lines, which indicated that Rab26 expression level may be related with genesis or development of adenocarcinoma, and Rab26 may serves as a potential marker of adenocarcinoma and hopeful for clinical diagnosis but more clinical supports were needed. Emerging evidence implicates alterations in the Rab small GTPases and their associated regulatory proteins and effectors in multiple human diseases including cancer (Cheng KW, 2005) [15]. For example, in a number of cancers (prostate, liver, breast) as well as vascular, lung, and thyroid diseases, the overexpression of select rab GTPases have been tightly correlated with disease pathogenesis. Since Rab26 is isolated from human brain, it could be believed that Rab26 might have important function in signal transduction of nerve cells, and alteration in the molecular itself or its expression pattern may cause diseases due to the abnormal function. Then finding cofunctioning molecule with Rab26 and identifying its role in kinds of diseases are of great interest in the future investigation.

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一个新的人 Rb26 基因 cDNA 的克隆、表达及其增强细胞吞噬功能研究 *

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摘要　Rab GTPase 家族成员基因及其调节囊泡膜运输途径功能已有许多报道，但 Rab26 蛋白分子的结构和功能仍不清晰. 通过生物信息学分析，并在人脑 cDNA 文库中克隆了一个新的 Rab26 基因全长 cDNA 序列，长 1 656 bp，与已发表的基因序列相比，在 48～50 位插入了 GCC，在 956 位 T 被 C 取代，而在 1 197 位 G 被 A 取代. 该序列包含一个 771 bp 完整的开放阅读框(ORF)，编码 256 个氨基酸残基的 Rab26 蛋白，分子质量为 27.9 ku(GenBank 登录号 No.AY646153)，而非如以往报告的 190 个氨基酸残基. GFP 荧光标记全长 Rb26 在哺乳动物细胞中表达显示，Rab26 主要呈现在细胞膜状结构相联系的分布，发现该基因高表达能显著增强 PE 标记的红色异源蛋白质的吞噬. 还应用逆转录 - 聚合酶链反应对多种人肿瘤细胞 Rb26 表达进行了研究，结果显示，Rab26 在 Acc2、SPC-A1，K562 以及 HeLa 等肿瘤细胞株呈高表达，而在 SMMOL/LC-7721、HepG2、Caco2 等肝和肠上皮细胞株中则不表达，值得进一步深入研究.

关键词　Rab 26，Rab GTP 酶，内吞作用
学科分类号　Q7

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