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RACK1 mRNA translation is regulated via a rapamycin-sensitive pathway and coordinated with ribosomal protein synthesis

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Abstract RACK1 has been shown to interact with several proteins, this suggesting that it may play a central role in cell growth regulation. Some recent articles have described RACK1 as a component of the small ribosomal subunit. To investigate the relationship between RACK1 and ribosome, we analyzed RACK1 mRNA structure and regulation. Translational regulation was studied in HeLa cells subjected to serum or amino acid deprivation and stimulation. The results show that RACK1 mRNA has a 5′ terminal oligopyrimidine sequence and that its translation is dependent on the availability of serum and amino acids in exactly the same way as any other vertebrate ribosomal protein mRNA.

Keywords: Translational regulation; TOP mRNA; Ribosome; Growth control

1. Introduction

Receptor for activated C kinase (RACK1) is a scaffold protein able to interact with several molecules, both soluble signaling proteins and membrane-bound receptors [1]. It was originally characterized by its homology to the guanine nucleotide-binding protein beta subunit and other proteins containing Trp-Asp (WD) repeat domains [2]. Later on, it was shown that RACK1 interacts with protein kinase C (PKC) beta, so increasing kinase activity and leading to the correct subcellular location [3]. RACK1 has also been shown to interact with Src, although in this case it acts as an inhibitor of the Src kinase activity [4]. An example of membrane-associated receptor that binds to RACK1 is the cytoplasmic domain of the integrin beta subunit [5,6]. An integrin beta-RACK1-PKC complex has also been observed; therefore, it seems that the interaction between RACK1 and the receptor results in the recruitment of other signaling molecules [7]. In addition to its well-established role as a receptor of signaling molecules, several studies identify RACK1 as a structural component of the small ribosomal subunit [8]. In fact, mass-spectrometry analysis of yeast and human ribosome showed that RACK1 is a component of the small subunit present in approximately equimolar concentration with the other ribosomal proteins (RP) [9,10]. Although some studies suggest that RACK1 (or the yeast homologs Asc1p and Cpc2p) may also exist in a non-ribosome-bound form [11,12], a more recent report showed that RACK1 is not present in a soluble form [10]. The role of RACK1 on the ribosome has been recently addressed by several groups: In mammals it may lead activated PKC to phosphorylate eukaryotic initiation factor (eIF6) [13]; in *Saccharomyces pombe* it promotes efficient translation [12], whereas in *Saccharomyces cerevisiae* it appears to repress gene expression [10].

It is well known that all vertebrate RPs along with other proteins involved in the ribosome synthesis and function are encoded by the terminal oligopyrimidine (TOP) genes which share structural and regulatory features [14]. Messengers encoded by TOP genes (TOP mRNAs) are identified by two major characteristics: (i) a 5′ terminal oligopyrimidine sequence of 5–15 nucleotides, and (ii) a growth-associated translational regulation. Within the TOP group, RP mRNAs represent an homogeneous subset defined by more stringent criteria: (i) 5′ consensus sequence CTYTTYYY, (ii) a non-coding first exon, (iii) a particularly short 3′ untranslated region (UTR) [15]. The pyrimidines are necessary for regulation but the 3′ UTR as well as other sequences in the 5′ UTR are probably also needed for full translational control [14,15]. Perturbation of cellular growth due to a variety of causes (development, mitogens, drugs, etc.), correlates with specific, rapid and reversible changes in the association of TOP mRNAs with polysomes [14,16]. Some putative TOP mRNA translational regulators have been identified: the autoantigen La, which binds the TOP sequence [17–20], and cellular nucleic acids binding protein (CNBP) which binds a downstream region [21]. TOP mRNA translational control has been shown to be dependent on PI3 kinase (PI3K)-mammalian target of rapamycin (mTOR)-S6 kinase (S6K) signaling pathway [22], although the model has been challenged by recent reports [23–25].

Given the number of different roles proposed for RACK1 in the literature, we thought it important to investigate RACK1 mRNA structure and regulation as an indirect approach in order to address RACK1 function. We found that according to the criteria developed by several laboratories in the past twenty years RACK1 mRNA, is indistinguishable from messengers coding for RP. In fact it starts with an oligopyrimidine sequence and its translation is regulated by growth factor and nutrient availability through signaling pathways involving PI3K and mTOR.

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Abbreviations: RACK, receptor for activated C kinase; TOP, terminal oligopyrimidine tract; PKC, protein kinase C; UTR, untranslated region; RP, ribosomal protein; PI3K, PI3 kinase; CNBP, cellular nucleic acid binding protein; S6K, S6 kinase; mTOR, mammalian target of rapamycin; eIF, eukaryotic initiation factor; FCS, fetal calf serum

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2. Materials and methods

2.1. Cell culture

HEK 293 and HeLa cells were grown at 37 °C in Dulbecco's modified Eagle Medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 50 u/ml penicillin and 50 μg/ml streptomycin, where indicated rapamycin was used at 20 nM and wortmannin at 200 nM.

2.2. RNA analysis

Polysome analysis was performed as described [26]. In short, the cells were lysed directly on the plate and transferred to a microfuge tube. After 2 min of incubation on ice with occasional vortexing, the extracts were centrifuged for 5 min at 13 000 rpm in the cold room. The supernatant was separated on a 15–50% linear sucrose gradient centrifuged in a Beckman SW41 rotor for 110 min at 37 000 rpm. Fractions were collected after monitoring the absorbance at 260 nm.

Total RNA was extracted from fractions by proteinase K method [27]. For Northern analysis, RNA was fractionated on formaldehyde-agarose gels and transferred to Gene Screen Plus membrane (NEN). Probes for human RACK1, RPS6, and β-actin were prepared by the random primer technique [27] using cDNA fragments isolated from plasmids. Quantification of Northern blots was performed using a PhosphorImager with ImageQuant software (Molecular Dynamics). For primer extension analysis a synthetic 20mer complementary to position −31 to −50 with respect to the ATG of the RACK1 gene sequence was 5′ end-labeled with 25 μCi of $\gamma[^{32}P]$-ATP and T4 polynucleotide kinase according to standard protocols [27]. Labeled primer was annealed to 20 μg of total RNA from HEK 293 cells and extended with MMLV reverse transcriptase.

3. Results

3.1. mRNA structure

A full-length RACK1 mRNA sequence was first obtained by EST database searching. This analysis identified a major transcription start site (TSS) at −115 with respect to the ATG. To obtain an experimental confirmation we performed a primer extension analysis by using a specific primer and total RNA from HEK 293 cells. The results, showed in Fig. 1, confirmed that the TSS is placed in the middle of a 17 bp long pyrimidine sequence.

3.2. Translational regulation induced by serum and amino acids

To address translational regulation of RACK1 mRNA and compare it to the regulation of RP mRNA, we used HeLa cells. Cells were incubated for 2 h in a serum-free or amino acid-free medium and then stimulated for 1 h in complete medium. In addition, we also performed the stimulations in the presence of rapamycin or wortmannin to establish the involvement of mTOR or PI3K in the regulation.

The translation of RACK1, RPS6 and β-actin mRNA was assayed under the different culture conditions by the standard polysomal association technique which measures the amount of mRNA associated with particles of different size (from heavy polysomes to light subpolysomal mRNP). The results are reported in two complementary ways: (1) as a linear plot of the percentage of mRNA present in each fraction of the extract separated on a sucrose gradient (Figs. 2A–D and 3A–D); the plot is superimposed on the absorbance profile of the gradient to allow the identification of the material present in each fraction; and (2) as a column graph of the percentage of mRNA on polysomes (Figs. 2E and 3E) obtained in each experiment by adding up the values of the polysomal fractions (fractions 1–5). The first representation allows, in addition to an approximate quantification of the translated mRNA, a qualitative analysis of the size of the polysomes with which the mRNA is associated under each culture condition. The second provides a more precise evaluation of the translational activity of each mRNA and allows a direct quantitative comparison

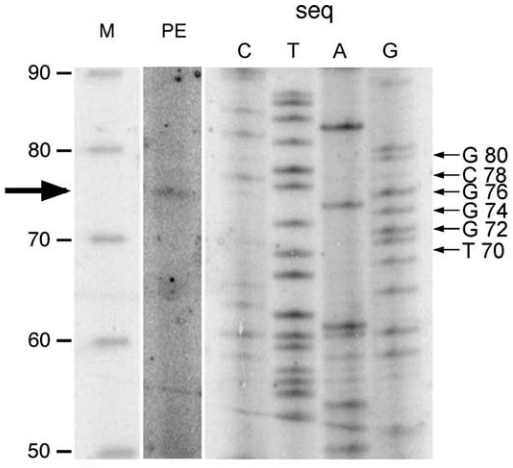

Fig. 1. *RACK1 mRNA 5′ end.* A 5′ end-labeled primer (underlined in the sequence shown in the lower part) was annealed to 20 μg of total RNA from HEK 293 cells and then extended with reverse transcriptase. The extension products were separated on 6% polyacrylamide gel and exposed for autoradiography. A major extension band of about 76 nt is indicated by the arrow. It corresponds to the C indicated in the sequence as a TSS. M, 10 bp size marker; PE, primer extension products; seq, unrelated sequence ladder with the size of few bands indicated on the right. The three parts of the figure are assembled from different pictures of the same gel to improve the visibility of the bands. The sequence complementary to the primer is underlined; the translation initiation codon ATG is in bold.

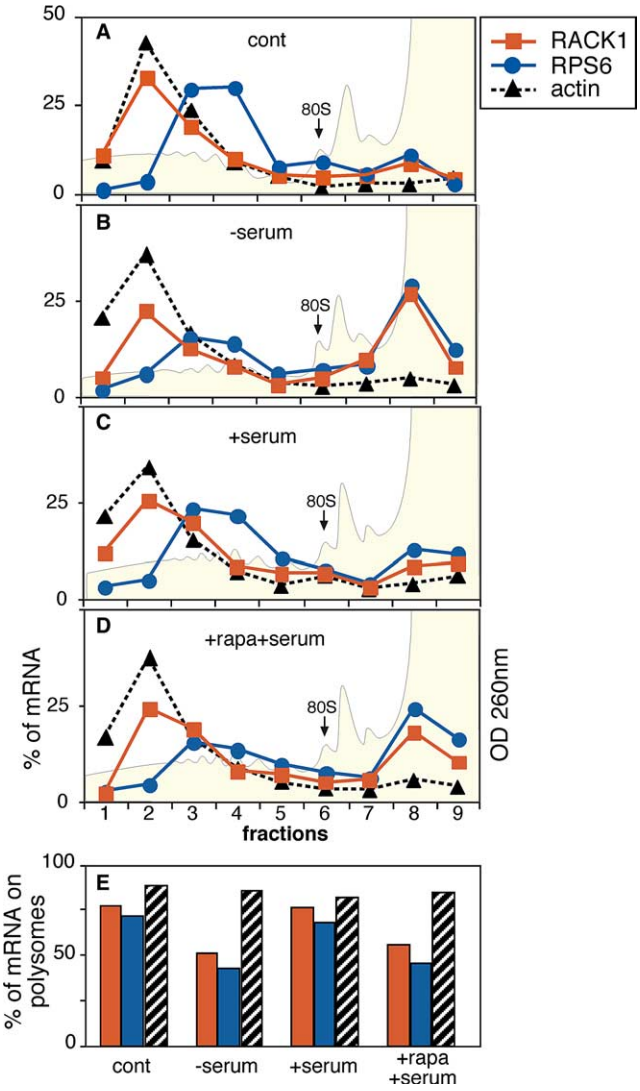

Fig. 2. *Serum effect on translation of RACK1 mRNA.* After overnight growth in complete medium (A, cont) cells were incubated for 2 h in medium lacking serum (B, −serum), then stimulated for 1 h with complete medium without (C, +serum) or with (D, +rapa + serum) rapamycin. Cytoplasmic extracts prepared from the cells were separated on sucrose gradients and fractions were collected while monitoring absorbance at 260 nm. RNA extracted from each fraction was analyzed on Northern blots with the indicated probes. Quantification of the signals is reported as a linear plot of the percentage of mRNA in each fraction (A–D) and as column plot of the percentage of mRNA on polysomes (**E**) obtained by adding up the values of fractions 1–5. In panels A–D the first fraction corresponds to the bottom of the gradient while the absorbance profile is outlined (yellow area) in the background of each plot with a black arrow indicating the position of 80S ribosomes.

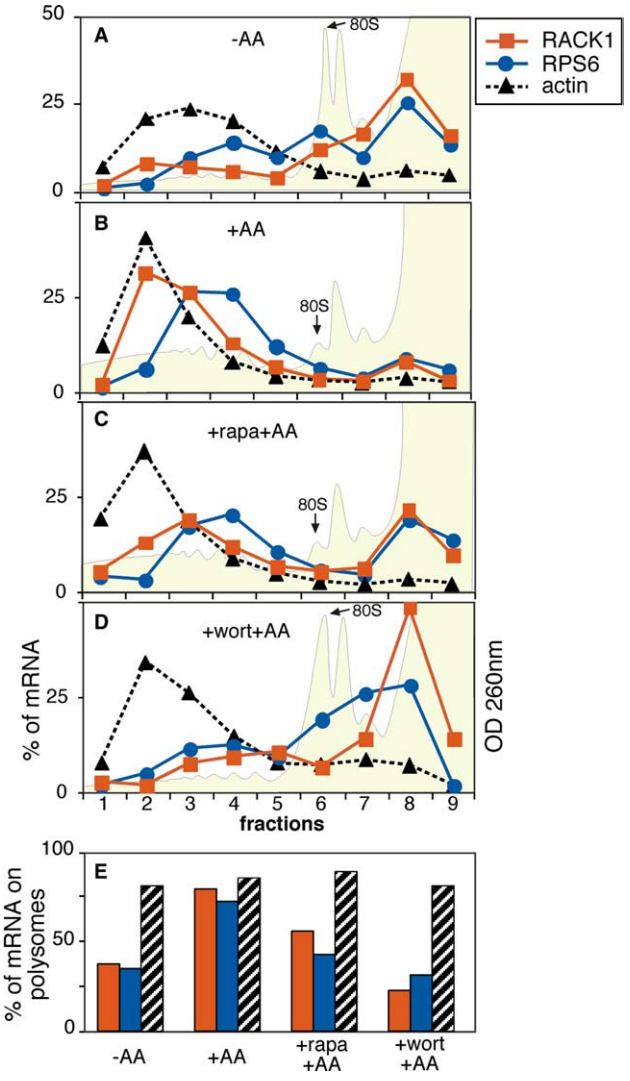

Fig. 3. *Amino acids effect on translation of RACK1 mRNA.* After overnight growth in complete medium (Fig. 2A) cells were incubated for 2 h in medium lacking amino acids (A, −AA), then stimulated for 1 h in complete medium without (B, +AA) or with rapamycin (C, +rapa + AA) or wortmannin (D, +wort + AA). Polysome separation, Northern blots and quantitation of the signals as in Fig. 2.

between the different conditions. The results (Figs. 2 and 3) show that RACK1 mRNA behavior follows a pattern similar to RPS6 mRNA. In fact RACK1 polysome association decreases during serum or amino acid deprivation and recovers during serum or amino acid stimulation. Rapamycin blocks the recovery (only partially in case of amino acid stimulation) whereas wortmannin effect is more complete At the same time β-actin mRNA does not show any relevant alteration of polysomal association.

4. Discussion

With the aim of collecting information on the function of RACK1 we investigated the similarities between RACK1 and RP addressing: (i) mRNA structure; and (ii) translational regulation associated to change in availability of growth factors and nutrients. We have recently shown that RP mRNA, as well as other TOP mRNAs, are translationally regulated in cultured cells by the serum and amino acid content of the medium [26]. We found that the structure and behavior of RACK1 and RP mRNAs is very similar in all our analyses. In fact RACK1 mRNA starts with an oligopyrimidines sequence and presents a short (45 nt) 3′ UTR, as all vertebrate RP mRNA do. Serum deprivation of HeLa cells induces a decrease of the percentage of RACK1 mRNA associated to polysomes whereas serum stimulation rapidly restores it to

the levels observed in exponentially growing cells. Moreover the mTOR inhibitor rapamycin blocks the recovery of RACK1 mRNA polysomal association. In all cases RACK1 mRNA behaves similarly to the control RPS6 mRNA and differently from the other control β-actin mRNA. Also in the case of deprivation and readdition of amino acids to the culture medium, RACK1 and RPS6 mRNAs exhibit a coordinate response in changing the fraction of mRNA associated with polysomes. In this case the recovery is blocked partially by rapamycin and totally by wortmannin as it has been shown for RP [26], suggesting that more than one signaling pathway is involved in this regulation. The overall conclusion from our experiments is that RACK1 synthesis is coordinated qualitatively and quantitatively with the production of RP. We think that this observation supports the idea, already proposed by other researchers, that RACK1 is a typical RP. It should be noted, however, that our findings do not necessarily rule out the possibility of secondary extraribosomal functions for RACK1 as at least a few of the ''standard'' RPs have been demonstrated or are presumed to have an additional function [28,29].

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