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RRas2, RhoG and T-cell phagocytosis

Balbino Alarcón* and Nuria Martínez-Martín
Centro de Biologia Molecular Severo Ochoa; Consejo Superior de Investigaciones Científicas; Universidad Autonoma de Madrid; Madrid, Spain

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Activating mutations and overexpression of classical Ras subfamily members (K-Ras, N-Ras and H-Ras) have been widely investigated as key events in the development of human cancers. The role in cancer of its closest relatives, the Ras-related (RRas) subfamily members, has been less studied despite the fact that one of its members (TC21 or RRas2) is strongly transforming in vitro. Nevertheless, and in spite the paucity of publications, several studies have shown that wild-type TC21 is overexpressed in different types of carcinomas and lymphomas. If the study of RRas members in cancer is still in its infancy, their role in physiological functions is even behind. For instance, T- and B-cell immunologists still use the vague term "Ras activation" without indication of what Ras family molecule is indeed intervening. In this view, we discuss the participation of TC21 in the specific process of T-cell antigen receptor internalization from the immunological synapse and acquisition of membrane fragments from the antigen presenting cells by phagocytosis.

Keywords: T cells, TCR, phagocytosis, trogocytosis, RRas2, TC21, RhoG, PI3K

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*Correspondence to: Balbino Alarcón;
Email: balarcon@cbm.uam.es

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The R-Ras subfamily of Ras-related proteins includes R-Ras1, R-Ras2 (TC21) and R-Ras3 (M-Ras) and show overall amino acid identity with the classical Ras subfamily (H-Ras, K-Ras and N-Ras) of 55–60%.[1] M-Ras is predominantly expressed in the brain whereas RRas1 and TC21 are more ubiquitously expressed. The effector loops of the R-Ras and classical Ras subfamilies (switch I and switch II) are basically undistinguishable, as well as the loops involved in the interaction with GDP and GTP (G1, G4 and G5) (**Fig. 1**). TC21 was found within a cDNA library derived from a teratocarcinoma cell line using a probe corresponding to part of the switch II loop.[2] Aside from the classical Ras members, TC21 is the only Ras superfamily member known to transform fibroblast and epithelial cell lines on its own[3,4] and that has been found in oncogenic versions in human cancers: an ovarian carcinoma and a leiomyosarcoma cell lines.[5,6] Nevertheless, TC21 has been most frequently found overexpressed in the wild type version in breast, oral cavity, esophagous and skin carcinomas as well as in lymphomas.[7-12] Furthermore, a polymorphism in the TC21 promoter has been associated with poor response to tamoxifen treatment in estrogen receptor-positive breast cancer.[13] Given the conservation of the effector loops it is not surprising that R-Ras and classical Ras members share a common set of upstream regulatory proteins and downstream effectors, although the overlap is not perfect.[14-16] Furthermore, although early findings pointed out to the Raf/MAP kinase cascade as required for fibroblast transformation,[17] others indicated that TC21-mediated transformation is Raf/MAP kinase independent[18] and that, indeed, TC21 transformation is mediated by PI3K.[19-22] Indeed, our own findings in TC21-deficient mice show that the PI3K pathway and not Raf/ERK are affected in T and B lymphocytes.[9] Therefore, TC21 seems to be specialized in the activation of PI3K pathway rather than Raf/ERK. Such difference could emerge from the distinct capability to interact and activate different catalytic subunits of type I PI3Ks.[1,23] Interestingly, Drosophila's genome encode for two Ras GTPases: one (dRas1, **Fig. 1**) that closely resembles the classical Ras members and another (dRas2, **Fig. 1**) that shares a higher

Figure 1. Sequence comparison of classical and Ras-related subfamily members. The amino acid sequence of the three classical human (h) Ras proteins is compared with these of the three R-Ras subfamily and with the two Ras subfamily members of *Drosophila melanogaster* (d). RRas is RRas1, TC21 is RRas2 and MRas is RRas3. The amino acid residues shared by at least two classical Ras members are in red. The positions in TC21 that differ from classical Ras are in bold blue type. The positions not conserved in classical Ras or TC21 are in purple. The sequences corresponding to the effector loops switch I and switch II and the G1, G4 and G5 loops are underlined in green. Drosophila's Ras1 resembles the classical Ras, whereas Drosophila's Ras2 resembles TC21.

identity with TC21 than with any other member of the Ras family. The expression of two distinct Ras GTPases, one of the classical type and another of the R-Ras type indicates these two subfamilies of GTPases became functionally specialized already in invertebrates.

The role of Ras in T- and B-cell biology has been traditionally studied by using dominant negative forms of a classical Ras and by ablation of genes downstream of Ras.[1,24] Expression of a dominant negative H-Ras mutant (H-Ras S17N) impaired differentiation of T cells during development in the thymus[25] as well as differentiation of mature T cells into IL4-secreting Th2 cells.[26] Likewise, expression of this transgene in B cells blocked B-cell development at a very early stage.[27] Surprisingly however, the ablation of H-Ras or N-Ras did not affect T-cell development, although

differentiation of mature T cells into IFNγ-producing Th1 cells in vitro and in vivo was impaired in the absence of either one of these GTPases.[28] The discrepancy of phenotypes resulting from expression of dominant negative H-Ras and H-Ras ablation could be explained by a possible dominant-negative effect on Ras family GTPases other than H-Ras and N-Ras. Our analysis of TC21-deficient mice has not shown any apparent deficiency on T- and B-cell development but an impaired survival and homeostatic control of mature T- and B-cells populations.[9] In addition, the germinal center (GC) response of mature B cells to antigen challenge in vivo is impaired in the absence of TC21. Therefore, the results with the H-Ras, N-Ras and RRas2 (TC21) knockouts indicate that classical Ras and R-Ras subfamilies of GTPases are likely to play non-redundant roles at different stages both during development and during antigen-induced responses in T and B cells. The specialized roles of each classical and RRas GTPase could originate from their intracellular localization, from their differential expression at different stages of differentiation or from their specialization in the activation of Raf/MAP kinase vs. PI3K pathways.

Our interest for TC21 originated from the initial finding that this GTPase interacts directly with a signaling sequence (known as Immunoreceptor Tyrosine-based Activation Motif, ITAM) that is present in each of the signaling subunits (CD3γ, CD3δ, CD3ε and CD3ζ) of the T-cell antigen receptor (TCR).[9] We found that TC21 interacts with the B-cell antigen receptor (BCR) as well, which can be explained by the presence of ITAMs in the Igα and Igβ subunits of the BCR. Furthermore, we found that TC21 plays an important role in the maintenance of housekeeping levels of PI3K activity in T and B cells.[9] TC21 accompanies the TCR to the contact area formed by T cells when they are primed by antigen-loaded presenting cells (APCs) and that it is known as the immunological synapse (IS). In the IS, the TCR is engaged by antigen, recruits signaling molecules such as the tyrosine kinase ZAP70 and the adaptor LAT, and triggers activation pathways. The TCR is triggered at the periphery of the IS and moves in a centripetal movement to an area known as central Supramolecular Activation Cluster (cSMAC) where it accumulates without apparent signaling activity and from where the TCR is internalized to promote its downregulation. In a more recent study, we have shown that overexpression of either a dominant negative mutant (S28N) or a constitutively active mutant (G23V) of TC21 in a T-cell line does not affect the localization of the TCR in the IS but prevents its internalization from the cSMAC by a clathrin-independent mechanism.[38] This effect was also detected in primary T cells from *RRas2*$^{-/-}$ mice. Using time-lapse fluorescent confocal videomicroscopy it is possible to visualize the formation of the IS following the accumulation of proteins like the TCR. Wild type TC21 and TCR were found to co-localize in the IS and to be co-internalized from the center of the IS, the cSMAC, into vesicular structures.[29] During IS formation, the T cell embraces the APC in a process that is reminiscent of a frustrated phagocytosis. Trying to pinpoint the nature of the internal vesicles originated from the cSMAC that contained TCR and TC21 we found partial co-localization with GTPases of the Rab subfamily previously shown to participate in endosomal trafficking. However, the co-localization of internalized TCR and TC21 with the small GTPase RhoG was striking. In spite its name, RhoG is a member of the Rho subfamily GTPases that is closer to Rac1 than to RhoA.[30] RhoG had been previously implicated in an evolutively conserved process of phagocytosis that is present in *C. elegans* and mammalian cells.[31,32] In macrophages, RhoG mediates the phagocytosis of apoptotic bodies.[33]

Expression of a dominant negative (T17N) or a constitutive active (Q61L) mutant of RhoG prevented the internalization of both the TCR and TC21 from the cSMAC, indicating that like TC21, RhoG mediated the internalization of the TCR from the IS.[29] This conclusion was backed by data generated with T cells from RhoG-deficient mice. Since RhoG has been involved in phagocytosis, we wondered whether TC21-mediated internalization of the TCR from the cSMAC was indeed a phagocytic process. To this end, we had first to demonstrate that T cells are endowed with the capacity to phagocytose. We found that latex beads of 1, 3 and even 6 μm diameter were phagocytosed by T cells if they were coated with stimulatory anti-TCR antibodies and not with control ones. This phagocytic process is dependent on both TC21 and RhoG. Interestingly, it has recently been published that TCR triggering with anti-TCR coated bead exerts a push force followed by a pulling force that leads to phagocytosis of the bead.[39] We proposed that when a T cell is contacted by an antigen-loaded APC the TCR is triggered in the IS and tries to phagocytose the APC. This attempt is frustrated probably due to the size of the APC. However, it has been long shown that T cells acquire fragments of the APC membrane through the IS, a process known as trogocytosis.[34,35] We found that TCR-triggered trogocytosis of APC membrane fragments was dependent on TC21 and RhoG and proposed that the frustrated phagocytosis of the APC resulted instead in the trogocytosis of APC membrane fragments.[29] Therefore, our current model is one in which the TCR is triggered by its pMHC ligand in peripheral areas of the IS and is translocated to the center of the IS, the cSMAC, where the high density of TCR triggers a phagocytic process resulting in both the internalization of the TCR from the cSMAC and the trogocytosis of an APC fragment.

Using pull-down assays with a glutathione-S-transferase (GST) fusion protein of the Ras-binding domain (RBD) of engulfment and motility protein (ELMO), an effector of RhoG, to measure the amount of active (GTP-bound) RhoG, we found that TCR-triggered activation of RhoG depended on both TC21 and PI3K activity. Since in other phagocytic systems, RhoG is placed downstream of PI3K because the RhoG activator TRIO is PI3K-dependent,[36] and TC21 directly contacts with the TCR and with the catalytic p110δ subunit of PI3K,[9] we propose that TC21 mediates the activation of RhoG by the TCR in conditions leading to phagocytosis. Indeed, the known interactions and activities of TC21 and RhoG in T cells and other cell types enable us to propose how they

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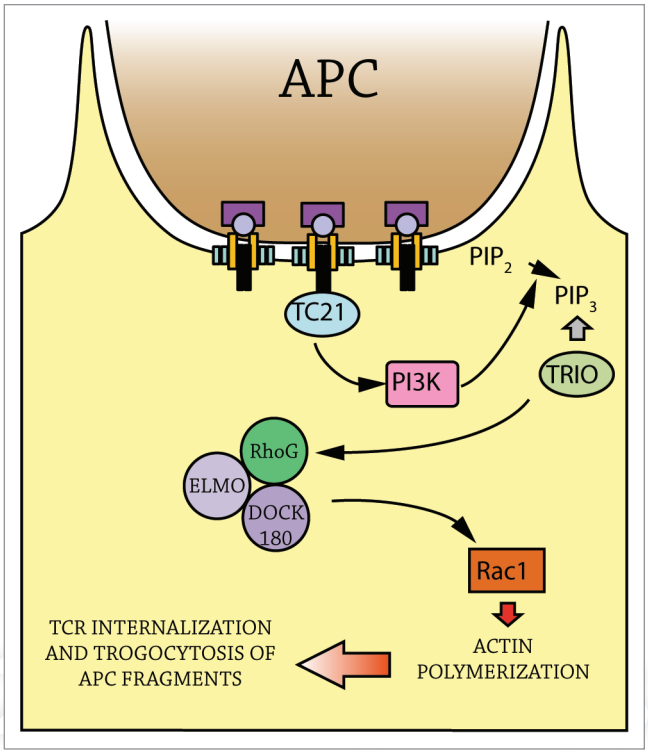

Figure 2. Hypothetical integration of TC21 and RhoG into a common phagocytotic pathway triggered by the TCR. TCR engagement of pMHC leads to the formation of an IS where the TCR and pMHC coalesce at the center of the structure (cSMAC). The activation of TC21 by the TCR leads to the direct recruitment of the p110δ subunit of PI3K, which is activated and phosphorylates phosphatidyl inositol-(4,5)-bisphosphate (PIP2) to phosphatidyl inositol-(3,4,5)-trisphosphate (PIP3). The direct recruitment of TC21 by the TCR followed by the direct recruitment of p110δ by TC21 increases the PIP3 concentration proximal to the site of TCR engagement. PIP3 recruits to the plasma membrane and activates the PH domain-containing protein TRIO, a GEF for RhoG, which is activated accordingly. RhoG forms a trimolecular complex with ELMO and DOCK180. The latter is a GEF for Rac1, which is activated and promotes the polymerization of the actin cytoskeleton necessary to trigger phagocytosis (trogocytosis) of a fragment of the APC membrane, together with the internalization of the TCR accumulated at the cSMAC.

might intervene in the phagocytic process (**Fig. 2**). RhoG is known to participate in the phagocytosis of apoptotic bodies in nematodes and in mammalian cells.[31,36] RhoG lies upstream of Rac1, which is necessary for actin polymerization in the phagocytic cup. On the other hand, TC21 directly activates the catalytic subunit p110δ of class I PI3Ks,[9] which plays an important role in the extension of the tip of the phagocytic cup around the phagocytosed particle.[37] TC21 directly binds to the TCR.[9] Therefore, ordering the intervention of these elements would suggest that the TCR accumulates at the cSMAC and promotes the activation of p110δ and RhoG via TC21 (**Fig. 2**).

In our opinion, the involvement of TC21 in TCR-driven phagocytic/trogocytic processes[29] and its specific role in the homeostasis of mature T- and B-cell populations[9] suggest that there is a functional specialization between classical Ras and Ras-related subfamilies, already found in insects, and that such specialization may derive from the preferential activation of signaling pathways, mainly Raf/ERK vs. PI3K, by such members. Such functional distinction, although not initially apparent given the conservation of effector loops, will require intensive investigation of sequence differences, regulated biological processes, and the identification of additional effectors.

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