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Review

Knocked out by Rho/Rac T-cell biology

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Summary. The Rho/Rac family is a group of Ras-related proteins with demonstrated roles in the regulation of proliferation and cytoskeletal structures in a number of cell lineages. Despite this, the actual role of these proteins in T-cells could not be addressed in vivo due to the lack of adequate animal models. Recently, the use of knockout and transgenic animals for Rac1, Rac2, and RhoA has provided a genetic proof of the importance of Rho/Rac protein in different aspects of T-cell signaling. These animals have also allowed us to get better views about the influence of these GTPases proteins on the maturation decisions of immature lymphocytes and on the signaling strategies these GTPases utilize to favor the generation of coherent and robust immune responses.

Key words: Rho/Rac GTPases, Transgenic mice, Knock-out mice, T-cell maturation, T-cell signaling

Introduction

The life of a lymphocyte is contingent on interactions with neighboring cells requiring extensive changes in the cytoskeleton. Positive and negative selection occurs during the migration of thymocytes through the thymus and their interaction with specific stromal subpopulations. Both the type of stromal cell making the contact with the thymocyte and the intensity of such an interaction determine whether the T-cell will live, die, or edit its TCR during the selection process. After maturation, T-cells must interact with endothelial cells and migrate across vessel walls to leave the blood stream and reach peripheral tissues. Finally, lymphocytes need to interact with antigen-presenting cells and target cells to assure antigen recognition and a sustained immune response. The dependence of all these processes on changes in the actin cytoskeleton has led to the study of the regulators that mediate actin dynamics. A group of proteins which is the main suspect for the coordination of this process is the Rho/Rac family of GTP-hydrolases. This family consists of twelve members that can be classified according to structural similarity in Rac, Rho, and Cdc42 subfamilies. Studies in many cell lineages have demonstrated that these GTPases work as molecular switches that sense the extracellular stimuli and translate them into changes of the cytoskeleton and the transcriptome that, in turn, will affect the adhesion, motility, gene expression, and cell cycle progression of the stimulated cell (for a review, Van Aelst and D'Souza-Schorey, 1997). The conservation of this function in T-lymphocytes was recognized very early on, when it was found that RhoA and Cdc42 had roles in cytotoxic responses and T-cell polarity in cultured cell lines, respectively (Lang et al., 1992; Stowers et al., 1995). Since then, these proteins have been linked to other T-cell functions, including second messenger production (Angkachatchai and Finkel, 1999), spreading (D'Souza-Schorey et al., 1998; Borroto et al., 2000), migration (del Pozo et al., 1999), chemotaxis (Haddad et al., 2001), and induction of cytokines (Angkachatchai and Finkel, 1999). Despite these observations, the study of the actual role of Rho/Rac-dependent pathways using animal models has been difficult because most of the animals homozygous for null mutations in *rho*/*rac* genes are embryonically lethal.

Until now, the most convincing genetic evidence for the implication of Rho/Rac GTPases in T-cell signaling derives from studies conducted with one of their upstream regulators, the Vav protein. Vav is a hematopoietic-specific nucleotide-releasing factor that catalyzes GDP/GTP exchange on members of the Rho/Rac family, leading to their activation during T-cell stimulation (for a review, Bustelo, 2000). Confirming the role of Rho/Rac proteins in cytoskeletal organization, *vav* (-/-) T-cells were found incapable of triggering actin polymerization upon TCR cross-linking. As a consequence, the stimulated lymphocytes show defects in receptor clustering and in the assembly of the immune synapse. In immature lymphocytes, these defects cause a defective transition of T-cell precursors from the $CD44^{-}CD25^{+}$ (double negative, DN3) to the $CD44^{-}CD25^{-}$

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(DN4) stage as well as an impaired positive and negative selection (Turner et al., 1997; Fischer et al., 1998). The defective signaling of mature T-cells induces low levels of interleukin-2 (IL2) secretion, resulting in lack of proliferative responses after TCR cross-linking (Tarakhovsky et al., 1995; Zhang et al., 1995; Turner et al., 1997; Fischer et al., 1998). The analysis of the signaling defects present in Vav-deficient cells revealed some unexpected results. First, *vav* (-/-) cells cannot induce Ca^{2+} during cell stimulation (Tarakhovsky et al., 1995; Zhang et al., 1995; Turner et al., 1997; Fischer et al., 1998). Secondly, Vav-deficient cells can activate some expected downstream elements of the Rho/Rac pathway (JNK, p38) but, instead, cannot fire the activation of the Ras-dependent ERK pathway (Costello et al., 1999). Although these signaling defects remain to be fully explained biochemically, it was assumed initially that they were due to the lack of activation of some Rac protein. However, this hypothesis has not been demonstrated experimentally up to now. Indeed, recent experiments conducted in Jurkat cells have given an unexpected twist to this story, since they suggest that both the stimulation of Ca^{2+} currents and the activation of IL2 gene transcription are independent on the exchange activity of Vav (for a review, Bustelo, 2001). Moreover, conflicting data still exist on the substrate specificity of Vav protein, with reports indicating specificity towards Rac proteins while others suggesting a generalized activity towards all members of the Rho/Rac family (Bustelo, 2000). At this point of the story, therefore, it is not known with certainty which parts of the phenotype of *vav* (-/-) animals are due to what GTPase and which parts are GTPase-independent. As we will discuss here, several reports by Flavell's and Cantrell's groups have apparently solved this signaling conundrum and, at the same time, established formal genetic proof of the key role of Rac1, Rac2, and RhoA proteins in different aspects of T-cell signaling. These studies have also allowed us to get better views about the influence of these GTPases on the maturation decisions of immature lymphocytes and on the signaling strategies these GTPases utilize to favor the generation of coherent and robust immune responses.

Animal models for Rho and Rac proteins

Flavell's group has reported for the first time the phenotype of T-cells derived from animals lacking a Rho/Rac subfamily member, the GTPase Rac2. Rac2 had been traditionally linked to hematopoietic responses, given its hematopoietic-specific expression and its implication in the oxidative burst of neutrophils. Rac2 mice were generated recently, confirming the important role of this GTPase in neutrophil responses (Roberts et al., 1999). The same knockout strain was utilized by Flavell's group to investigate the role of this GTPase in T-cells (Yu et al., 2001). Unlike *vav* (-/-) animals, Rac2-deficient mice show no detectable alterations in T-cell development and positive/negative selection. However, mature T-cells do show a very similar, although significantly milder, phenotype to that displayed by *vav* (-/-) animals. This work also shows that the milder effect of the Rac2 deficiency is probably due to a compensatory effect by the related Rac1 protein, since this GTPase becomes overexpressed in several T-cell populations of *rac2* (-/-) animals. These results appear to tilt the balance of the signaling basis of the Vav phenotype towards the idea that it is caused by a generalized defect in the activation of both Rac1 and Rac2 GTPases. In addition to the implication of Rac2 in the early responses of T-cells to antigens, Rac2 has been previously shown to be important for the differentiation of T^{H1} cells, probably by regulating the production of IFN-γ (Li et al., 2000). Taken together, these results indicate that Rac2 mediates both the proliferation of primary T-cells as well as the differentiation of activated helper cells into the T^{H1} lineage.

Two other studies recently done by Cantrell's laboratory have shed light on the role of two other GTPases, Rac1 and RhoA, in T-cell signaling. To approach the function of these GTPases in T-cells, they have used transgenic mice expressing constitutively-active forms of Rac1 ($Rac1^{Q61L}$) or RhoA ($RhoA^{G14V}$) in thymocytes. The phenotypic analysis of these mice indicates that Rac1 and RhoA have become specialized in different aspects of T-cell biology, with Rac1 having an active role in pre-T cell biology and RhoA in more mature thymic populations. $Rac1^{Q61L}$ animals show an enhanced transition of pre-T cells from the DN3 to the DN4 maturation stage and an exacerbated negative selection of double-positive (DP) cells that totally eliminates the generation of helper and cytotoxic T-cells (Gomez et al., 2000). When these animals were crossed with *rag1* (-/-) mice that cannot rearrange the TCR genes, $Rac1^{Q61L}$ could trigger the transition of DN4 to DP cells (Gomez et al., 2000), indicating that this GTPase can substitute the pre-TCR to induce some aspects of this maturational shift (see below). In contrast, it has been shown by Cantrell's group that RhoA has no noticeable effect on pre-TCR signaling in the same type of experiments (Corre et al., 2001). This result is somewhat surprising, since previous publications by the same group using transgenic mice expressing the C3 exoenzyme, a RhoA inhibitor, showed that this GTPase is important for the survival of some pre-T cell subpopulations and for DN4 cell proliferation (Galandrini et al., 1997; Henning et al., 1997). These contradictory results can be reconciled assuming that RhoA is necessary, but not sufficient, to promote such biological effects or, alternatively, that some of these maturational defects are artifacts derived from the inactivation of uncharacterized signaling molecules sensitive to the action of this bacterial inhibitor. The final elucidation of this riddle will be only answered when the rhoA knockout becomes available. Despite the relative "inactivity" of RhoA in DN cells, $RhoA^{G14V}$ has significant effects in TCR signaling, resulting in enhanced positive selection of DP cells and more robust

proliferation of single positive (SP) T-cells upon anti-CD3 stimulation (Corre et al., 2001).The signaling mechanism by which this occurs has not been explored in that report. One interesting experiment to carry out in the future is to generate transgenic mice with switch mutants of $RhoA^{G14V}$ that cannot engage specific signaling pathways in order to identify the effector molecules that are important in this process. A similar approach has been done with Rac1 with very interesting results (see below). Since positive selection is mainly driven by activation of the ERK cascade (Sugawara et al., 1998), another possible experiment is to cross $RhoA^{G14V}$ animals with ERK1 knockouts to test the dependency that RhoA has on ERK to promote positive selection. Given the important role of the effector Rock in proliferative signals in other cell types (Van Aelst and D'Souza-Schorey, 1997), another alternative is to see the effect of available Rock inhibitors in both positive selection and T-cell proliferation using organotypic or in vitro culture experiments, respectively. All these experiments should give us in the future a much better view of what is going on in these biological processes.

Role of Rho and Rac proteins in T-cell development

The availability of these mice has also made it possible to dissect for the first time the intricacies of the biological processes involved in T-cell differentiation. For instance, it is known that the transition from DN4 to DP cells involves the activation of several genetic programs, including the stimulation of proliferation and cell cycle progression of DN4 cells, the up-regulation of CD4, CD5, and CD8 markers, and the downmodulation of CD25 gene expression. Using complementation studies with *rag1* (-/-) mice, Cantrell's group has recently shown that $Rac1^{Q61L}$ signaling is sufficient for the regulation of only some of these pathways. Thus, $Rac1^{Q61L}$ is capable of inducing the expression of CD4, CD8, and CD25 in the absence of pre-TCR. Instead, $Rac1^{Q61L}$ cannot upregulate the CD5 marker or trigger enough levels of proliferation of DN4 cells to restore the low cellularity of *rag1* (-/-) thymi back to wild type levels (Gomez et al., 2000). Thus, these two responses seem to require the separate stimulation, or co-engagement, of other unrelated signaling pathways.

Role of Rho and Rac proteins in T-cell signaling: clues about pathways and their cross-talks during receptor activation

Cantrell's group has also used these transgenic strains to solve the problem of the identity of the signaling pathway responsible for the abnormal pre-T cell maturation found in *vav* (-/-) mice. To this end, they crossed the Rac1 and RhoA transgenic animals with *vav* knockout mice to perform rescue experiments. It was found that $Rac1^{Q61L}$, but not Rho^{AG14V}, could restore the abnormal DN3 to DN4 transition of Vav-deficient thymocytes (Gomez et al., 2000; Corre et al., 2001). The importance of these findings is two-fold. On one hand, they demonstrate that the regulation of this maturational transition is dependent on the exchange activity of Vav and not on other unrelated pathways. On the other hand, they give a firm genetic demonstration that Rac, but not Rho or Cdc42 proteins, are the main targets of Vav in this biological process. Collectively, all these results indicate that Rac and Rho proteins promote quite different signaling pathways and biological outcomes in immature T-cells. Unfortunately, the lack of SP cells in Rac1-expressing animals made it impossible to extend the comparative analysis of Rac1 and RhoA to mature T-cells (Gomez et al., 2000).

The study of GTPase function has indicated that the generation of cellular responses to extracellular stimuli requires the stimulation of multiple intracellular pathways. The synchronization of these routes to give rise to a coherent cell response appears to be achieved using two regulatory strategies. On one hand, there is the phenomenon known as signaling divergence, in which a single GTPase can induce the activation of several effector molecules. At the same time, the stimulation of cells leads to processes of signaling convergence, where the pathways that have been stimulated by different GTPases establish synergistic interactions to yield more robust signaling responses. For example, the activation of Cdc42 in fibroblasts triggers a signaling cascade that leads to the sequential activation of Rac1 and RhoA. Rac and Ras pathways also have synergistic interactions at the level of Rac GEFs, PAK, Raf, and MEK (for a review, Bar-Sagi and Hall, 2000). The results obtained with transgenic and knockout approaches have also contributed to the understanding of how these signaling mechanisms work in the lymphocyte. In regard to signaling divergence, we are realizing that the GTPases may require only a fraction of their effectors to induce specific T-cell responses. For instance, the phenotype induced by $Rac1^{Q61L}$ in the thymus can be reproduced when these studies are done with a $Rac1^{Q61L}$ mutant that cannot activate PAK, JNK, p38, or the cyclin D1 promoter ($Rac1^{Q61L+Y40H}$) (Gomez et al., 2000). These results suggest that only the pathway mediated by the Rac1 effector POR1 (or other proteins binding to the same region) is the important one to elicit the complex developmental program of a pre-T cell. Indeed, unrelated studies suggest that GTPases utilize complex signaling strategies to make sure that only a subset of their effectors are activated during specific phases of the immune response. Perhaps the most interesting example of this type of strategy is the regulation of JNK activity. This kinase plays two opposite and antagonistic roles during T-cell stimulation. At the beginning of the immune response, JNK exerts an inhibitory role over the secretion of IL2 and, therefore, plays a negative influence on T-cell proliferation. At later time-points, JNK activity plays positive roles in the differentiation of helper lymphocytes to TH1 and TH2 subtypes (Dong et al., 2000). To bypass the inhibitory action of JNK at the initial stages of the immune response, lymphocytes

maintain JNK at low levels and only increase its concentration when helper T-cell differentiation has to take place (Yang et al., 1998; Dong et al., 2000; Weiss et al., 2000). All these results indicate that the signaling output of a particular GTPase depends both on the availability and concentration of the antigen and on the "signaling history" of the T-cell where such stimulation occurs. In regard to the possible convergence of independent pathways during T-cell stimulation, it has been demonstrated that the cross talk between Rac/RhoA and Rac/Ras found in other cell types is also conserved in T-cells. Thus, the expression of the C3 exoenzyme in $Rac1^{Q61L}$ mice leads to the inhibition of all the biological effects induced by $Rac1^{Q61L}$ in T-cell differentiation. However, C3 does not abolish the effect of Rac1 on negative selection, indicating that this synergism is limited to specific signaling events (Corre et al., 2001). These results give us some extra confidence about the physiological significance of the results obtained with the C3 exoenzyme, since Rho^{AG14V} does not trigger negative selection (Corre et al., 2001). According to these results, it seems that the Rac1/RhoA pathway is conserved both in fibroblasts and pre-T cells but not in DP cells. What the authors of this work have not addressed is the nature of the interconnection between Rac1 and RhoA in those cells. In principle, there are two possible explanations for this functional interaction: the direct activation of RhoA by Rac1 or, alternatively, a synergism between different signaling elements activated by these two proteins. These possibilities can be explored in the future biochemically using these animals. For instance, if the first case is at work, we must expect that $Rac1^{Q61L}$-expressing cells should have the endogenous RhoA protein in the activated, GTP-bound state. This can be easily verified by performing pull-down experiments with GST fusion proteins containing the RhoA binding domain of some of its known effectors (i.e., rhotekin). In the same functional context, the examination of *rac*2-deficient T-cells has demonstrated that the Rac and Ras pathways are interconnected in T-cells, since these cells show defective activation of the Ras/ERK pathway upon TCR stimulation (Yu et al., 2001). Unfortunately, nobody has looked so far at the basis of this signaling alteration either in these mice or in the previously generated *vav* (-/-) animals. Using reagents available in the market, experiments can be designed to test the type of connection between the Vav/Rac2 pathway and Ras. Among the possible pathways to be explored, these animals can be tested for the activation status of Ras, PAK, Raf or MEK. The results of these experiments will tell us whether the points of interaction between the Rac and Ras previously found in other cell types are conserved in the lymphocyte (Bar-Sagi and Hall, 2000). Moreover, we can also explore whether T-cells may utilize tricks of their own to activate this route. For instance, Rac2-deficient cells have impaired activation of PLC-γ proteins, as inferred from the low levels of Ca^{2+} generation during T-cell stimulation (Yu et al., 2001). This suggests that the activation of Ras in this cell system can be accomplished via the activation of PLC-γ, generation of DAG, and stimulation of RasGRP (also known as CalDAGII), a DAG-dependent, Ras-specific exchange factor that has essential roles in TCR signaling (Dower et al., 2000).

The fact that much of the information discussed here was discovered only after the generation of transgenic and knockout mice highlights the importance of these approaches to understand the complexities of T-cell regulation. Taken the present and previous studies together, we now know that Rho/Rac proteins have specialized functions that depend on the maturational stage of the T-cell and the stage of the immune response in which their signaling occurs. Some of these functions are related to the regulation of actin dynamics, while others rely possibly on other routes that mediate the activation of downstream effectors and the synergism with independent pathways. Depending on the GTPase involved, these signaling events influence the maturation and selection of thymocytes, the proliferation of mature lymphocytes to antigens, and/or their effector functions. Arguably, the tissue-specific knockouts and knock-ins of Rac1, RhoA, Cdc42, and other GTPases will give us a new perspective on the involvement of each member of this GTPase family in all aspects of T-cell biology.

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