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The regulation and function of p21^{ras} during T-cell activation and growth

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The delivery of signals that control the growth of T cells is a key event for effective co-ordination of T-cell-dependent immune responses. It is now recognized that guanine nucleotide binding proteins play an important role in signal transduction by the T-cell receptor (TCR) and cytokine receptors. Here, Manolo Izquierdo Pastor, Karin Reif and Doreen Cantrell review the numerous recent advances in understanding how the p21^{ras} guanine nucleotide binding protein couples the TCR to the T-cell signalling cascade.

The ability of the T-cell receptor (TCR) and the interleukin 2 receptor (IL-2R) to regulate the guanine nucleotide binding cycle of the low-molecular-weight G protein p21^{ras} suggests that these proteins might be important in immunological responses. T-cell growth can be divided into two phases: the first phase, termed T-cell activation, is initiated by the interaction of antigen with the TCR, which triggers the G0–G1 transition of the cell cycle, as well as the induction of IL-2 secretion and IL-2R expression. The second phase involves G1–S phase progression and mitosis, and is controlled by IL-2–IL-2R interactions. In this review, we wish to discuss current understanding of the regulation and function of p21^{ras} in the immune system, using the paradigms of TCR and IL-2R control of T-cell activation and growth.

The regulation of p21^{ras} by the TCR and IL-2R

Triggering of the TCR- or the IL-2R-coupled protein tyrosine kinases (PTKs) perturbs the guanine nucleotide binding cycle of p21^{ras}, and results in a rapid and prolonged accumulation of active p21^{ras}–GTP complexes. The TCR is a multichain complex comprising the polymorphic $\alpha\beta$ chains, the invariant CD3 γ , δ and ϵ chains, and the ζ (16 kDa) subunits¹. The intracellular tails of the CD3 and ζ molecules couple the TCR to intracellular PTKs such as ZAP-70, p59^{lyn} and p56^{lck}. This PTK regulation is absolutely required for all subsequent TCR responses, including p21^{ras} activation¹. Triggering of the IL-2R also activates p21^{ras} in a PTK-dependent response². The IL-2R comprises three subunits, α , β and γ , of which the β and γ chains are mem-

bers of the haematopoietin receptor family. Deletion analysis of the IL-2R β subunit has identified a membrane-distal acidic domain that binds *src*-family kinases such as p56^{lck}, as well as a membrane-proximal serine-rich domain that binds Janus family kinases (JAKs)³. Both of these domains of the IL-2R β chain are required for p21^{ras} activation².

The adapter molecule Grb2 and associated proteins in T cells

One mechanism for p21^{ras} activation is the stimulation of guanine nucleotide exchange proteins such as the homologue of the *Drosophila* 'son of sevenless' (Sos) gene product^{4,5}. In fibroblasts, growth factors regulate p21^{ras} by stimulating Sos *via* a mechanism involving the adapter protein Grb2/Sem5 (Ref. 6). Grb2 comprises one *src*-homology 2 (SH2) domain and two SH3 domains. The SH3 domains of Grb2 bind to the C-terminal proline-rich domain of Sos, whereas the SH2 domain interacts with tyrosine-phosphorylated molecules that regulate the cell localization/function of this p21^{ras} exchange protein⁶. Studies of Grb2 in T cells have identified two proteins that, when tyrosine phosphorylated, can potentially bind to the Grb2 SH2 domain. These are the SH2 domain-containing adaptor protein Shc, of which two isoforms of 46 and 52 kDa are expressed in T cells, and a membrane-located tyrosine phosphoprotein of 36 kDa (Ref. 5).

In TCR-activated cells, both Shc and p36 are tyrosine phosphorylated and, *in vitro*, can be shown to bind Grb2 SH2 domains. Shc has an SH2 domain that can

Fig. 1. A summary of the immediate membrane-proximal signals triggered by TCR ligation. TCR-coupled tyrosine kinases, such as ZAP-70, phosphorylate a novel adaptor molecule p36, which binds to PLC, thus regulating inositol phospholipid metabolism and calcium/PKC signalling pathways. p36 also binds the SH2 domain of the adaptor protein Grb2. The SH3 domains of Grb2 bind a novel 75 kDa molecule or Sos, a guanine nucleotide exchange protein for p21^{ras}. p36-Grb2-Sos complexes may thus link the TCR to the p21^{ras}/Raf-1/ERK kinase cascade. The TCR also regulates p21^{ras} via inhibition of p21^{ras} GAPs. Solid lines indicate interactions based on protein phosphorylation. Dashed lines indicate uncharacterized pathways. Abbreviations: TCR, T-cell receptor; PLC, phospholipase C; PKC, protein kinase C; SH2, src-homology domain 2; Sos, Drosophila 'son of sevenless'-like protein; ERK, extracellular-signal-regulated kinase; GAP, GTPase-activating proteins; MKK, mitogen-activated protein kinase kinase.

bind to the tyrosine-phosphorylated TCR ζ chain, hence tyrosine-phosphorylated Shc could potentially recruit Grb2 and Sos to the TCR complex in activated cells⁷. However, no Shc-Grb2-Sos complexes are detected in TCR-activated cells⁵. Moreover, it appears that the Shc interaction with ζ chains is of relatively low affinity as compared with the binding of tyrosine kinases such as ZAP-70 or p59^{lyn} to ζ (N. Osman, D. Cantrell, unpublished). This may ensure that the Shc interaction with ζ is transient and perhaps only sufficient to bring Shc to the TCR complex, where it can be tyrosine phosphorylated, but is insufficient for the formation of stable TCR-Shc complexes. Tyrosine-phosphorylated Shc may then dissociate from the receptor and form a complex with cytoplasmic adaptor molecules. The major tyrosine phosphoprotein to associate with Grb2 SH2 domains in TCR-activated cells is a 36 kDa molecule (p36), which is proposed to be an adaptor that links the TCR-activated PTKs to Grb2-Sos (Refs 5,8). The failure to find tyrosine-phosphorylated Shc binding to Grb2 and Sos in TCR-activated cells may be the consequence of competition between p36 and Shc for the SH2

domain of Grb2. However, it seems unlikely that Shc recruits Sos to the TCR complex or that Shc is involved in TCR coupling to Grb2-Sos or p21^{ras}. This does not mean that Shc is unimportant for TCR signalling: there is the possibility that Shc can form a complex with other adaptor molecules and hence regulate an effector system distinct from the Sos/p21^{ras} pathway in response to TCR ligation (Fig. 1).

In contrast to the failure to see Shc-Grb2-Sos complexes in TCR-activated cells, there are data suggesting that Shc may be important in coupling the IL-2R to p21^{ras}. Thus, in IL-2-activated cells, there is no tyrosine phosphorylation of p36, rather there is a high level of Shc tyrosine phosphorylation and rapid formation of Shc-Grb2-Sos complexes⁹. The IL-2R can be co-precipitated with Shc in IL-2R-activated T cells, but it is not clear whether this association also results in recruitment of Grb2 and Sos to the IL-2R complex⁹: for example, Sos co-precipitation with the IL-2R has not been described. Therefore, it must be considered a possibility that Shc allosterically regulates the activity of Grb2-associated Sos (Fig. 2).

Fig. 2. Signalling pathways that induce IL-2-responsive genes and T-cell growth. Signalling to the nucleus may occur by direct, JAK-mediated phosphorylation of STATs, in combination with other pathways. IL-2-activated src-family tyrosine kinases, such as p56^{lck}, tyrosine phosphorylate the adapter protein Shc, which then complexes to the SH2 domain of Grb2. The interaction between Shc and Grb2 couples the IL-2R to at least two Grb2-associated effector molecules: the p21^{ras} guanine nucleotide exchange protein Sos and a novel 75 kDa molecule. IL-2 activates p21^{ras} and Raf-1 but its ability to stimulate kinases such as ERK2 is determined by a balance of the positive p21^{ras}-induced signals and negative-feedback mechanisms mediated by MAP kinase phosphatases such as PAC-1. Abbreviations: IL-2, interleukin 2; JAK, Janus family kinases; STATs, signal transducers and activators of transcription; MAP kinase, mitogen-activated protein kinase. For all other abbreviations see Fig. 1.

Intracellular PTKs that are important for TCR signal transduction include the *src*-family kinases p56^{lck}, p59^{lyn} and ZAP-70 (Ref. 1), whereas the IL-2R most notably regulates the activity of p56^{lck} (Ref. 3). Shc is tyrosine phosphorylated both by the TCR- and IL-2R-induced PTKs and, on this basis, it would be predicted that Shc is a substrate for the *src* kinases, although a role for IL-2-stimulated JAKs in Shc phosphorylation cannot be excluded. By contrast, p36 is only tyrosine phosphorylated in response to TCR triggering and a simplistic analysis would suggest that it is probably a substrate for the kinase ZAP-70, which is activated by the TCR but not by the IL-2R. Recently, it was observed that p36 also exists in a complex with phospholipase C in TCR-activated cells, suggesting that p36 may be an adaptor that couples the TCR both to p21^{ras} and to Ca²⁺/protein kinase C (PKC) signalling pathways that originate from the TCR (Ref. 10; Fig. 1).

In summary, the current hypothesis for the regulation of p21^{ras} in T cells is that the IL-2R regulates Sos, a guanine nucleotide exchange protein for p21^{ras}, via the adaptors Shc and Grb2 (Fig. 2). The TCR may also regulate the p21^{ras} guanine nucleotide binding cycle via

Grb2–Sos, but it is an unknown 36 kDa tyrosine phosphoprotein rather than Shc that links the TCR to the adaptor Grb2 (Fig. 1). However, it must be emphasized that the purported role of Grb2 and Sos in TCR or IL-2R regulation of p21^{ras} is based on correlative data and remains to be proven by genetic studies. Moreover, two molecules in addition to Sos are found complexed to the SH3 domains of Grb2 in T cells. These two proteins, of 75 and 116 kDa respectively, are constitutively associated with the SH3 domains of Grb2, analogous to the Grb2–Sos association^{8,11}. p75 and p116 are substrates for TCR-activated, but not IL-2R-activated, PTKs. On the basis of their association with the Grb2 SH3 domains, p75 and p116 are candidates for Grb2 effector molecules. Whether they are involved in p21^{ras} regulation remains to be determined.

Ras-GTPase-activating proteins (GAPs) and other p21^{ras} regulatory molecules

It has been suggested that p95^{vav} is a guanine nucleotide exchange protein for p21^{ras} in T cells¹², even though its structure suggests that p95^{vav} is a more likely candidate for a *rho* or *rac* exchange protein¹³. p95^{vav} is tyrosine

phosphorylated in response to TCR triggering and, *in vitro*, this appears to stimulate the guanine nucleotide exchange activity of the protein¹³. However, tyrosine phosphorylation of p95^{vav} does not correlate with p21^{ras} activation: for example, in T cells derived from peripheral blood, p59^{vav} is not phosphorylated in response to IL-2 even though IL-2 activates p21^{ras}. Conversely, stimulation of T cells *via* CD28, with the B7-1 ligand, induces a strong, persistent tyrosine phosphorylation of p95^{vav} but does not activate p21^{ras} (Ref. 14). Instead, the failure of CD28 to regulate the p21^{ras} guanine nucleotide binding cycle correlates with the failure of CD28 to induce the tyrosine phosphorylation of p36, Shc or any other protein capable of recruiting the Grb2-Sos complex to the membrane¹⁴. Moreover, the ability of p95^{vav} to function as a p21^{ras} guanine nucleotide exchange factor is not supported by analyses of p95^{vav} function in fibroblasts, where no evidence for its regulation of p21^{ras} can be detected¹⁵. Thus, p95^{vav} may well have important functions in haematopoietic cells but possibly not as a guanine nucleotide exchange protein for p21^{ras}.

Undoubtedly, the current trend in studies on p21^{ras} regulatory function is to focus on receptor-mediated control of p21^{ras} guanine nucleotide exchange proteins. However, the importance of negative-regulatory p21^{ras} GTPase-activating proteins (GAPs) for p21^{ras} activation should not be ignored. The GTPase activity of p21^{ras} is controlled by GAPs such as p120-GAP and neurofibromin⁶, and experiments have shown that TCR triggering inhibits the activity of such p21^{ras}-GAPs (Ref. 16). The mechanism by which the TCR inhibits these proteins is not understood but it is probable that simultaneous regulation of guanine nucleotide exchange proteins and GAPs by the TCR contributes to p21^{ras} regulation. By contrast, IL-2 activation of p21^{ras} is not associated with any detectable regulation of p21^{ras}-GAP proteins and it is predicted therefore that the IL-2R regulates p21^{ras} *via* the activation of p21^{ras} guanine nucleotide exchange proteins¹⁷.

p21^{ras} function in TCR-mediated signal transduction

The function of p21^{ras} in T cells has been explored using transient transfection protocols that examine the consequences on T-cell activation of expressing mutated, constitutively active or dominant inhibitory p21^{ras} molecules. The expression of a dominant negative p21^{ras} suppresses IL-2 gene induction, and a constitutively active p21^{ras} protein can activate transcriptional factors such as AP-1 and synergize with a calcium signalling pathway to activate the IL-2 gene^{18,19}. One point to emphasize regarding the role of p21^{ras} in T-cell activation is that p21^{ras} function is essential, but not sufficient, for a full activation response²⁰. For example, IL-2 gene expression requires the coordinate interaction of multiple transcriptional factors, which reflects the convergence of multiple signalling pathways triggered by both the TCR and costimulatory receptors, only some of which involve p21^{ras} (Fig. 3). The regulation of lymphokine expression is one important facet of T-cell activation, but equally important are the signals that regulate the expression and function of lymphokine receptors and adhesion molecules. Indeed, p21^{ras}

may have a role in this aspect of TCR function, as demonstrated by observations that p21^{ras} regulates the expression of CD69, a T-cell activation marker²¹.

The transmission of signals from p21^{ras} to the nucleus is proposed to involve the regulation of the activity of mitogen-activated protein (MAP) kinases or extracellular-signal-regulated (ERK) kinases such as ERK1 and ERK2 (Ref. 22). Two intracellular pathways for ERK2 regulation co-exist in T cells: one mediated by p21^{ras} and the other by PKC (Ref. 23). The TCR stimulates both p21^{ras} and PKC, although it appears that p21^{ras} rather than PKC couples the TCR to the regulation of MAP kinases^{23,24}. The MAP kinases are activated by a kinase cascade involving a MAP kinase kinase (MKK) that phosphorylates and stimulates ERK1 and 2 directly²⁵. The activity of the MKK is itself controlled by phosphorylation and hence a MAP kinase kinase (MKKK) plays a crucial role in the regulation of ERK1 and 2 (Ref. 26). In some cells, the serine/threonine protein kinase Raf-1 has been identified as the MKKK that plays a key role in coupling p21^{ras}, and hence receptors that stimulate p21^{ras}, to the MAP kinases²⁷. The N-terminal regulatory domain of Raf-1 can interact directly with 'activated' GTP-bound p21^{ras} (Ref. 28). It has also been shown that constitutively active Raf-1 can mimic the effect of activated p21^{ras}, stimulating ERK2 and synergizing with calcium signals to induce the IL-2 gene^{29,30}. These observations collectively suggest that Raf-1 is an effector molecule for p21^{ras} in T cells, but they do not rule out that other p21^{ras} effectors may also exist.

p21^{ras} regulation of the IL-2 gene is apparently mediated by nuclear factor of activated T cells (NF-AT1). NF-AT1 is a complex comprising AP-1 and NF-ATp, a member of the *c-rel* family of transcriptional factors³¹. NF-ATp is cytoplasmic in quiescent cells and translocates to the nucleus in response to calcium/calcieneurin-dependent signals. p21^{ras} and calcineurin synergize for NF-AT1 induction and the role of p21^{ras} in NF-AT1 regulation can probably be explained by p21^{ras} effects on AP-1 (Refs 20,32). One level of AP-1 regulation is *via* induction of *fos*-family proteins. The phosphorylation of the transcriptional factor Elk1 by ERK2 appears obligatory for induction of the *c-fos* gene³³. Accordingly, the role of p21^{ras} in coupling the TCR to ERK2 and the ability of ERK2 to translocate to the nucleus, where it can directly modulate transcriptional factors such as Elk1, could explain the role of p21^{ras} in TCR signal transduction. However, there is as yet no functional proof that ERK2 is the link between p21^{ras} and the induction of lymphokine gene expression. Furthermore, it is known that AP-1 activity also requires *jun*-family proteins and the regulation of *c-jun* phosphorylation mechanisms is complicated by the involvement both of positive and negative phosphorylations that control transcriptional activity³⁴. Kinases other than ERK1 and ERK2 can phosphorylate *c-jun* and hence regulate AP-1. One of these, JNK-1, is activated synergistically by the TCR and the costimulatory receptor CD28. p21^{ras} may play a role in the TCR synergy with CD28 for JNK-1 activation^{35,36} and it should also not be excluded that other p21^{ras}-regulated ERKs will exist. NF-AT1 binding sites have been described in the promoters of a number of cytokine genes³⁷ and

Fig. 3. Signalling pathways that control T-cell activation. At least three essential signalling pathways are involved in TCR-mediated signalling: those mediated by p21^{ras}, calcium/calcineurin signals and PKC. The costimulatory molecule CD28 does not regulate these pathways but activates PI 3-kinase and a MAP kinase family member JNK-1 (see also Ref. 44). The nuclear target for each signalling pathway is a transcriptional factor such as AP-1, NF- κ B or NF-ATp. Cytokine genes such as IL-2 require the coordinate action of multiple transcriptional factors for transcriptional activation. This explains why a single intracellular signal is insufficient for IL-2 gene induction. The known key convergence points for these intracellular signals are indicated by an asterisk. Abbreviations: NF-ATp, cytosolic component of nuclear factor of activated T cells; PI 3-kinase, phosphoinositide 3-kinase; JNK, Jun kinase; [Ca²⁺]_i, intracellular calcium; PtdIns(4,5)P₂, phosphatidylinositol (4,5)-bisphosphate; PtdIns(1,4,5)P₃, phosphatidylinositol (1,4,5)-trisphosphate; Ins(1,4,5)P₃, inositol (1,4,5)-trisphosphate; DAG, diacylglycerol.

For all other abbreviations, see Figs 1 and 2.

p21^{ras} may therefore play a general role in controlling cytokine expression in lymphocytes.

p21^{ras} function in IL-2R-mediated signal transduction

Although IL-2R triggering is associated with p21^{ras} and Raf-1 activation, there are discrepant reports as to whether IL-2-mediated stimulation of ERK2 occurs^{38,39}. Recently, it has been shown that cell phosphatase activity may be rate limiting for regulation of ERK2. In particular, a dual specificity phosphatase PAC-1 has been identified that dephosphorylates ERK2 *in vivo*, thus terminating or preventing its activation in T cells⁴⁰. PAC-1 expression is upregulated only after T-cell activation, so in the initial stages of TCR signalling it can play no role. However, its upregulation in response to the TCR may explain the transience of TCR-mediated activation of ERK2. IL-2Rs are expressed only in activated T cells and, since the constitutive expression of PAC-1 suppresses activation of ERK2, the presence of PAC-1 in

activated (i.e. IL-2R expressing) T cells could explain the inability of IL-2 to stimulate ERK2 (Ref. 40). Thus, it is becoming clear that the expression of MAP kinase phosphatases is an important factor that will determine the cellular outcome of p21^{ras} stimulation of the ERK1 and 2 pathway. However, it is not yet known whether p21^{ras} couples the IL-2R to a downstream kinase cascade analogous to the role of p21^{ras} in TCR coupling to ERK2. Lymphocyte cell growth is dependent on cytokine production, which means that p21^{ras} function would be, albeit indirectly, indispensable for T-cell growth. It remains to be established whether p21^{ras} function has a more direct impact on T-cell growth by playing an essential role in IL-2R control of mitogenesis.

Concluding remarks

The ability of the TCR and the IL-2R to regulate a common signal transduction molecule such as p21^{ras} may seem at odds with the observations that there are

distinct patterns of gene expression regulated by the TCR and IL-2R (Ref. 41). However, p21^{ras} signalling may be more complicated than is frequently depicted, and the potential exists for multiple p21^{ras} effectors. The only p21^{ras} pathway identified in T cells to date is the p21^{ras}/Raf-1/MAP kinases cascade, but other pathways may exist. It is also clear that p21^{ras} activation is only one of a complex series of biochemical responses triggered by the TCR or IL-2R. In particular, biochemical signalling pathways specific to either the TCR or the IL-2R exist. One well-documented TCR-mediated signalling response that is not shared by the IL-2R is the activation of phosphatidylinositol hydrolysis, intracellular calcium mobilization and PKC activation⁴². Similarly, the IL-2R but not the TCR activates a signal transduction pathway that involves JAKs as well as signal transducers and activators of transcription (STAT) proteins⁴³. The synergistic interaction between p21^{ras} signals and calcium/PKC signals is essential for T-cell activation. The interaction between p21^{ras} signals and the JAK/STAT pathway is likely to be required for T-cell cycle progression and clonal expansion.

Note added in proof: Swan, Perlmutter and colleagues [Swan, K.A. *et al.* (1995) *EMBO J.* 14, 276–285] have recently described the effects of expressing a dominant-negative Ras mutant in thymocytes. Their data confirm that p21^{ras} function is important for TCR signal transduction. Furthermore, the involvement of p21^{ras} can distinguish positive and negative selection in thymocytes.

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