The pharmacology of cell differentiation
R A Rifkind editor

Role of Ras proteins in T cell activation

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INTRODUCTION

The ras proto-oncogenes, Ha-, Ki- and N-*ras*, encode 21,000 molecular weight GTP binding proteins that are critical regulatory proteins in all eukaryotic cells. In most mammalian cell types, ras proteins are essential for cell growth and when constitutively activated by point mutation cause cellular transformation. In some cell types ras proteins have been implicated in differentiation pathways. Their activity is normally regulated by a cycle of binding GTP to give the biologically active form of the protein followed by hydrolysis of bound GTP to GDP. The GDP bound form of the protein is inactive: it is reactivated by exchange of bound GDP for free cytosolic GTP.

RECEPTORS AND INTRACELLULAR SIGNALS THAT REGULATE P21^{RAS}

While ras proteins had long been postulated to couple extracellular signals to intracellular targets, it was not until 1990 that the first physiological stimulus to control the activity of p21^{ras} was identified in studies showing that activation of T lymphocytes via the T cell antigen receptor (TCR) caused a very rapid stimulation of p21^{ras} as measured by its conversion from the GDP to the GTP bound state in activated cells [1]. Since this initial finding a number of cell surface receptors have been identified in various cell systems which upon triggering cause stimulation of p21^{ras}

ACTIVATION OF P21^{RAS} IN CELLS OTHER THAN T LYMPHOCYTES

Ras proteins can be regulated in fibroblasts by signals generated by triggering the receptors for platelet derived growth factor (PDGF) [2,3], epidermal growth factor (EGF) [4] and insulin [5]. In addition, fibroblasts which have been engineered to express the colony stimulating factor 1 (CSF-1) receptor also respond to this factor with a stimulation of the amount of GTP bound to p21^{ras} [6]. Common features of the receptors so far found to regulate p21^{ras} in fibroblasts are that they all regulate cell growth in a positive manner and they all possess tyrosine kinase activity.

Ras is known to be important in regulating cell differentiation in neuronal cells. The best characterised system is the rat pheochromoytoma cell line PC12: this is induced to differentiate to a neuronal phenotype in response to nerve growth factor (NGF). This process has been known for some time to rely on endogenous ras proteins. It has recently been shown directly that NGF activates p21^{ras} in PC12 cells [7, 8]. Interestingly, EGF also activates p21^{ras} in these cells with a very similar time course; however, EGF does not promote differentiation but instead cell growth in this system. Clearly events other than ras activation must also be important in determining the final cellular response to these two receptor tyrosine kinases.

In mast cells a number of stimuli have been directly demonstrated to activate p21^{ras} including Interleukin 3 (IL3), Granulocyte/Macrophage colony stimulating factor (GM-CSF) and Steel factor (SLF), the ligand for the c-kit [9]. In B cells ras proteins can be activated by IL3 [10]. In erythroleukemia cells ras is activated by erythropoietin (EPO) [11]. All of the above stimuli have in some way been linked to activation of tyrosine kinase activity [12]. One system that does not fit this pattern is the recently reported activation of ras in response to transforming growth factor β (TGFβ) [13].

Table1. Extracellular stimuli that activate p21^{ras}

Stimulus	Cell type	GTP/GTP+GDP on p21^{ras} (%) basal	stimulated
Phorbol ester	T cell	5	85
anti-TCR	T cell	5	50
anti-CD2	T cell	5	50
IL-2	T cell	2	8
IL-3	B cell	3	16
IL-3	Mast cell	6	40
GM-CSF	Mast cell	3	16
Insulin	Fibroblast [a]	20	70
PDGF	Fibroblast	7	15
PDGF	Fibroblast [b]	0.5	1.0
EGF	Fibroblast [b]	0.5	2.0
NGF	PC12	10	30
EGF	PC12	10	40
EPO	HEL	5	30
SLF	Mast cell	3	25

[a] Rat-1 cells overexpressing insulin receptor.

[b] Swiss 3T3 cells overexpressing normal c-Ha-*ras*.

ACTIVATION OF P21^{RAS} IN T LYMPHOCYTES

In T cells, it has been shown that p21^{ras} proteins are regulated by receptors that function at different stages in cell cycle progression: the T cell antigen receptor (TCR) and CD2 antigens that initiate G_0 to G_1 transition [1, 14] and the IL-2 receptor that controls G_1 to S transition and ultimately controls T cell mitosis [10]. In T lymphocytes, phorbol esters and diacylglycerols that activate protein kinase C (PKC) can mimic TCR and CD2 antigen triggering and are potent stimulators of p21^{ras}. Since the TCR and CD2 antigen can activate PKC it was accordingly postulated that PKC mediates all or part of the p21^{ras} activation seen upon triggering these receptors [1, 14]. However, this initial model for p21^{ras} regulation was complicated by the discovery that activation of PKC by phorbol esters does not result in p21^{ras} activation in cell types such as fibroblasts and mast cells even though p21^{ras} proteins can be activated via growth factor receptors in these cells. Furthermore, it was recognised that, even in the T cell, not all the receptors known to regulate p21^{ras} can also activate PKC. For example the insulin, IL-3 and IL-2 receptors can stimulate p21^{ras} in fibroblasts, mast cells and T cells respectively but these receptors probably do not activate PKC. There must therefore be an additional, non-PKC route for controlling p21^{ras}. The molecular details of this alternative p21^{ras} control mechanism are not yet known. However, in fibroblasts and haemopoietic cells all the stimuli known to activate p21^{ras} are activators of tyrosine kinases. A second route by which p21^{ras} proteins can be stimulated could thus be mediated by tyrosine kinases.

In T lymphocytes there are indications that at least two mechanisms for p21^{ras} regulation co-exist: one PKC mediated and one which is not but involves tyrosine kinases [15]. Briefly, this second mechanism has been characterised in streptolysin O permeabilised peripheral blood lymphoblasts. In this system, activation of ras in response to PKC and TCR agonists can still be demonstrated [1]. However, it has now been shown that TCR agonists will still partially activate ras in this system under zero calcium conditions in which there is no activation of PKC [15]. Pseudosubstrate peptide inhibitors of PKC are capable of fully blocking the phorbol ester response but not the TCR agonist response. The non-PKC dependent component of the TCR ras activation is inhibitable by herbimycin A, an inhibitor of lymphocyte tyrosine kinases. The TCR and CD2 antigen can both stimulate PKC and regulate pathways of tyrosine

phosphorylation and could work via both the PKC dependent and independent mechanisms to activate $p21^{ras}$. These data do not exclude that the TCR and CD2 antigen *can* regulate ras via PKC but do suggest that these receptors also use a PKC "independent" mechanism. The IL-2 receptor, which does not activate PKC but does activate tyrosine kinases, must act on $p21^{ras}$ proteins entirely through this second pathway or possibly even a third one: the IL2 response does not work in permeabilised cells and so cannot be characterised at this level. However, it is known to be inhibitable in whole cells by herbimycin A (M. Izquierdo, D. Cantrell and J.D., unpublished observations).

GTPASE ACTIVATING PROTEINS REGULATE $P21^{RAS}$ IN T LYMPHOCYTES

There are two possible ways in which increases in GTP levels on $p21^{ras}$ proteins could be achieved: by stimulation of the rate of guanine nucleotide exchange onto $p21^{ras}$ or by a decrease in $p21^{ras}$ GTPase activity. The GTPase activity of $p21^{ras}$ is controlled by GTPase activating proteins (GAPs). The best characterised of these is $p120^{GAP}$, which was the first such activity to be identified [16]. Another related protein with GAP activity that has been recently identified is neurofibromin, the product of the *NF 1* gene: this is a candidate tumour suppressor gene, damage to which may give rise to the hereditary disease neurofibromatosis type 1. In T cells, the observed stimulation of $p21^{ras}$ in TCR or phorbol ester stimulated cells correlated with a rapid decrease in the level of GAP activity measurable in cell extracts. It is not yet known whether $p120^{GAP}$ or neurofibromin are inhibited upon T cell activation since both are known to be present in these cells. A study using the detergent dodecylmaltoside which inhibits the catalytic activity of the NFI protein but not that of $p120^{GAP}$ suggests that both proteins are inhibited upon PKC activation of T cells [17]. However, the interpretation of these results could be complicated if other $p21^{ras}$ GTPase activating proteins exist .

It has been shown that purified ras proteins exchange nucleotide only slowly with a turnover rate of the order of one hour [18]. In contrast, kinetic studies of nucleotide exchange and hydrolysis on $p21^{ras}$ proteins in permeabilised T lymphocytes have revealed that the rate at which guanine nucleotide exchanges on $p21^{ras}$ is very rapid with a half life of about 1 minute [1]. A number of proteins have been described that will stimulate the exchange

of nucleotide on $p21^{ras}$ *in vitro*; these have been termed GDS for GDP dissociation stimulators [19, 20, 21, 22, 23, 24]. Recently a mammalian guanine nucleotide exchange factor for $p21^{ras}$ has been cloned by a functional complementation assay in budding yeast where the ras exchange factor CDC25 has been known for some time [25]. The elevated exchange rate of nucleotides on $p21^{ras}$ in permeabilised T cells suggests that GDS proteins have high constitutive activity in T lymphocytes. Guanine nucleotide exchange on $p21^{ras}$, although rapid, is unchanged in both PKC and TCR activated cells. It appears therefore that the principal control mechanism for $p21^{ras}$ stimulation is mediated via inhibition of its GTPase activity: due to the high rate of nucleotide exchange on $p21^{ras}$ this is very rapidly translated into accumulation of GTP on $p21^{ras}$.

MECHANISMS OF REGULATION OF RAS-GAPS IN T CELLS

From the data discussed above it seems likely that $p21^{ras}$ is controlled in the T cell by regulation of GAP like proteins; however, it is still unclear how the GTPase activating proteins themselves are controlled. The most straightforward mechanism for regulating GTPase activating proteins in T lymphocytes would be by direct phosphorylation by PKC or by another kinase involved in the PKC independent stimulation of $p21^{ras}$. However, available data show that $p120^{GAP}$ is not a substrate for PKC (J.D., unpublished data). Furthermore, although $p120^{GAP}$ can associate with activated tyrosine kinases via SH2 domains, it is phosphorylated on tyrosine residues at vanishingly low stoichiometry (about 0.1%, J.D., unpublished data). Similarly, preliminary analysis of the phosphorylation of neurofibromin in T cells reveals no obvious modulation. An alternative and perhaps more likely mechanism for the regulation of GTPase activating proteins might involve the phosphorylation of proteins that regulate $p120^{GAP}$ or neurofibromin. For $p120^{GAP}$ two such proteins that stably associate with the GTPase activating protein have been found: membrane associated p62 which is phosphorylated on tyrosine at high stoichiometry in response to receptor stimulation and cytosolic p190 which has a very low level of phosphorylation on tyrosine and considerably more on serine and threonine [26]. Association of $p120^{GAP}$ with p190 inhibits its GTPase activating ability, so if this interaction increased upon T cell activation, inhibition of GAP activity

would result [27]. Neither of these two proteins seem to be greatly altered in their level of phosphorylation or of binding to $p120^{GAP}$ upon activation of the T cell. There is currently no data available regarding NFI associated proteins. Nevertheless, the role of GAP associated proteins is an issue that needs to be addressed to further our understanding of the molecular mechanisms of $p21^{ras}$ regulation in T cells.

An alternative possibility for the control of GAP activity in T cells could be through the ras-related low molecular weight GTP binding protein $p21^{rap1}$. This protein is encoded by the K-rev1 gene which is known to be able to reverse $p21^{ras}$ transformation when expressed at high levels [28]. GTP bound $p21^{rap1}$ has been shown to competitively inhibit the interaction of $p21^{ras}$ with $p120^{GAP}$ *in vitro* [29, 30]. As yet there is no evidence available that $p21^{rap1}$ is involved in the normal regulation of $p21^{ras}$ in T cells or any other cell type. Another mechanism whereby GAP activity could be regulated involves the production of mitogenic lipids. Lipids and lipid metabolites such as phosphatidic acid, polyphosphoinositides and arachidonic acid strongly inhibit the activity of the NF 1 protein and, to a lesser extent, that of $p120^{GAP}$ [17, 31]. There are also reports of a GTPase inhibitory protein (GIP) that blocks the action of GAP proteins on $p21^{ras}$ and can be activated by diacylglycerols and the lipids that inhibit GAP proteins [32]. Studies to date suggest that neither these lipids, nor GIP, play a major role in GAP regulation in T cells. Firstly, phorbol ester and IL2 treatment of T lymphocytes do not stimulate the production of these lipids within the time frame of $p21^{ras}$ activation, so they are unlikely to account for the protein kinase C or IL2 mediated activation of $p21^{ras}$. Secondly, while lipids such as phosphatidic acid are produced in response to TCR stimulation, experiments in permeabilised cells indicate $p21^{ras}$ activation can occur efficiently under conditions in which cellular phospholipases are inactive [15]: the PKC independent regulation of $p21^{ras}$ in T cells is therefore also unlikely to rely on lipid fluxes.

FUNCTION OF $P21^{RAS}$ IN T LYMPHOCYTES

A likely role for $p21^{ras}$ in T cells is to couple receptors such as the TCR to intracellular signalling pathways that ultimately control the expression of T cell activation induced genes such as those encoding lymphokines and their receptors. A distal gene regulation function for $p21^{ras}$ in T lymphocytes is

compatible with observations in fibroblasts that $p21^{ras}$ can control nuclear transactivating factors such as SRF, c-jun and NFκB [33, 34], all of which have been implicated in the regulation of gene expression in T lymphocytes. The details of the intracellular signalling routes that could link plasma membrane associated $p21^{ras}$ to the nucleus are not known. In fibroblasts, $p21^{ras}$ has been described to modulate the function of serine/threonine kinases including PKC and c-raf [35, 36]. In T cells it is believed that PKC is an upstream regulator of $p21^{ras}$ and not an effector molecule. It is conceivable, however, that c-raf operates downstream of $p21^{ras}$ since receptors such as the TCR and IL-2 receptor that rapidly activate $p21^{ras}$ are known to have a subsequent regulatory effect on c-raf protein.

In T cells it has now been shown directly that activated $p21^{ras}$ is capable of stimulating gene expression [37, 38]. Mutationally activated ras proteins potentiate the ability of phorbol esters and TCR agonists to induce expression the interleukin 2 gene in the EL4 murine thymoma cell line. They also synergize strongly with a calcium ionophore. Furthermore, it has been shown [38] that dominant negative mutants of ras (Asn17) prevent the activation of IL2 gene transcription by phorbol esters and by TCR agonists. The data is compatible with a system in which ras mediates a signal which is necessary but not sufficient for activation of gene transcription in response to the T cell receptor or protein kinase C. There thus must exist a ras-dependent PKC mediated signal. Other pathways which must also exist are a calcium signal and a ras-independent PKC mediated signal. It is not yet known whether the effects of TCR agonists in these experiments are mediated by PKC or by a PKC-independent tyrosine kinase mechanism.

It has been suggested that GAP proteins may be $p21^{ras}$ effectors, either alone or as a $p21^{ras}$/GAP complex [39,40]. This conclusion is based on the fact that $p21^{ras}$ interacts with these proteins via its effector regions. The ability of $p21^{ras}$ to interact in this way with $p120^{GAP}$ and neurofibromin, which have homology in their catalytic GAP-related domains but are otherwise structurally distinct, indicates that it is possible for $p21^{ras}$ proteins to interact with multiple effector molecules. One speculation in this context is that the receptor that induces $p21^{ras}$ activation might, by a separate regulatory event, be able to direct the interaction of $p21^{ras}$ with a different effector molecule. For example, the TCR and IL-2 receptor both activate $p21^{ras}$ but if the additional signals generated by these receptors controlled $p21^{ras}$ association with different proteins then the

$p21^{ras}$ effector complex generated by triggering the TCR or the IL-2 receptor would be different, as would the subsequent signalling pathways operating downstream of $p21^{ras}$. The only way to examine this hypothesis and gain insight regarding the role of $p21^{ras}$ in T cells activation is to identify these immediate proximal $p21^{ras}$ effector molecules. This is the ultimate goal and biggest challenge of studies of $p21^{ras}$ in the T lymphocyte system.

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Discussion - ROLE OF RAS PROTEINS IN T CELL ACTIVATION

J. León

I assume that you have done these experiments with the PC 12 and ras cells, using panreacting monoclonal antibody against the three Ras proteins, so what are the chances that the GTP binding varies among the three members of the family?

J. Downward

Well, we have looked at that in some cell systems, but not, I would have to admit, with PC 12. The ability to look at nucleotide bound to ras, in vivo as it were, relies on having extremely good antibodies and the vast majority of antibodies are not capable of bringing down nucleotide bound to ras, or at least not in the quantities you need to do these analyses. There are, however, two antibodies that will do that, the one we normally use is 259 which recognises all three types of ras and probably not any other ras family members, and another one is 238 which recognises Kirsten and Harvey but not N. In some systems, particulary in T cells, it has allowed us to differentiate between what is happening to Harvey ras, what's happening to Kirsten ras, and what's happening to N ras. In that system they all seem to be affected equally, but we have not extended that analysis to other systems at the moment.

J. Massagué

In your permeabilized cell system, do you have cytosolic components left or are they extensively washed? Could you see the same effects in just membrane preparations?

J. Downward

You do lose cytosolic components progressively as the experiment goes on, but you probably retain a lot that you would lose in a membrane preparation, and we have never managed to see anything like that working in just a membrane preparation.

J. Massagué

You can in fact induce the loss of most of the cytosol by extensive washing. Have you done the experiment under such conditions, and if so, is the effect independent of the cytosolic components?

J. Downward

Yes, essentially it is independent. You can wash a lot and you still keep the activity, and that's true in both systems, both the T cell system, where it seems to be going through GAP, and the fibroblast system, where it seems to be going through the exchange factors. In both cases you keep that ability to regulate and if you look at the retention of either of the exchange factors or the GAP and NF1 proteins they all still seem to stay in the cells, you don't get any appreciable loss of them.

S.H. Friend

I have a question related to tissue specificity. If patients with NF1 have one missing copy throughout their body we can assume that they are probably getting inactivations in that second copy in most of their cells. In trying to understand why only certain cells seem to undergo dramatic proliferation, one wonders whether there is any evidence that other factors, whether it's exchange factors or other things, vary from cell to cell so that we can get some clues as to why these Schwannomas are more common.

J. Downward

Well, we don't really have evidence of that sort at the moment. It's like the situation with most of these tumour suppressive genes, the pattern of tumours they form is very hard to understand really. It's the same story with retinoblastoma, obviously. Presumably there are only a certain number of cell types in the body where it actually matters if you lose all your NF1 and I'm sure it is going to crop up elsewhere. It has been found in a few tumours from non NF1 patients that there are deletion of, or mutation in NF1 in both alleles, so it probably can contribute to tumour formation under other circumstances in quite different tissue types. Examples could

be colon carcinoma and myeloid dysplasia, but we have not managed to correlate it with anything we can look at, at the moment.

S.H. Friend

Another question refers to why does a cell stop growing. When you look at the Schwannomas in tissue cultures and those cells are no longer growing, do you have a clue whether they are trying to differentiate or whether there is apoptosis or something? What it is they have undergone or how they have changed?

J. Downward

What seems to be happening is very similar to what we see when we put in the NF1, that is, when you are blocking the growth through blocking ras. One sees the same programmed cell death, so it's very specific and very characteristic. In the microscope it is very clear, looks like programme cell death. That way you can't look at DNA laddering and things like that because you can't do any biochemical analysis, but putting in anti-ras, antibodies and putting in NF1 looks identical. So, from that analysis, it looks like NF1 is just acting as a negation of ras.

H.F. Lodish

In any of the systems you cited, is there any indication of how a GTP - GDP exchanger can be turned on by a protein tyrosine kinase? In fact, is there any sort of biochemical evidence that this is a direct as opposed to a second order effect or receptor?

J. Downward

It is early days on that at the moment. We are studying possible phosphorylations and exchange factors, but we have only really had any antibodies for a very short period of time. So far we can't see any indication, for example, of tyrosine phosphorylation of these exchange factors. However, there does seem to be an association with tyrosine phosphorylated proteins. We are not quite sure what they are at the moment, but we could envisage quite a simple connection if it really was

just the tyrosine kinase phosphorylating something that binds to the exchange factors. I think that is a possibility from what we know at the moment, but I don't think anyone has had any antibodies against these for more than a few months, so it's hard to say.

G.E. Francis

Is loss of heterozygosity the critical event in the formation of these tumours?

J. Downward

Well, it's most commonly the case that you get reduction to homozygosity. There are a few cases in which there seems to be some sort of deletion in the second allele, that is, a different event in the second allele, but most of them, I think the vast majority, when you look at them are homozygous for the NF1 allele.

M. Crescenzi

About the apparent paradox of EGF and NGF both activating ras in PC12 cells, it is possible the specificity is provided by a second pathway being activated by each one of the growth factors. Do you have any evidence for that?

J. Downward

No, I don't have any evidence for that, but it would seem to be, certainly the most likely thing that was going on, that the NGF receptor track is causing some other phosphorylation that's quite different from what EGF is doing. I believe, although I have not done the experiments myself, that if you look at the tyrosine phosphorylated substrates after EGF treatment versus NGF treatment, they are pretty different, so clearly that could be the explanation for why the effects are quite different. The ras activation could give you one signal and then how it is interpreted would depend on what accompanying signals there are.

I.B. Weinstein

Isn't NM23 a protein that has been implicated in influencing nucleotide levels in ras activation. Could you comment on that?

J. Downward

That is related to GDP kinases or NDP kinases. I think the evidence is all fairly indirect at the moment. There was a lot of talk about the fact that NDP kinases could directly activate G proteins or possibly even ras family members by somehow phosphorylating the GDP while it was still bound to the ras or the G protein. Recently all that work has been shown to be artefactual. So I think that makes it less comprehensible quite how the NDP kinases or related proteins could be working. Clearly they could still be working through gross alterations in GTP/ GDP ratios in the cell, but it seems like a very blunt tool indeed to use to control ras activity, even artificially.