Cell cycle: **Routine role for Ras**

Julian Downward

The Ras proteins are key mediators of the early cellular response to mitogens; the way in which they influence the later events in the cell cycle is beginning to fall into place.

Address: Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX, UK. E-mail: downward@icrf.icnet.uk

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The Ras proteins are encoded by a closely related set of genes, initially identified in acutely transforming sarcoma viruses and subsequently in human tumour cell lines [1]. The transforming *ras* oncogenes are activated by point mutation of the normal proto-oncogenes, so that they encode a protein that is locked in the active, GTP-bound state. As expression of a *ras* oncogene is sufficient to cause transformed growth of established cell lines in culture, it is clear that Ras can profoundly influence the passage of cells through the cell cycle. Much has been learnt in the past five years about the immediate signalling targets of Ras: these include not only the well characterized Raf/mitogen activated protein (MAP) kinase cascade, but also phosphatidylinositol 3-kinase and the guanine nucleotide exchange factor Ral-GDS [2]. It has remained very unclear, however, how these Ras-controlled pathways feed into regulation of the cell-cycle machinery. Recent observations are beginning to shed light on this important question, which has significance for our understanding of both normal and transformed cell growth.

In addition to its transforming ability, the importance of Ras in cell-cycle regulation has been established in several ways. Seminal experiments showed that microinjection of activated Ras protein into quiescent fibroblasts would cause entry into S phase. The critical role of the endogenous Ras protein in normal growth was established by microinjection of neutralizing antibodies against Ras: this resulted in the arrest of cells growing in serum. This treatment was also able to inhibit the growth of fibroblasts that had been transformed by oncogenes encoding tyrosine kinases. These experiments, and others involving temperature-sensitive mutant forms of Ras and cell-cycle inhibitors, suggested that Ras function was required throughout G1 phase, but that, once cells had entered S phase, Ras became dispensable until the next cell cycle [3,4], with the exception of a possible requirement in G2 in some cell types and organisms. The need for Ras function both early and late in G1 is reminiscent of the well established model for cell-cycle control by growth factors, in which 'competence' factors, such as platelet-derived growth factor (PDGF), act to release quiescent cells from the quiescent state, G0, while 'progression' factors found in plasma are required until a 'restriction point' is reached late in G1, at which time cells become mitogen-independent [5,6].

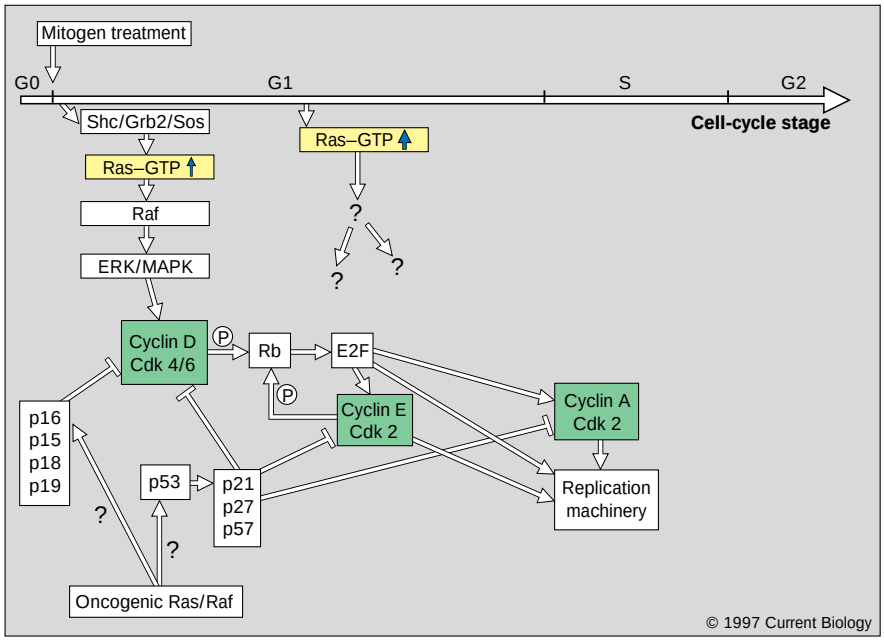
Work over the past decade has established that the activation state of normal Ras proteins is regulated in response to treatment of cells with a very wide variety of mitogens, including what would normally be regarded as both competence and progression factors. However, because of technical limitations of the methods used to measure the activation state of Ras, which relied on metabolic labelling of cells with ^{32}P-orthophosphate and subsequent immunoprecipitation of Ras along with its bound nucleotide, the stimulation of cells was usually only followed for short periods, at most a couple of hours. Recently, a new method has been perfected for measuring Ras activation without the need for radioisotopic labelling, by affinity precipitation using its target binding partner Raf, which only binds to the active, GTP-bound form of Ras [7]. This has allowed the activation state of Ras to be followed throughout the cell cycle.

The use of this new method showed that, unexpectedly, in NIH 3T3 cells released from quiescence by treatment with serum, the activation of Ras achieved rapidly after serum addition is much less than a second activation some five hours later in mid-G1 phase [7]. The mechanism whereby this second peak of Ras activity is achieved is unclear, but it appears to require protein synthesis and not to use the well characterized pathway involving the linker proteins Shc and Grb2, and the Ras nucleotide exchange factor Sos, which mediates the short-term activation of Ras by serum factors [8]. The mid-G1 peak of Ras activity may explain the requirement for Ras function throughout most of G1, and one might speculate that transition through the restriction point does not occur until after this peak of Ras activity has been reached. Interestingly, only the early phase of Ras activation correlates with activation of the Raf/MAP kinase pathway, while at later times high levels of Ras–GTP occur without significant MAP kinase activity, possibly because that pathway is down-regulated by inducible expression of specific phosphatases.

Although the signalling pathways acting immediately downstream of Ras are reasonably well understood, as is the basic cell-cycle machinery, the connections between the two are still unclear. Recent work has begun to illuminate this area. For example, activated Ras expression has been shown to increase cyclin D1 expression levels and shorten

Figure 1

Influence of Ras on G1 progression and S phase entry. Two peaks of endogenous Ras activity are seen, on release from G0 and more strongly in mid G1. The first peak of Ras activity may be responsible for cyclin D induction *via* MAP kinase. The second peak of Ras activity may act through other effectors to influence later, unidentified events in mid-to-late G1. Constitutively activated, oncogenic Ras or Raf induce expression of the Cdk inhibitor p21 *via* p53 to arrest primary (Schwann) cells. Deletion of the gene encoding Rb or the Cdk inhibitor p16 partially removes the ability of a block in Ras function to arrest primary cell-cycle progression, implicating cyclin D as a major, though probably not the only, cell-cycle target for endogenous Ras. P in a white circle indicates phosphorylation.



G1 phase [9–11]. This effect appears to occur through the Raf/MAP kinase pathway [12], which is stimulated by the initial phase of Ras activation. It seems unlikely, however, that cyclin D1 expression accounts for all the growth stimulation provided by Ras, as cyclin D1 cooperates with activated Ras in transformation of primary murine cells under some circumstances [13,14]. One of the principal targets of the cyclin D-associated kinases, cyclin-dependent kinase 4 (Cdk4) and Cdk6, is the retinoblastoma tumour suppressor protein (Rb); phosphorylation of Rb results in release of bound E2F transcription factors, which play a key role in transition from G1 to S phase.

Very recent work from Peeper *et al.* [15] and Mittnacht *et al.* [16] shows that the ability of neutralizing antibodies against Ras, or a dominant-negative mutant form of Ras, to inhibit cell-cycle progression in asynchronously growing murine fibroblasts depends on the presence of Rb. Cycling cells lacking Rb fail to arrest when Ras function is inhibited, although release of quiescent cells from G0 is inhibited by dominant-negative Ras even in the absence of Rb. Expression of dominant-negative Ras causes loss of Rb phosphorylation, which can be corrected by forcing the expression of cyclin D1. The ability to induce Rb phosphorylation through cyclin D-dependent kinases is thus a major part of the link between Ras and the cell-cycle machinery (Fig. 1). It seems likely, however, that Rb does not account for all aspects of Ras regulation of the cell cycle, as primary mouse embryo fibroblasts lacking Rb are only partially protected from the effects of inhibiting Ras. More complete effects are seen in established cell lines, but this may be due to other events that have occurred during cell culture.

This illustrates a major problem with much of the work done on Ras and the cell cycle — the variability of Ras effects in different cell types, particularly in established cell lines. For example, expression of activated Ras in BALB c/3T3, but not NIH 3T3, cells fails to increase the activity of Cdk4 and Cdk6, because of the presence of the Cdk inhibitor $p27^{Kip1}$ [11]. Ras by itself fails to induce S phase in BALB c/3T3 cells, but does induce S phase in NIH 3T3 cells. It has long been known that, unlike in NIH 3T3 and similar cell lines which tend to have lost some Cdk inhibitors, activated Ras alone will not transform primary murine fibroblasts, Schwann cells or the REF52 cell line, but requires a cooperating oncogene to do so. By itself, Ras causes growth arrest in these cells [17–19].

From recent work by Lloyd *et al.* [20], it is now apparent that the growth inhibitory effect of Ras in Schwann cells is due to the ability of the Raf/MAP kinase pathway to induce expression of the Cdk inhibitor $p21^{Cip1/Waf1}$. Cooperating oncogenes, such as SV40 large T antigen, block p53 function, which is required for induction of $p21^{Cip1/Waf1}$ expression, thus allowing activation of cyclin E-dependent kinase activity. Down-regulation of $p21^{Cip1/Waf1}$ with antisense oligonucleotides also relieves growth arrest by Ras. It therefore appears that, in certain primary cells, oncogenic Ras can induce Cdk inhibitor expression by a p53-dependent pathway, providing an explanation of oncogene cooperation, and perhaps also the frequent occurrence of both Ras activation and p53 loss in common human tumours such as colon carcinoma. Conversely, forcing the expression of high levels of the Cdk inhibitors $p16^{Ink4}$ [21] or $p21^{Cip1/Waf1}$ [22] blocks the

ability of oncogenic Ras to cause proliferation and transformation in murine fibroblast cell lines, whereas loss of $p16^{Ink4}$ allows Ras transformation of primary mouse embryo fibroblasts [23].

A concern with the studies that try to establish the role of Ras in normal cell-cycle regulation by using activated mutant forms of Ras is that high levels of constitutively activated Ras may behave differently from the relatively low levels of activated Ras found in a normal cell. Furthermore, in the normal cell cycle, the Ras activation state fluctuates with progression through the cycle [7]: it is not inconceivable that the apparent ability of oncogenic Ras or Raf to induce the expression of some Cdk inhibitors is a protective or stress response of the cell to receiving a Ras signal at an inappropriate stage in the cell cycle.

This view is supported by recent work by Serrano *et al.* [24] showing that expression of activated Ras in primary mouse embryo fibroblasts, primary human diploid fibroblasts or REF52 cells causes growth arrest and premature cellular senescence associated with increased expression of p53 and $p16^{Ink4}$. Inactivation of either p53 or $p16^{Ink4}$ allows Ras to transform the mouse cells, rather than cause senescence, but to achieve this in human cells, further loss of tumour suppressor gene function is required; this can be achieved by adenovirus E1A, which causes loss of both Rb and p53 function.

Thus, induction of cellular senescence is likely to be another mechanism by which organisms can protect themselves from the damaging effects of activated oncogenes. By contrast, Mittnacht *et al.* [16] found that cycling primary mouse embryo fibroblasts lacking $p16^{Ink4}$, but not $p21^{Cip1/Waf1}$, show partial protection from Ras-antibody-induced cell-cycle arrest. Hence, loss of Cdk inhibitors can release cells from cell-cycle arrest caused both by too little Ras signal and also by too much Ras signal.

Although the work described above indicates that a major point at which Ras controls the cell cycle is through regulation of cyclin D expression, there is clearly reason to think that this is not the whole story. The major peak of Ras activation seen by Taylor and Shalloway [7] occurs after cyclin D expression is already at a high level. Also, this late Ras peak does not appear to be activating MAP kinase, which has been implicated in the control of cyclin D expression. What is the effect of activated Ras in mid-G1 on cell-cycle progression? And what downstream effector pathway is Ras using? To what upstream signals is Ras responding at this stage of the cell cycle? Ras at this point may be more directly influencing later events in the cell cycle, such as cyclin E or cyclin A expression, and could be responding to environmental cues other than serum factors. For example, Ras might be involved in the checkpoint control that monitors cell adhesion late in G1 [25]. It is possible that Ras is exerting its effects through the phosphatidylinositol 3-kinase pathway, which is also required for cell-cycle progression [26]. Whatever the details, it is likely that the connections between Ras and the control of both the normal and transformed cell cycle will be greatly clarified in the near future.

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